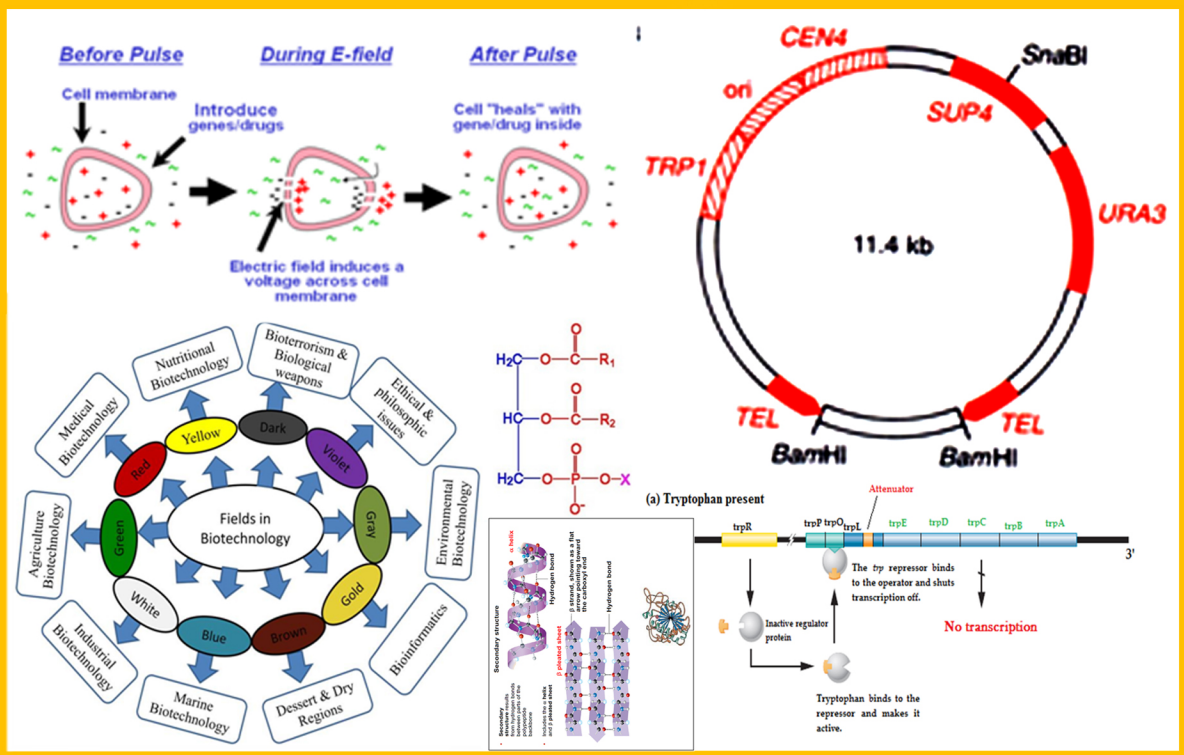
MSCBOT-604

M. Sc. III Semester

MOLECULAR BIOLOGY AND BIOTECHNOLOGY



DEPARTMENT OF BOTANY
SCHOOL OF SCIENCES
UTTARAKHAND OPEN UNIVERSITY

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Block-1

Molecular Biology

UNIT-1-Biomolecules: Classification

Contents:

1.1 OBJECTIVES

- After reading this unit you would be able to:
    - Introduce and Classify the biomolecules;
    - Define and enumerate the carbohydrates, lipids, nucleic acids, and proteins on the basis of their structure and functions;
    - Explain the important functional group and structural features of biomolecules.
    - Describe the importance of biomolecules like carbohydrates, lipids, proteins and nucleic acids.

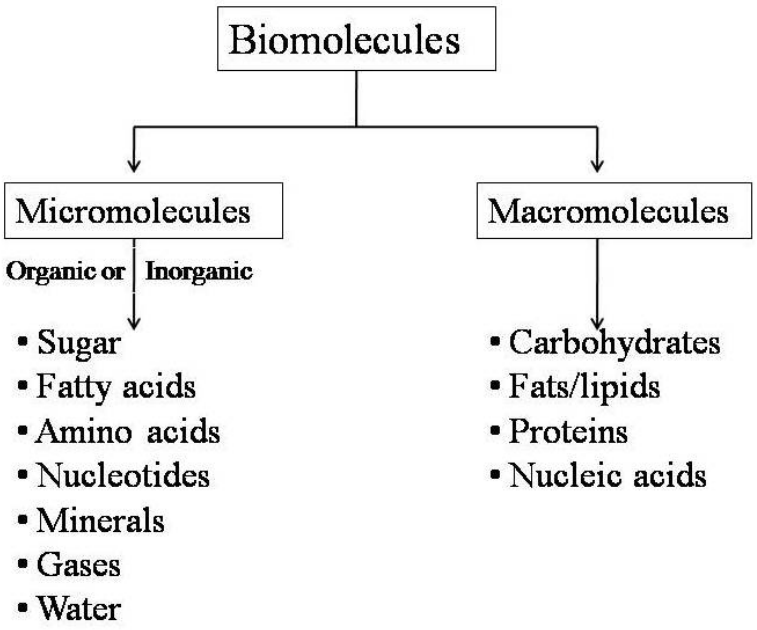
1.2 INTRODUCTION

The cells carry out life-sustaining essential functions with the help of several organic molecules present in them. These organic molecules are referred to as biomolecules. A Biomolecule, also known as a biological molecule, is a broad term for molecules found in organisms that are necessary for one or more distinctive biological processes, such as cell division, morphogenesis, or development. Large macromolecules i.e. proteins, carbohydrates, lipids, and nucleic acids and small molecules such as primary metabolites, secondary metabolites, and natural products are examples of biomolecules.

Although biomolecules are organic compounds, they differ from other organic molecules in that they share the same chemical kinds, reactions, and physical principles. The biomolecules have a broad range of sizes and structures, and they are involved in a wide range of life functions. They are made of more than 25 naturally occurring elements, mainly of carbon and hydrogen with nitrogen, oxygen, sulphur, and phosphorus. Carbon compounds have major involvement in the formation of biomolecules. They covalently bind with other elements to form several other compounds. Some biomolecules are considered derivatives of hydrocarbons; they are produced by replacing hydrogen atoms from functional groups like alcohols, amines, aldehydes, ketones, and carboxylic groups (Kumar et al., 2016). The present unit introduces you to about Introduction, classification, chemical nature and biological role of carbohydrate, lipids, proteins and nucleic acids.

1.3 CLASSIFICATION OF BIOMOLECULES

Based on molecular weight and solubility, biomolecules are divided into two categories: micromolecules and macromolecules. Micromolecules typically have tiny sizes, molecular weights between 18 and 800 Da (dalton), and high solubilities. Organic or inorganic compounds can make up micromolecules (minerals, gases and water, amino acid and nucleotides etc). Macromolecules are large sized. With the exception of lipids, they have a high molecular weight of 800–1000 dalton and are poorly soluble. Carbohydrates, proteins, nucleic acids, and lipids are macromolecules.



Different biomolecules can be classified as aldehyde, ketones and aromatic compounds as chemical forms. There are four major classes of biomolecules:

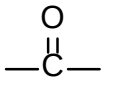
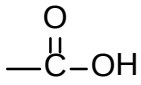
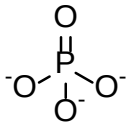
	Micromolecules (Small sized)	**Macromolecules (large sized)**
i	Sugars	Carbohydrates
Ii	Fatty acids	Fats/lipids
iii	Amino acids	Proteins
iv	Nucleotides	Nucleic acids

1.4 CHEMICAL BACKGROUND OF BIOMOLECULES

Biomolecules made up of carbon and hydrogen, including large molecules such as proteins, polysaccharides, lipids and nucleic acids. Generally these biomolecules known as the derivatives of hydrocarbons and some of the hydrogen atoms replaced by various types of functional groups such as hydroxyl, methyl, carbonyl, carboxyl, amino, phosphate and sulfhydryl to formed different bioorganic molecules or biological molecules (Table 1.1). Numerous biomolecules are polyfunctional, which means they contain two or more functional groups that can affect how reactively they interact with one another. Basically functional group of biomolecules contain carbon, hydrogen, oxygen, nitrgen, phosphorus and sulphur besides these biomolecules also contain several heterocyclic and homocylic ring in their structure for example indol ring in amino acid tryptophan, phenanthrene ring in steroids. Pyrole is the basic unit of porphyrins found in several biomolecules like hemoglobine, chlorophyll etc. while thiophene ring is the part of

vitamin-biotin. The amino acid histadine contain the ring structure of imodazole. Pyrimidines and purine are the basic elements of the nucleic acids.

Table 1.1 Some important functional group of biomolecule.

Functional group		Properties	General feature	Example of bimolecule
Name	Structure			
Hydroxyl	—OH	Polar, Hydrophilic, Capable of hydrogen bond interactions	Characterized by presence of H and O	Carbohydrates
Carbonyl		Polar, Carbonyl group of ketones and aldehydes is strongly polar group capable of acting as a hydrogen bond acceptor	Characterized by central C and O Double bond to oxygen increases the polarity	Carbohydrates
Carboxyl		Ionized to release H^+. Since carboxyl groups can release H^+ ions into a solution, they are considered acidic. Polar, Capable of hydrogen bond interactions	Characterized by central C bound to O and OH	Fatty acids
Amino	—NH_2	Capable of hydrogen bond interactions as a base, accepts H^+ to form NH_3^+. Since amino groups can remove H^+ from solution, they are considered basic.	Characterized by presence of N	Protiens
Phosphate		Charged, ionizes to release H^+. Since phosphate groups can release H^+ ions into solution, they are considered acidic.	Characterized by presence of P	Nucleic acids
Sulfhydryl	—SH	Polar	Characterized by presence of S	Coenzyme-A

Due to their enormous size, the majority of biomolecules have distinctive 3-dimensional shapes. The molecule's three-dimensional structure is maintained via a large number of non-covalent

interactions between its atoms. The structure of a biomolecule is flexible rather than static due to the weak nature of most noncovalent bonds and interactions between the biomolecule and the solvent. Biomolecules also exhibit stereochemistry as organic compounds. When the molecule contain stereogenic (or chiral or asymmetric) carbon then it can exist in two different isomeric enantiomers or diastereomers forms that have different configurations in space and have different properties.

1.5 CARBOHYDRATES

In the biological system, carbohydrates are the most abundant class of organic molecules found on the earth because they are one of the major classes of biomolecules, along with proteins and lipids. In general, carbohydrates are found in both plants and animals. Carbohydrates are the form in which the solar energy that is used by algae, certain bacteria, and green plants during photosynthesis is stored. Everyday foods like rice, bread, potatoes, sugar, soft drinks, jams and fruit juices, salad, etc. contain them in various forms. Carbohydrate is an organic compound, it comprises of only oxygen, carbon and hydrogen. Originally it referred to compounds of general formula $C_n(H_2O)_n$. Where *n* is the number of carbons in the molecule represents carbohydrates. In other words, the ratio of carbon to hydrogen to oxygen is 1:2:1 in carbohydrate molecules. So, carbohydrates are hydrates of carbon; technically they are polyhydroxy aldehydes and ketones. Carbohydrates are also known as saccharides, the word saccharide comes from Greek word *Sakkron* which means *Sugar*. They are the major source of energy in many organisms, serve to store energy, and are structural components in some organisms.

The carbohydrates are poly functional compounds and contain functional group Alcoholic hydroxy group (-OH), Aldehyde group (-CHO) and Ketone (>C=O). Thus carbohydrate can be defined as Polyhydroxyaldehydes such as D-glucose, polyhydroxy ketones such as D-fructose, and large molecules such as sucrose that produce these compounds on hydrolysis.

1.5.1 Classification and nomenclature of carbohydrates

The carbohydrates are divided into three major classes depending on the number of monomer units or upon whether or not they undergo hydrolysis and if they do, on the number of products formed. The simplest carbohydrates in this classification are monosaccharides since they cannot be divided into smaller carbohydrates. Only one unit (mono-single) makes up these molecules. Oligosaccharides (oligo- few) consists of 3-20 monosaccharide units. Polysaccharides (poly- many) are the most abundant carbohydrate found in food, it has more than 20 to 100 or 1000 of monosaccharide units bound together.

1. Monosaccharides: The monosaccharides are polyhydroxy aldehydes or polyhydroxy ketones which cannot be decomposed by hydrolysis to give simpler carbohydrates. Examples: Glucose, Fructose, Galactose etc. On the basis of number of carbon in main chain monosaccharides are also classified as trioses, tetroses, pentoses, hexoses and heptoses (Table 1.2).

Table 1.2 Clasification of monosacarides

No. of Carbon Atoms	Emprical Formula	Aldose		Ketose	
		Types	Example	Types	Example
3	$C_3H_6O_3$	Aldotriose	Gleceraldehyde	Ketotriose	Dihydroxyacetone
4	$C_4H_8O_4$	Aldotetrose	Erythrose	Ketotetrose	Erythrulose
5	$C_5H_{10}O_5$	Aldopentose	Ribose	Ketopentose	Ribulose
6	$C_6H_{12}O_6$	Aldohexose	Glucose	Ketohexose	Fructose
7	$C_7H_{14}O_7$	Aldoheptose	Glucoheptose	Ketoheptose	Sedoheptalose

2. Oligosaccharides: Oligosaccharides (Oligo: few) made of short chains of monosaccharide units, or residues, joined by characteristic linkages called glycosidic bonds and upon the hydolysis gives definite number (2-10) of monosaccharide molecules. Disaccharide and trisaccharide are the names for oligosaccharides with two and three monosaccharide units, respectively.

(i) Disaccharides: The oligosaccharides containing two monosaccharaides units are called disaccharide, which yield two monosaccharide's molecules on hydrolysis. Its molecular formula is C12H22O11.Examples Sucrose, maltose etc. Sucrose (C12H22O11) is disaccharides because on hydrolysis it gives one molecule of glucose and one molecule of fructose.

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{H^+} \underset{\text{Fractose}}{C_6H_{12}O_6} + \underset{\text{Glucose}}{C_6H_{12}O_6}$$

$$\underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{H^+} \underset{\text{Glucose}}{2C_6H_{12}O_6}$$

ii) Trisaccharides: The oligosaccharides containing three monosaccharaides units are called trisaccharide, which yield three monosaccharides molecules on hydrolysis and have molecular formula is $C_{18}H_{32}O_{16}$. Example- Raffinose, Melezitose.

$$C_{18}H_{32}O_{16} + H_2O \xrightarrow{H^+} C_6H_{12}O_6 + C_6H_{12}O_6 + C_6H_{12}O_6$$

Raffinose — Fractose, Glucose, Galactose

iii) Tetrasaccharides: The oligosaccharides containing four monosaccharaides units are called tetrasaccharide, which yield four monosaccharides molecules on hydrolysis and have molecular formula is C22H42O21. Example- Stachyose.

$$(C_6H_{10}O_5)_n + H_2O \xrightarrow{H^+} nC_6H_{10}O_6$$

Starch or Cellulose — Glucose

3. Polysaccharides: The carbohydrates which have higher molecular weight, which yield more than 10 monosaccharide units on hydrolysis. Some polysaccharides, like cellulose, are linear chains; others, such as glycogen, are branched. Example- Starch, Glycogen, Dextrin, Cellulose etc.

$$(C_6H_{10}O_5)_n + H_2O \xrightarrow{H^+} n\,C_6H_{12}O_6$$

Glucose

In general, polysaccharides are amorphous, insoluble in water, and tasteless, whereas monosaccharides and oligosaccharides are crystalline solids, soluble in water, and sweet to taste. These substances are collectively referred to as sugars. Differences between monosaccharides, oligosaccharides, and polysaccharides are shown in Table 1.3.

Table 1.3 Differences between monosaccharides, oligosaccharides and polysaccharides.

Character	Monosaccharides	Oligosaccharides	Polysaccharides
No. of sugar molecules	1	2-10	More than 10
Glycoside bond	Absent	Present	Present
Molecular weight	Low	Moderate	High
Taste	Sweet	Minimally sweet taste	No teste
Solubility in water	Soluble	Soluble	insoluble
Nature	Always reducing sugar	May or may not be	Always non reducing sugar
Example	Glucose, Fructose, Glactose	Sucrose, Maltose	Starch, Cellulose, Glycogen

Additionally, the carbohydrates can be categorised as either reducing sugars or non-reducing sugars. Reducing sugars are all carbohydrates that may reduce Fehiling's solution and Tollen's reagents, whereas non-reducing sugars are all other carbohydrates. Reducing sugar has the ability to convert cupric ions from Benedict's or Fehling solution to cuprous ions by carrying a free aldehyde (-CHO) or ketonic [RC(=O)R'] group. Other than sucrose, all monosaccharides and disaccharides (such as lactose, melibiose, cellobiose, and gentiobiose) are reducing sugars.

1.5.2 Structure and Stereochemistry of Carbohydrates

Wedge-and-dash structures and Fischer projections are frequently used to illustrate the chemical structures of carbohydrates (Fig. 1.1). Numerous stereocenters exist for carbohydrates. For instance, glyceraldehyde only has one. However, as you get to more complex carbohydrates, you will see an increase in stereocenters. A glucose molecule has four chiral carbons. This suggests that the glucose molecule contains a total of 16 stereoisomers. The number of stereoisomers is equal to 2^n, where "n" is the number of chiral centres. Stereoisomers are molecules that are identical chemically but differ only in their spatial arrangement. Asymmetric (chiral) carbon atoms are found in all monosaccharides, with the exception of dihydroxyacetone, and they all contain isomeric forms that are optically active. Aldose carbohydrate glyceraldehyde has two distinct optical isomers, or enantiomers, called d- and l-glyceraldehyde because it has a chiral carbon atom in the centre. Enantiomers are paired chiral compounds or steroisomers that are not mirror reflections of one another. Such compounds have the opposite orientation of all chiral carbon atoms. By convention, one of these two enantiomers is designated as the D isomer, the other as L isomer.

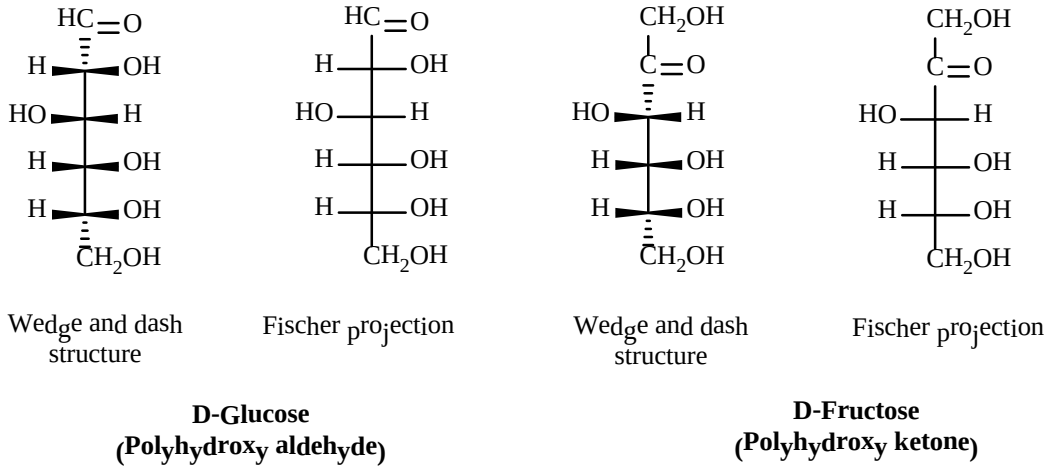


Figure 1.1 Wedge-dash structures and Fischer projections of carbohydrates.

A. Open Chain form of carbohydrates

The D and L notation, which describes the arrangement of the final chiral carbon in the chain, is used to explain the stereochemistry of carbohydrates. It is used to give the name of molecule by relating it to glyceraldehyde. Fischer's projection is used to distinguish between D and L carbohydrates. The carbohydrate is given the D configuration in Fischer's projection if the hydroxyl group is located to the right of the last stereocenter, and the L configuration in Fischer's

projection if it is put to the left of the final stereocenter carbon. It simply provides information on how carbohydrates are configured; it makes no mention of the direction in which plane-polarized light rotates or the fact that molecules with dissimilar atomic configurations are known as asymmetrical molecules. The D and L arrangement does not provide absolute stereochemistry, but the anomeric carbon centre can by comparing its orientation to the glyceraldehyde. The D-L system is significant because it provides the relative arrangement of the molecules. Enantiomers, for instance, can be quickly designated using the D- and L- notation. The enantiomer of L-glucose is called D-glucose. Enantiomers can be quickly identified using the D- and L- notation; for instance, D-glucose is the enantiomer of L-glucose (Fig. 1.2).

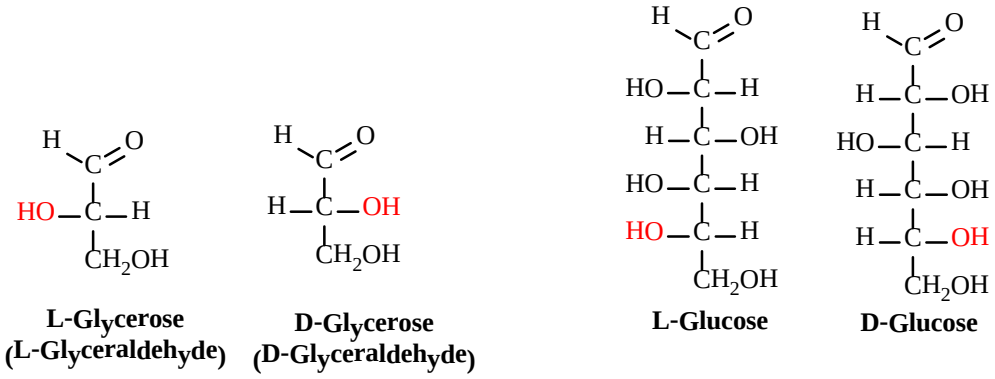


Figure 1.2 Open chain form of carbohydrates and D, L nomenclature.

Additionally, the molecule has optical activity due to the presence of asymmetric carbon atoms. When a beam of plane-polarized light is passed through a solution of an optical isomer, it will be rotated either to the right, dextrorotatory (+); or to the left, levorotatory (-). The direction of rotation is independent of the stereochemistry of the sugar, so it may be designated D (-), D (+), L (-), or L (+). The D (-) isomer of fructose is one example of a sugar that occurs naturally.

B. Cyclic form of carbohydrates (Hemi-acetal structure)

Carbohydrates can generate intramolecular (cyclic) hemiacetals because they have carbonyl and alcohol functional groups. There are no intramolecular cyclic forms for smaller carbohydrates because they are required to be at least a tetrose in order to do that. A 5-membered ring results from the cyclization of the -OH on the fourth carbon. A 6-membered ring results when the -OH is provided by the fifth carbon. The 5-membered rings are known as furanoses, and the 6-membered rings are known as pyranoses. These names are derived from the analogy with the widely used heterocyclic chemicals furan and pyran, which contain oxygen. Here is an illustration of how the ubiquitous sugar D-galactose can take on two distinct cyclic forms (Fig.1.3).

The majority of monosaccharides with five or more carbon atoms in aqueous solutions have a ring structure. The ring structure is formed by an intramolecular (within the molecule) reaction between hydroxyl groups and aldehyde group of aldose or ketone group of ketose. A covalent bond is formed between the carbonyl group and the oxygen of a hydroxyl group along the chain. It leads to the formation of cyclic (ring) adducts (product formed due to addition of the reactants)

that are known as hemiacetal and hemiketals, respectively. These cyclic structures now contain an additional asymmetric carbon atom and therefore, can exist in two steroisomeric forms. The asymmetric carbon atom is known as anomeric carbon.

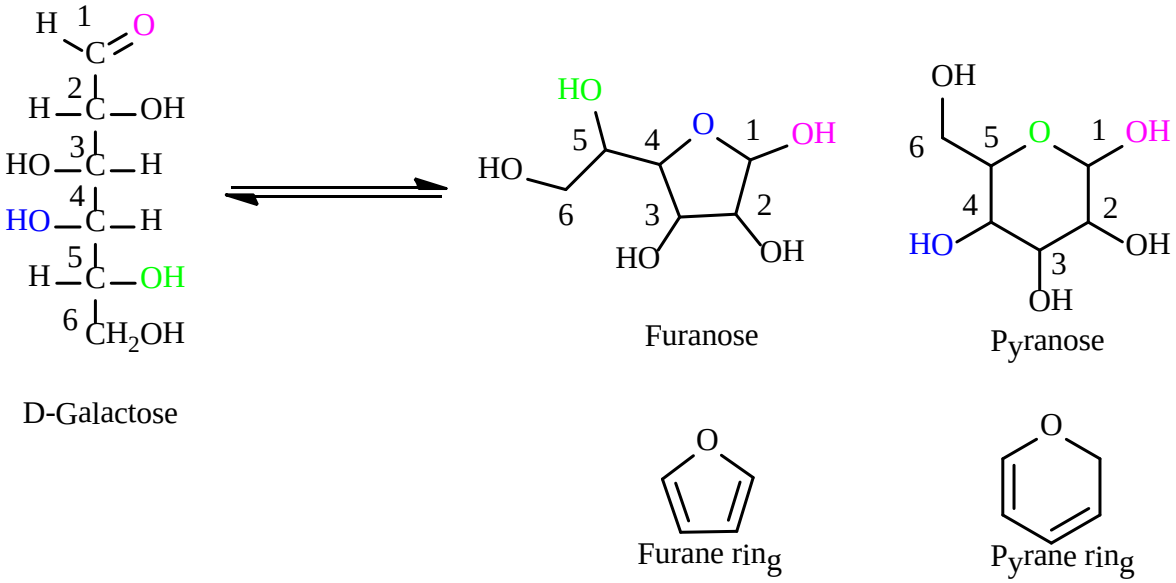


Figure 1.3 Cyclization of galactose (produced two different cyclic products)

C. Haworth Projections (α and β anomers)

The cyclic forms of carbohydrates are frequently represented by a certain sort of illustration. They are referred to as forms or Haworth projections. Basically, a Haworth projection is a cyclic structure with, traditionally, carbon 1 to the right and the bottom portion of the structure oriented towards the observer. C1 in a cyclic carbohydrate is called anomeric carbon. This carbon used to be a carbon of the C=O in the open-chain structure prior to the cyclization. Anomeric carbon is unique since it has no fixed stereochemistry and can exist in either an α-form or a β-form. The α- and the β-forms are defined as *trans* or *cis* isomers of the cyclic carbohydrates where we look at the anomeric -OH and the C 5 or C6 for furanoses or pyranoses correspondingly.

Cyclization produces an anomeric carbon (the former carbonyl carbon), which results in the formation of the sugar's and α and β configurations, such as α-D-glucopyranose and β-D-glucopryanose. In a Haworth projection formula for the α-configuration, OH (C1) is *trans* to the CH2OH (C5) group, but in the α-configuration, OH (C1) is *cis* to the CH2OH (C5) group. They are referred to as diastereomers because the forms are not mirror images. Thus monosaccharaides, differing only in this configuration around the carbon atom to which the carbonyl group is attached (the anomeric carbon), are called anomers (Fig.1.4).

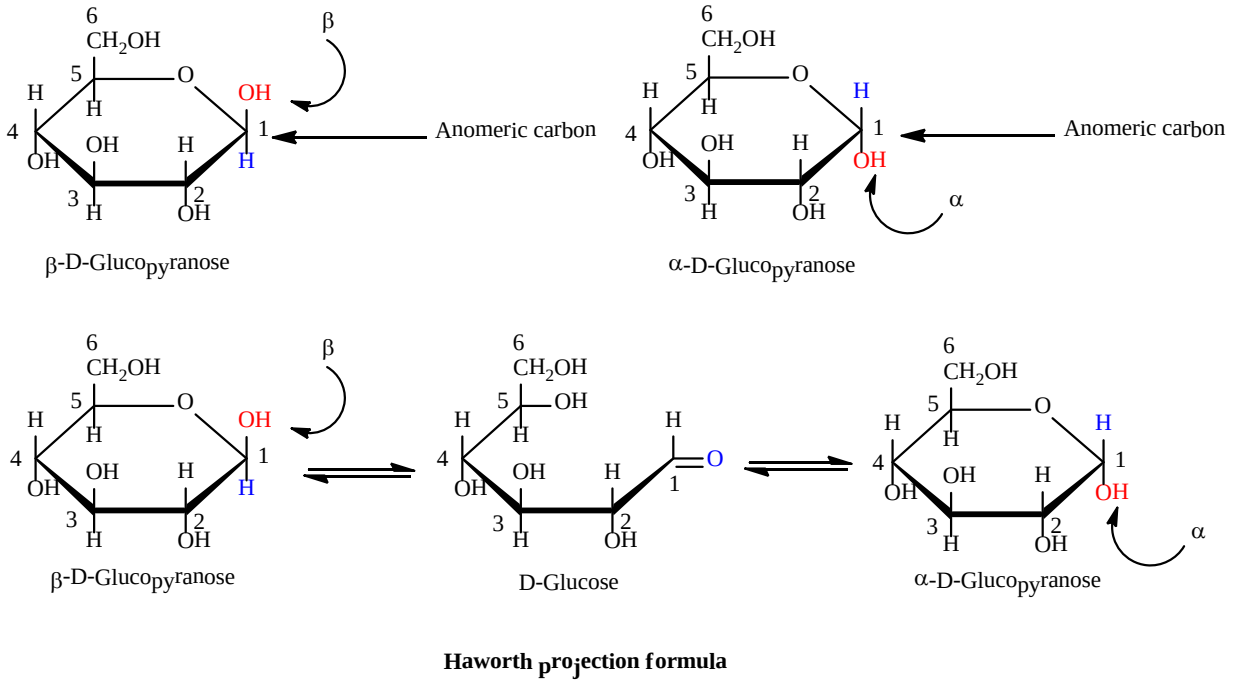
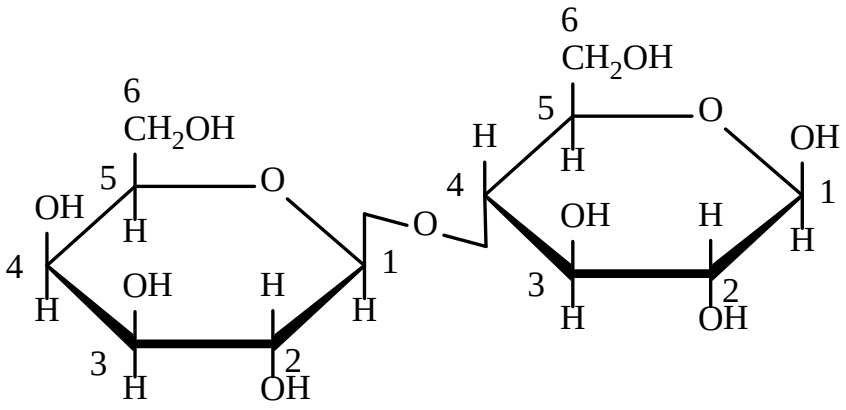


Figure 1.4 The α- and the β-forms of Glucopyranoses

D. Glycosidic bonds

Glycosidic linkages combine monosaccharides or longer sugar chains with other carbohydrates to make disaccharides, oligosaccharides, and polysaccharides. It is a specific kind of covalent bond. Important disaccharides include lactose (galactose + glucose), sucrose (glucose + fructose) and maltose (glucose + glucose). The bonds that link sugars are called glycosidic bonds. These monosaccharide sugar chains have the capacity to make additional glycosidic linkages with alcohols and amines to yield sugar acetals/glycosides and nucleosides. Glycosidic bonds between sugars are named according to the numbers of the connected carbons and also with regard to the position of the anomeric hydroxyl group of the sugar involved in the bond. An O-glycosidic bond is formed when the anomeric carbon of the sugar joins up with the oxygen atom in the hydroxyl group of the alcohol. Instead, an N-glycosidic bond is referred to as such if the anomeric carbon of the sugar forms a link with the nitrogen atom of an amine. If this anomeric hydroxyl is in the **α** configuration, the linkage is an **α-bond**. If it is in the **β** configuration, the linkage is a **β-bond**.

- Example- lactose, forming a glycosidic bond between carbon 1 of β-galactose and carbon 4 of glucose. The linkage is, therefore, a **β (1→4)** glycosidic bond (Fig.1.5).



D-Galactosyl1-β (1-4)-D-glucose

Figure 1.5 β (1→4) Glycosidic bond

E. **Epimer**

Compounds that have the same chemical formula but have different structures are called isomers. For example, fructose, glucose, mannose, and galactose are all isomers of each other, having the same chemical formula, $C_6H_{12}O_6$ (Fig. 1.6).

- Carbohydrate isomers that differ in configuration around only one specific carbon atom are defined as **epimers** of each other.
- For instance, the only structural difference between glucose and galactose, which are both C-4 epimers, is the location of the -OH group at carbon 4. [Note: The carbons in sugars are numbered beginning at the end that contains the carbonyl carbon-that is, the aldehyde or keto group].
- Glucose and mannose are C-2 epimers. However, galactose and mannose are **not** epimers-they differ in the position of -OH groups at two carbons (2 and 4) and are, therefore, defined only as isomers.

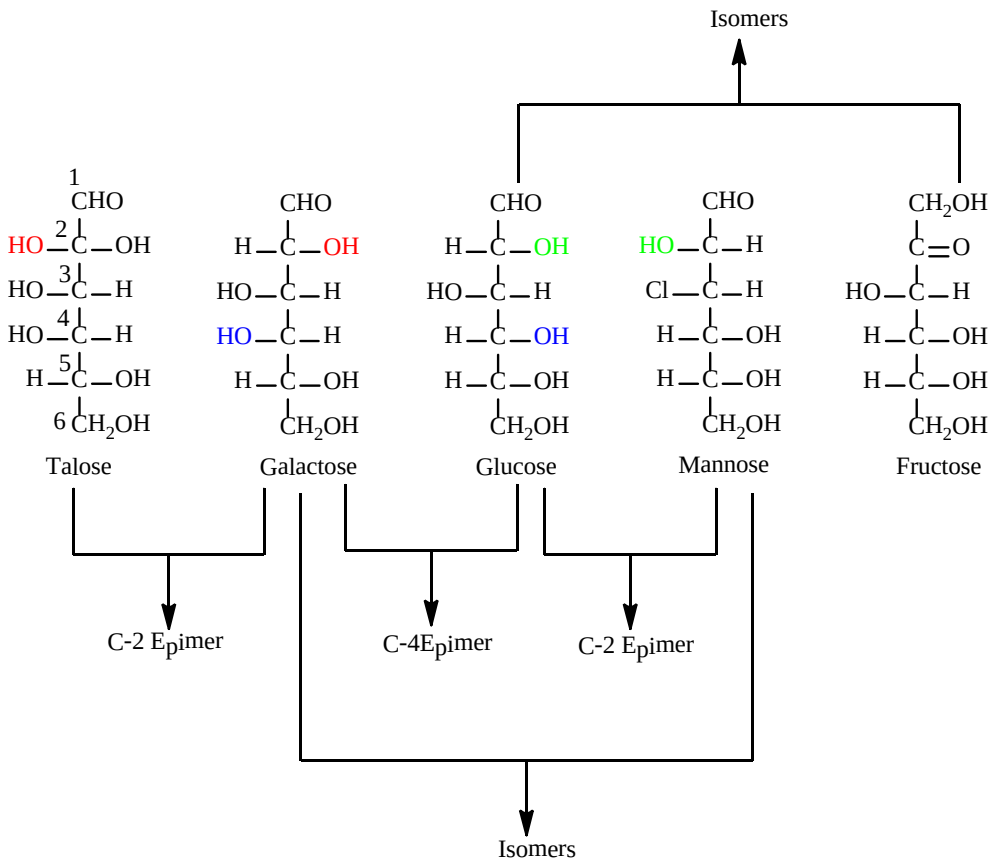


Figure 1.6 Epimers of carbohydrate with chemical formula, $C_6H_{12}O_6$.

I. Mutarotation

Mutarotation is defined as the changes in specific optical rotation by inter conversion of α and β form of D-glucose to an equilibrium mixture. Enzymes that accelerate the attainment of this equilibrium are called mutarotases and can be incorporated in assay reagents in order to speed up the equilibrium formation.

Mutarotation is the change in the optical rotation because of the change in the equilibrium between two anomers, when the corresponding stereocenters interconvert. Cyclic sugars show mutarotation as α and β anomeric forms interconvert.

- The optical rotation of the solution depends on the optical rotation of each anomer and their ratio in the solution.
- Mutarotation was discovered by French chemist **Sir Dubrunfaut** in 1846, when he noticed that the specific rotation of aqueous sugar solution changes with time.
- Sugars in the ring form can exist in two states, one where the C-1 hydroxy group is above the plane of the ring (β) and one where it is below (α).
- In aqueous solution there is a constant interchange between the various conformations via the breaking open of the hemi acetal structure and its subsequent reforming.

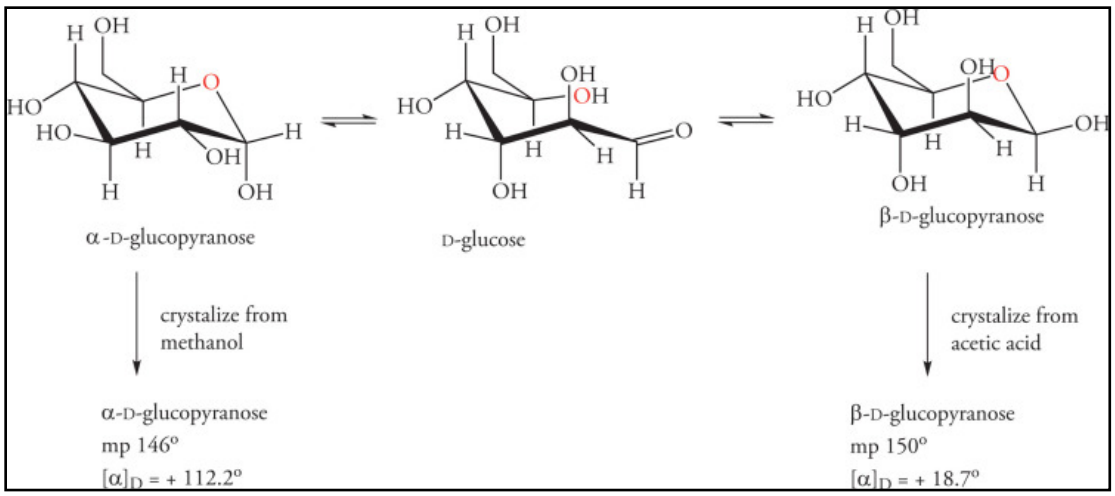


Figure 1.7 The figure shows the mutarotation of glucose anomers (Source: Mutarotation 2020).

1.5.3 Properties of Carbohydrates

General properties

- Carbohydrates serve as a source of stored energy, fuel, and metabolic intermediates.
- The structural framework of the genetic material, RNA and DNA, is made up of the sugars ribose and deoxyribose.
- Proteins and lipids involved in cell interactions are connected to carbohydrates.
- Cell walls of bacteria and plants are made of polysaccharides like cellulose.
- Organic molecules known as carbohydrates are aldehydes or ketones with many hydroxyl groups.

Physical Properties

- Optical Activity: It is the rotation of plane polarised light that produces (+) and (-) glucose.
- Steroisomerism: compounds with the same structural formula but different spatial arrangements. Example: In terms of the penultimate carbon atom, glucose contains two isomers. They are D- and L-glucose, respectively.
- Diastereoisomers: It is the configurational alterations of glucose's C2, C3, or C4. Mannose and galactose are examples.
- Annomerism - It is the spatial configuration with respect to the first carbon atom in aldoses and second carbon atom in ketoses.

Chemical Properties

- Osazone formation: Three moles of phenylhydrazine and one mole of aldose combine to form a crystalline substance which is known as phenylosazone (Scheme-1). Phenylosazones are helpful derivatives for identifying sugars since they crystallise easily (unlike sugars).

- Benedicts test: Simple carbohydrates can detect using Benedict's Test The Benedict's test finds reducing sugars (monosaccharides and some disaccharides) that include free ketone or aldehyde functional groups.
- Oxidation: Sugars are referred to as reducing sugars because they easily oxidise to form carboxylic acids. Because aldehydes contain an open C=O link, they are simpler to oxidise. Ketones, however, cannot be oxidised unless they first tautomerize to generate an aldose.
- Reduction to alcohols: By using Sodium borohydride (NaBH4), the carbonyl group in aldoses and ketoses can be converted to 1^o and 2^o alcohols, respectively. An *alditol*, a polyalcohol, is the end result of this process. In both arrangements, the reduction of the ketoses generates a new chiral centre.

1.5.4 Functions of Carbohydrates

Carbohydrates have a variety of functions such as:

- In many animals, carbohydrates are the principal source of energy and are a quick supply of power. The glycolysis and Kreb's cycle breaks down glucose to produce ATP.
- The source of energy storage is glucose. Animals store it as glycogen, while plants store it as starch.
- Stored carbohydrates act as energy source as an alternative of proteins.
- In the production of proteins and lipids, carbohydrates act as intermediaries.
- Carbohydrates combine with lipids and proteins to produce surface antigens, receptor molecules, vitamins, and antibiotics.
- Carbohydrates are also involved in detoxification, e.g. glucuronic acids.
- As in the cell walls of plants and microbes, they compose structural and defensive components.
- They are an essential component of connective tissues in mammals.
- Act as structural elements, such as chitin in insects, cellulose in plants, and glycosaminoglycans in humans.
- They take part in biological transport, cell-cell communication, and growth factor activation.
- Fiber-rich carbohydrates aid in preventing constipation.
- Additionally, they aid in immune system modulation.
- Non digestable carbohydrates like cellulose, be used as dietary fibres.
- Constituents of nucleic acid RNA and DNA. E.g. Ribose and deoxyribose sugar.

1.5.5 Example of Carbohydrates

- **Monosaccharides**: Erythrose, Fructose, Galactose, Glucose, Glycerose, Ribose, Ribulose,
- **Oligosaccharides:** Lactose, Maltose, Raffinose, Sucrose, Stachyose.

- **Polysaccharides:** The composition, occurrences and functions of some important polysaccharides are given below (Table 1.4):

Table 1.4 Examples of polysaccharides with occurrences, composition and functions.

Polysaccharide Name	Occurrence	Composition	Functions
Cellulose	Plant cell wall	Polymer of glucose	Cell wall matrix
Chitin	Bodywall of arthropods. In some fungi also	Polymer of glucose	Exoskeleton Impermeable to water
Glycogen	Animals	Polymer of glucose	Storage of reserve food
Gums and mucilages	Gums - bark or trees. Mucilages - flower	Polymers of sugars and sugar acids	Retain water in dry seasons
Hemicellouse	Plant cell wall	Polymer of pentoses and sugar acids	Cell wall matrix
Heparin	Closely related to chrondroitin	Connective tissue cells	Anticoagulant
Hyaluronic acid	Connective tissue matrix, Outer coat of mammalian eggs	Polymer of sugar acids	Ground substance, protection
Inulin	In roots and tubers	Polymer of fructose	Storage of reserve food
Lignin	Plant cell wall (dead cells like sclerenchyma)	Polymer of glucose	Cell wall matrix
Murein	Cell wall of prokaryotic cells	Polysaccharide cross linked with amino acids	Structural protection
Pectin	Plant cell wall	Polymer of galactose and its derivatives	Cell wall matrix

1.6 LIPIDS

Lipids are a heterogeneous group of organic molecules that are hydrophobic and are structurally made up of a chain of hydrocarbons ($–CH_2–CH_2–CH_2–CH_2–$). Typically, these are fats, oils, steroids, waxes, or substances that are similar. The term "Lipid" was coined in 1943 by German chemist Bloor (the Greek word "lipos" meaning "fat"). It refers to a diverse class of biomolecules relatively insoluble in water but soluble in organic solvents such as chloroform, ether and benzene. Chemically, lipids are more diverse than other biomolecules and comprise various types of esters of different alcohols. They are mainly involved in energy storage and membrane structure. Lipids can be categorised into a number of different groups. The majority of lipids serve as molecules that store energy or as parts of the structure of membranes. Some are also pigments, vitamins, and harmones.

1.6.1Classification of lipids

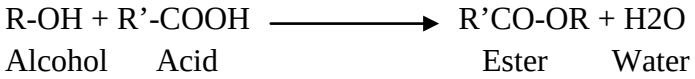
Henri Braconnot divided lipids into the two categories of solid grease and fluid oil for the first time in 1815. However, T. P. Hidlich, who split the simple lipids into grease and waxes, provided the correct categorization in 1947. Lipids can be classified in four ways, depending on chemical composition, fatty acids, requirements and sources.

1.6.1.1 Based on the Chemical Composition

On the basis of chemical composition lipids are mainly classified into three types: Simple, Complex, and Derived lipids.

1) Simple Lipids:

Simple lipids are esters of fatty acids with different alcohols. These are triglycerides, esters of fatty acids, and wax esters. The hydrolysis of these lipids gives glycerol and fatty acids. Neutral fats or Triacylglycerols are esters of fatty acids with glycerol, whereas waxes are esters of fatty acids (saturated or unsaturated) with high molecular weight.



A. Fatty acids

Fatty acids are the simplest form of lipids. They are a long chain of hydrocarbons (4 to 36 carbons long) with one carboxyl group (Kumar & Mina, 2016). Fatty acids are amphipathic, having both polar and nonpolar ends. The alkyl chains present in their structure can either be saturated or unsaturated (Kumar & Mina, 2016). Most naturally occurring fatty acids are composed of even number of carbon atoms since their biosynthesis requires a concatenation of C2 units. Most commonly found fatty acids have carbon atoms between 16 and 20. In higher plants and animals, half of the fatty acids are polyunsaturated. Predominantly found saturated fatty acids (Fig. 1.8) include Palmitic acid (C16), Stearic Acid (C18) and Arachidic acid (C20).

Based on the hydrocarbon, fatty acid chains are given systematic names The notation used to represent unsaturated fatty acids is C: n where C represents the number of carbon atoms and n represents the number of double bonds in the fatty acid. Numbering begins with the first carbon (carboxy terminal). The 2, 3, and 4 carbons next to the carboxyl carbon are referred to as **α, β** and **γ** carbons, respectively. If the hydrocarbon chain is monounsaturated, the unsaturated chain is given the suffix "enoic acid" after the name, while the saturated chain is given the suffix "anoic acid" or "anoate". In the unsaturated fatty acids, the double bonds are always found in the *cis* configuration. When there are many double bonds, as in polyunsaturated fatty acids, the prefix di, tri, etc. is used before the word "enoic acid." One or more double bonds can be found in unsaturated fatty acids (Fig. 1.8), as in Oleic acid (18:1) , Linoleic Acid (18:2), Linolenic acid (18:3) and Arachidonic Acid (20:4).

i) Monoenoic acids: Unsaturated fatty acid having a single double bond.
e.g., 18:1(Δ9) Oleic acid (C17H33-COOH)
CH3-(CH2)7-CH=CH-(CH2)7-COOH

ii) Dienoic acids: Unsaturated fatty acids having two double bonds
e.g., 18:2(Δ9, 12) Linoleic acid (C17H31-COOH)
CH3-(CH2)4-CH=CH-CH2-CH=CH-(CH2)7-COOH

iii) Trienoic acids: Unsaturated fatty acids having three double bonds
e.g., 18:3(Δ9, 12, 15) Linolenic acid (C17H29-COOH)
CH3-CH2-CH=CH-CH2-CH=CH-CH2-CH=CH-(CH2)7-COOH

iv) Polyenoic acid: Unsaturated fatty acids having two or more double bonds, otherwise known as polyunsaturated fatty acids (PUFA)
E.g. 20:4(Δ5, 8, 11, 14) Arachidonic acid (C19-H31-COOH)
CH3-(CH2)14-(CH=CH-CH2)3-CH=CH-(CH2)3-COOH.

A number of conventions utilize the symbol Δ to denote the number and position of double bonds in a hydrocarbon chain for example, 18:0 represents a fatty acid having 18 carbon long chain with no double bond, while18:1 (Δ9) represents a fatty acid having 18 carbon chain length and one double bond at position 9 and 18:2 (Δ9, 12) represents a fatty acid having 18 carbon chain length and two double bonds, one at position 9 and the other at 12 starting from the carboxylic end.

The Most Abundant Fatty Acids in Animal Fats, Vegetable Oils, and Biological Membranes

Saturated fatty acids			Unsaturated fatty acids		
Lauric	12C	$CH_3(CH_2)_{10}COOH$	Palmitoleic	16C:1	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$ *cis*
Myristic	14C	$CH_3(CH_2)_{12}COOH$	Oleic	18C:1	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$ *cis*
Palmitic	16C	$CH_3(CH_2)_{14}COOH$	Linoleic	18C:2	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$ **ω-6** *cis* *cis*
Stearic	18C	$CH_3(CH_2)_{16}COOH$	Linolenic	18C:3	$CH_3(CH_2CH{=}CH)_3(CH_2)_7COOH$ **ω-3** all *cis*
Arachidic	20C	$CH_3(CH_2)_{18}COOH$	Arachidonic	20C:4	$CH_3(CH_2)_4(CH{=}CHCH_2)_4CH_2CH_2COOH$ **ω-6** all *cis*

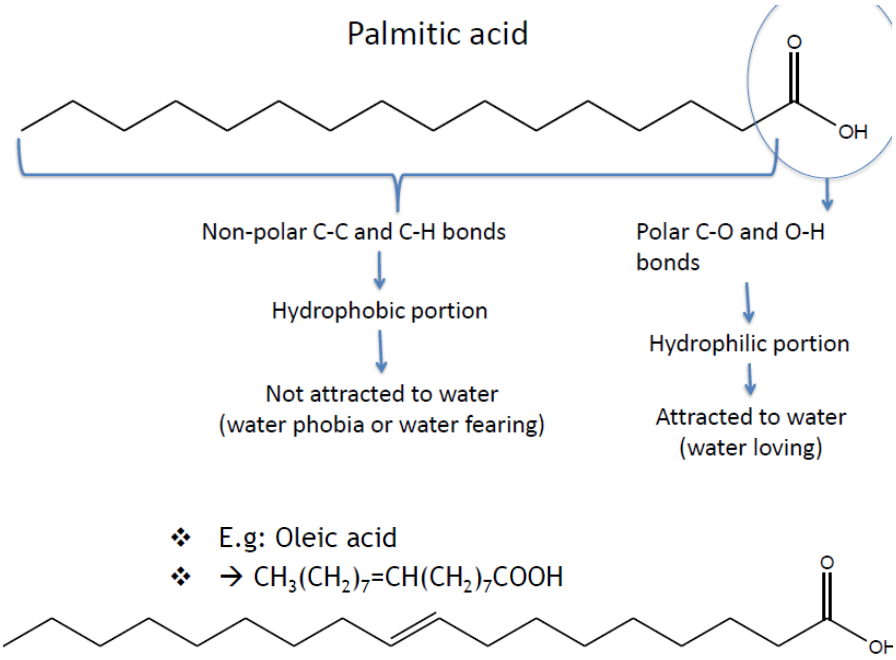


Figure 1.8 Saturated and unsaturated fatty acid

B. Triglycerides (TG) or Neutral fat

In both animals and plants, glycerides serve as the primary fatty acid storage form. Triacylglycerols are tri-esters of glycerol and fatty acids, often known as triglycerides. They have a hydrophobic character and are nonpolar. They are referred to as neutral lipids because they are charge-free. Triacylglycerol contains varying lengths of fatty acids that can be saturated or unsaturated. Triacylglycerols can be divided into two categories: simple and mixed. Simple triglycerides are those with just one type of fatty acid, while mixed triglycerides are those with two or more different types of fatty acids (Kumar & Mina, 2016).

Triacylglycerols are fatty acid esters of glycerol that are insoluble in water (Fig. 1.9). The three fatty acids that are esterified to the three alcoholic groups of glycerol give them their distinctive characteristics. They act as the cell's energy reserves, because lipids are less oxidised than carbohydrates and proteins, and more energy is released when the same amount of fat is oxidised, they are useful as energy reserves in the cell. Additionally, they are stored in anhydrous form because they are nonpolar, as opposed to carbohydrate polymers like glycogen that bind twice as much water.

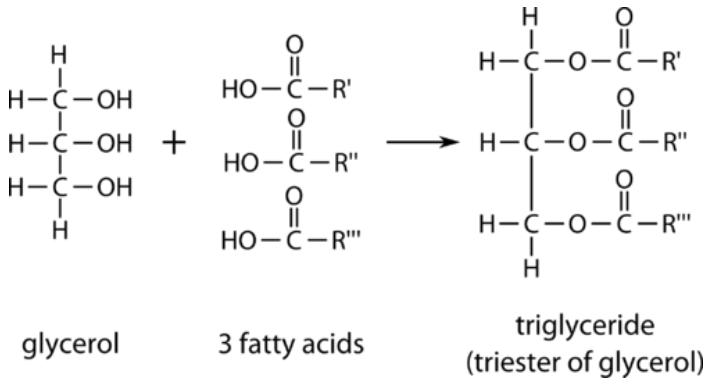


Figure 1.9 Triacylglycerols (fatty acid esters of glycerol)

C. Waxes

Waxes are long-chain fatty acid and long-chain alcohol esters (Fig. 1.10). They are totally insoluble in water and solid at normal temperature. They are synthesized through the esterification of high molecular weight monohydroxy alcohol with long-chain fatty acids. The popularly known beeswax contains triacontanyl palmitate as a major molecule. The hydrophobic nature of waxes allows them to function as water repellents on leaves of some plants, feathers, and cuticles of insects. They also act as energy reservoirs for higher aquatic organisms and planktons.

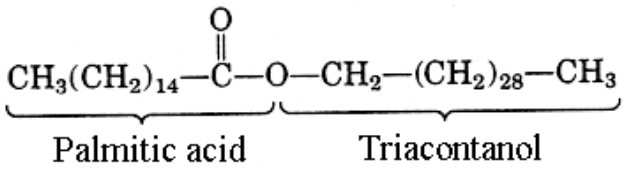


Figure 1.10 The molecular structure of beeswax

2) Complex Lipids:

The complex or compound lipids contain some other organic molecules in addition to fatty acids and glycerols. Phospholipids, glycolipids, and lipoproteins are some of them.

A) Phospholipids

The four elements that make up phospholipids are fatty acids, glycerol or sphingosine, phosphate, and alcohol bound to phosphate. It contains sphingophospholipids, ether glycerophospholipids or phosphoglycerides. These compounds have an amphipathic character.

i) Glycerophospholipids: Glycerophospholipids (also known as phosphoglycerides) contain glycerol, two fatty acid molecules, a phosphate group at C-3 and alcohol attached to it (Fig. 1.11). They are derivatives of phosphatidic Acid. They are the most abundant phospholipids found in the cell membrane, among all the other phospholipids. Phosphatidic acid is the most fundamental type of phosphoglyceride. The hydroxyl groups at the C1 and C2 carbons of glycerol are structurally esterifies with the carboxyl groups of two fatty acid chains, while the hydroxyl group at the C3 carbon is structurally esterifies with phosphoric acid. Phosphoglycerides frequently contain serine, ethanolamine, choline, glycerol, and inositol as their alcohol moieties.

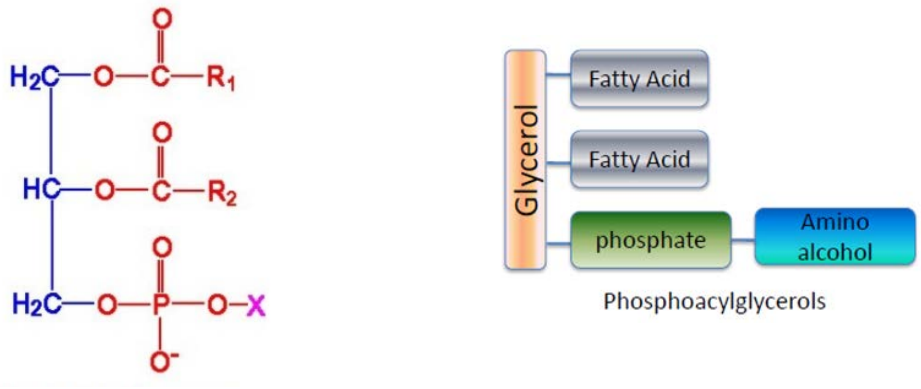


Figure 1.11 Structure of phosphoglycerides

ii) Sphingolipids: The majority of biological membranes are made up of sphingolipids. These lipids are derived from the C18 amino alcohols sphingosine and dihydrosphingosine. In a sphingolipid, the amino group at the C-2 position of sphingosine is connected to a fatty acid residue that can be saturated or monounsaturated with 16, 18, 22, or 24 carbon atoms via an amide bond. A phosphodiester or a glycosidic bond connects the C1 position to a polar head group. Its parent structure consists of ceramide, which is a fatty acid joined to sphingosine via an amide linkage. One example of sphingophospholipids is sphingomyelin which is a major constituent of the nervous system in higher animals. The backbone of sphingosine, the structure of ceramide, and a typical sphingolipid are illustrated in fig. 1.12.

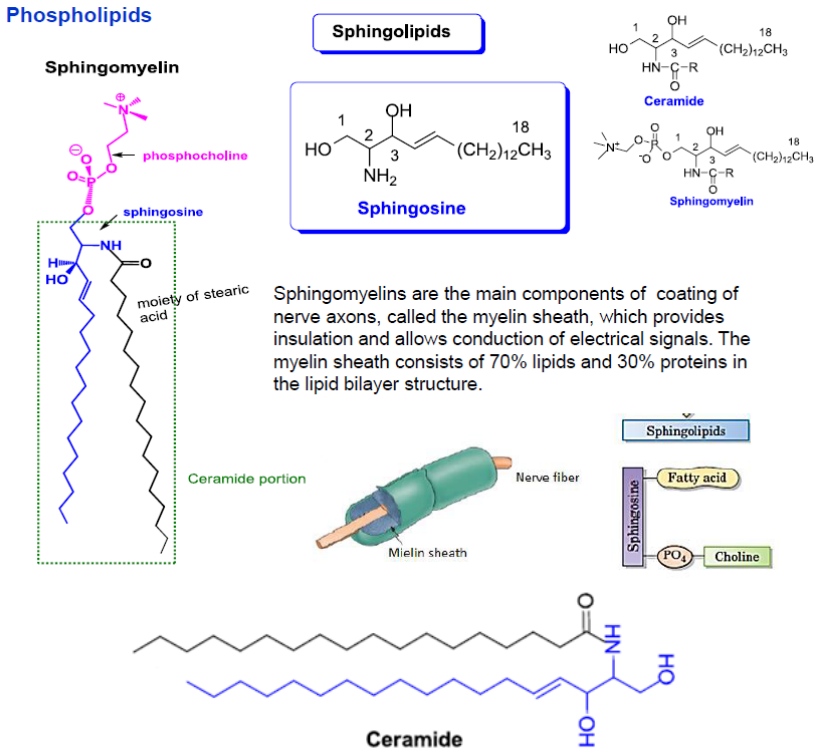
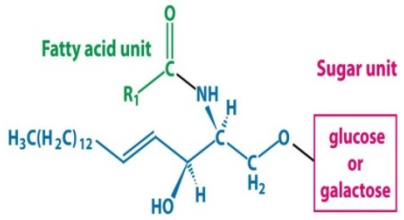


Figure 1.12 Example of Sphingolipids.

B. Glycolipids

Glycolipids are molecules in which the carbohydrate moiety is attached with glycerol and fatty acids. The head group of the molecule contains sugar (one or more) connected directly to the hydroxyl group at C1 of the ceramide moiety. It is the third major class of membrane lipids and generally found on the extracellular face of eukaryotic cellular membranes, and function to maintain stability of the membrane and to facilitate cell–cell interactions. Glycolipids can also act as receptors for viruses and other pathogens to enter cells. Some examples of glycolipids are cerebrosides, gangliosides, sulfolipids and lipoproteins (proteins covalently linked to lipids). Cerebroside that has a single sugar moiety attached to ceramide; globoside, having multiple sugar moiety attached to ceramide; and ganglioside, which is a globoside with the head group containing one or more residues of N-acetylneuraminic acid (sialic acid).



Cerebroside
(a glycolipid)

C. Lipoproteins

The lipid and protein complexes are known as lipoproteins. They support the movement of lipids produced in one organ to throughout the body, such as phospholipids, cholesterol, and cholesterol esters. Lipoproteins soluble in the blood are categorized into four groups based on their densities such as chylomicrons, Very Low-Density Lipoproteins (VLDL), Low-Density Lipoproteins (LDL) and High-Density Lipoproteins (HDL). High-density lipoprotein (HDL), often known as "good" cholesterol, and low-density lipoprotein (LDL), sometimes known as "bad" cholesterol, are the two primary subgroups of lipoproteins.

3) Derived Lipids

Derived lipids are the hydrolyzed forms of simple and complex lipids. Its include fatty acids, fatty aldehydes, ketone molecules, steroids, hormones and lipid-soluble vitamins.

A. Steroids

Steroids are cyclic lipids and complex derivatives of triterpenes. It consists of four fused rings called steroid nucleus known as ‘cyclopentanoperhydrophenanthrene’ (CPPP) nucleus (Fig. 1.13). Three of these rings have 6 carbons while the fourth ring has five carbons. It’s a planar ring system and relatively rigid. Any movement around the C-C bonds in this system is restricted. Cholesterol is weakly amphipathic in nature as it has a polar hydroxyl group attached to the 3rd carbon atom and a long non polar aliphatic chain attached to the 17th carbon atom. Cholesterol is the main sterol which serves as not only an important structural component of

biological cell membranes but is also a precursor to major steroid hormones in the body. Steroids regulate many important physiological processes in the body as well as carbohydrate metabolism.

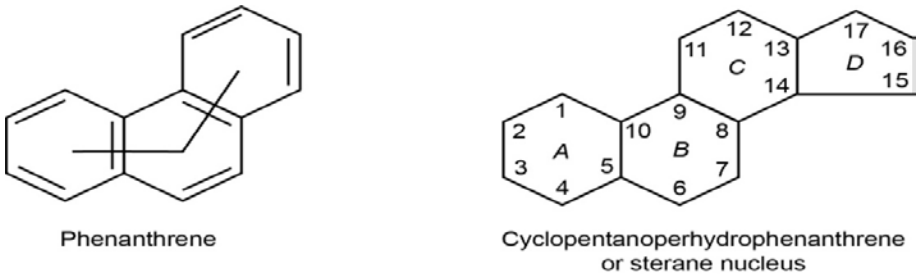


Figure 1.13 Parent compounds of steroids- A: Phenanthrene; B: Sterane

1.6.1.2 Based on Fatty Acids

Based on the type of fatty acids they contain, lipids are categories into two groups: saturated and unsaturated fatty acids.

A. Saturated Fatty Acids

There are no double or triple bonds in saturated fatty acids. They are a simple, unbranched, and linear chain of CH2 groups connected with a carbon-carbon single bond and one carboxylic acid at its end. In general, they have the formula CH3 - (CH2)n - COOH, where n is the number of methylene groups.Some examples of saturated fatty acids are lauric, myristic, palmitic, stearic, behenic, and lignoceric acids.

B. Unsaturated Fatty Acids

Unsaturated fatty acids have one or more double or triple bonds. Therefore, they can either be monounsaturated or polyunsaturated. Natural fatty acids often have a cis rather than a trans structure. Only a few fatty acids containing triple bonds are found in nature, and they are frequently derived from plants, such as stearolic acid. The unsaturated fatty acids are named referring to the number of carbons they contain with the suffix *-anoic* (for saturated fatty acids) and *-enoic* (for unsaturated fatty acids). For example, stearic acid contains 18 carbons and is named octadecanoic acid (18:0). Here, 18:0 refers to 18 carbon fatty acids with zero double bonds. Some examples of monounsaturated fatty acids are palmitoleic acid, oleic acid, gadoleic acid and some common polyunsaturated fatty acids include linoleic acid, linolenic acid, and arachidonic acid.

1.6.1.3 Based on Requirements by the Human Body

Essential and non-essential fatty acids are the two categories of lipids that are classified based on nutritional needs.

A. Essential Fatty Acids

The term "essential fatty acids" refers to fatty acids that our bodies are unable to generate or syntheses. These fatty acids need to be taken through a diet to fulfill the body's requirement for different metabolic functions. Some essential fatty acids are linoleic acid, linolenic acid, and arachidonic acid.

B. Non-essential Fatty Acids

Non-essential fatty acids include those lipids that are synthesized by our body. They are not needed to be taken through any outside food source. Example- palmitic acid, oleic acid, and butyric acid.

1.6.2 Properties of lipids

Physical Properties

- Lipids are relatively insoluble in water. They are soluble in non-polar solvents, like ether, chloroform, and methanol.
- Lipid molecules have no ionic charges
- Pure fats and oils are colorless, odorless, and tasteless.
- Lipids have a high energy content and burn calories during metabolism. They act as electrical insulators and protect nerve axons.
- Lipids are greasy in texture and stored in adipose tissues inside the body
- Lipids can either be present in saturated (having only single bonds) or unsaturated (having one or more double bonds) structural form.
- Fats contain saturated fatty acids, which are solid at room temperature such as animal fats. Plant fats are unsaturated and are liquid at room temperatures.
- The melting point of fats depends on the length of the chain of the constituent fatty acid and the degree of unsaturation.
- Geometric isomerism, also known as cis-trans isomerism, is a property of lipid molecules caused by the presence of a double bond in the unsaturated fatty acid.
- Fats have insulating properties and are poor heat conductors.
- Emulsification is the process by which a lipid mass is divided into numerous tiny lipid droplets. Before the fats can be absorbed by the intestinal walls, the process of emulsification takes place.

Chemical Properties

- **Saponification:** Saponification is the term for the hydrolysis of lipids (Triglycerides) by an alkali (NaOH/ KOH) or lipase enzymes. Glycerol and soap-like fatty acid salts are produced as a result of this reaction.
- **Hydrolysis of triglycerides:** Triglycerides (neutral lipids) on reacting with water form carboxylic acid and alcohol (Sagar, 2008)
- **Hydrogenation:** The breakage of double bonds occurs after the reaction of unsaturated fatty acids with hydrogen. This turns the molecules into saturated fatty acids.
- **Halogenation:** Free or combined fatty acids in the reaction with halogens gain double bonds and cause decolorization of halogen solutions.
- **Rancidity:** Oxidation and hydrolysis of fats and oil to generate a disagreeable odor – this is known as rancidity (Sagar, 2008).

1.6.3 Biological Significance of Lipids

Lipids being a significant biomolecule in organisms, serve a variety of functions. Here is a list of the major roles that lipids play in living things.

i) **Energy storage:** Triacylglycerols, often known as triglycerides, are an important source of energy for both plants and animals and are found in adipose tissues. The complete breakdown of fatty acids releases about 38 kJ/g (9 kcal/g) caloric content. The enzyme lipase regulates the lipid breakdown process within the body.

ii) **The structural component of the cell membrane:** The plasma membrane of cells is made of a lipid bilayer with proteins embedded in it. Glycerophospholipid molecules that are amphipathic make up the lipid bilayer. The glycolipids and phospholipids in the cell membrane serve as structural components of the membrane.

- There are also certain non-glyceride lipids in the cellular membrane, such as sphingomyelin and sterols, which are important for membrane flexibility.

iii) **Chemical Messenger**: Different kinds of lipids serve as cellular messengers or signalling molecules. They activate different signaling pathways either by binding with G-coupled receptors or nuclear receptors. The following are a few examples of lipid compounds with signalling roles:

- **Diacylglycerol and phosphatidylinositol phosphate:** They are involved in calcium-mediated activation of protein kinase C.
- **Estrogen, testosterone, and cortisol:** These are hormones that regulate a variety of processes, including as blood pressure, reproduction, and metabolism.
- **Prostaglandins:** It is an eicosanoid, involved in inflammation and immunity.
- **Phosphatidylserine:** It is involved in signaling phagocytosis of apoptotic cells by exposing themselves to the outer leaflet of the bilayer cell membrane.
- **Sphingosine-1-phosphate:** It's a potent messenger molecule, involved in calcium mobilizing regulations, cell growth, and apoptosis.

iv) **Other Functions**

- Fatty acid absorption: Phospholipids play an important role in the absorption and transportation of fatty acids.
- Lipids also play important role as pigments (carotene), hormones (vitamin D derivative and sex hormone), cofactors (vitamin K), and detergents (bile salt).
- Vitamin carriers: Natural fat-soluble vitamins including vitamin A, D, and E are carried by lipids.
- Lipids in the subcutaneous layer of the skin act as an insulator and safeguard against the cold. Additionally, fats have a role in regulating body temperature.
- Prostaglandins stimulate uterine contraction, lower blood pressure, vasodilation, inflammation, and pain.
- Thromboxanes function as vasoconstrictors and stimulate platelet aggregation.
- Prostacyclins act as antagonists of thromboxanes – it's a potent vasodilator.
- Leukotrienes play functional roles in chemotaxis, inflammation, and allergic reactions.

- Antibiotic agent: *Squalamine*, a steroid from the blood of sharks, has been shown to be an antibiotic and antifungal agent of intense activity. This seems to explain why sharks rarely contract infections and almost never get cancer.

1.7 PROTEINS

The term "protein" refers to a class of large biomolecules, or macromolecules, made up of one or more long chains of amino acid residues and they are known as building blocks of life. The most prevalent macromolecules inside of cells are proteins. They provide structural support, storage, transport, movement, cellular communications, and defense against foreign substances. Protein's function is highly governed by its stable structure. The stability of a protein's structure heavily influences how it works. There are four layers in this structure: primary, secondary, tertiary, and quaternary. The sequence of amino acids connected by peptide bonds makes up the primary structure. Secondary structure is the local folding of a part of polypeptide, while the tertiary structure is mixture of α-helix and β-sheets. Quaternary structure is the subunit composition of a protein. Proteins regulate and catalyze the body chemistry in the form of hormones, enzymes, immunoglobulin's etc. In this section, we will have deeper insights into the protein structure, classification and its importance.

1.7.1 Structure of Proteins

There are only 20 distinct amino acids that can be polymerized to produce linear chains to form proteins. Proteins are the polymers of L-α-amino acids. They are divided into four categories based on how they are structurally organized such as primary, secondary, tertiary, and quaternary (Fig. 1.14).

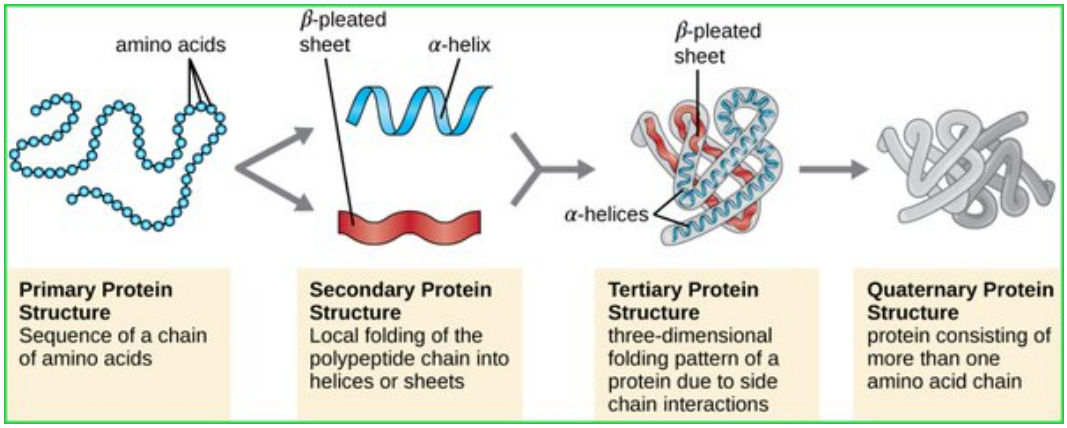


Figure 1.14 Four structure of a protein (Source: microbenotes.com/protein-structure)

A. Primary structure

The primary structure of a protein is formed by the formation of a peptide bond between amino acids. Each peptide has its own unique sequence of amino acids (Fig. 1.15). It is held together by covalent peptide bonds, which are formed during the process of protein biosynthesis or translation. Post-translational modifications such as disulfide formation, phosphorylations and

glycosylations are usually also considered a part of the primary structure. As with naming of peptides, the assignment of positions of the amino acids in the sequence starts at the N-terminal end. Examples of protein with a primary structure are *Hexosaminidase,* and *Dystrophin*.

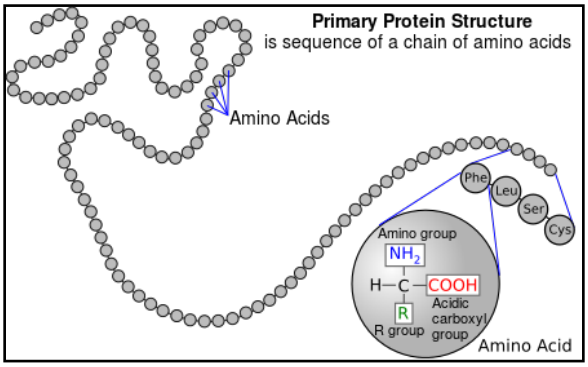


Figure 1.15 Primary structure of a protein (Source: commons.wikimedia.)

B. Secondary structure

Proteins can be arranged differently by twisting their polypeptide chains, which is known as secondary structure. It is a folded structure within a polypeptide that is due to the formation of hydrogen bonds between amide hydrogen and the carbonyl oxygen of the peptide backbone. The alpha helix and the beta sheet (Fig.1.16), first proposed by **Linus Pauling** in 1951, are the two primary kinds of secondary structure seen in proteins. *Myoglobin* is an illustration of a protein with a secondary structure.

- **α-helix:** The most common type of secondary structure of a protein is the α-helix exists in hair protein, keratin. Various conformations can be assumed for a protein by rotation around single bonds and rigid peptide bonds. The simplest arrangement of a polypeptide chain is α-helix. The polypeptide coils around an imaginary axis with side groups protruding out from the helix. The single turn of the helix is 5.4 Angstrom which is the repeating unit of the α-helix. Every winding turn in an alpha helix has 3.6 amino acids residues. In α-helix protein, a hydrogen bond is formed between the N−H group to the C=O group of the amino acid. The alkyl groups of the alpha-helix chain are not involved in the H bonds but maintain the alpha-helix structure. Intra-hydrogen bonding stabilizes the α-helix. This bond forms between the first amino acid and the fourth amino acid. The charge of the side chains can destabilize the helix. All the amino acid side chains point outward from the helix.
- **β-sheet:** The β-sheet is zig-zag extended conformation of a polypeptide. Three to ten amino acids are combined to create a beta-strand polypeptide. Beta sheets are involved in forming the fibrils and protein aggregates observed in amyloidosis. Alike alpha-helix, the residue hydrogen bond between the adjacent strands is separate from each other. In this case, the orderly alignment of protein chains is maintained by intermolecular or intramolecular hydrogen bonds. The β-sheet structure can occur between molecules when polypeptide chains run parallel (all N-terminal ends on one side) or antiparallel (neighboring N-terminal ends on opposite sides). β-Pleated sheets can also occur intramolecularly, when the polypeptide chain makes a U-turn, forming a hairpin structure, and the pleated sheet is antiparallel. In all secondary structures, the

hydrogen bonding is between backbone –C=O and H-N-groups. Differences between α-Helix and β-sheet type of secondary structure of Protein are given below:

Table 1.5 Differences between α-Helix and β-sheet type of secondary structure of Protein

α-Helix	β-Sheet
Amino acids exist in the right-handed coiled rod-like structure.	Amino acids exist in an almost entirely extended conformation, i.e. linear or sheet-like structure.
Intramolecular hydrogen bonding forms within the polypeptide chain to create a spiral structure.	β-sheets are formed by linking two or more beta strands by intermolecular hydrogen bonds.
3.6 amino acid residues are winded to form an α-helix polypeptide.	Three to ten amino acids are combined to form a β-strand polypeptide.
α-helix can be a single chain polypeptide.	β-sheets cannot be in a single chain Polypeptide. There must be two or more beta-strands.
Alkyl groups of α-helix are oriented outside of the helix.	Alkyl groups are oriented both inside and outside of the sheet.
Example: Myoglobin, Haemoglobin and Keratin.	Example: Skin Fibres or Fibroin.

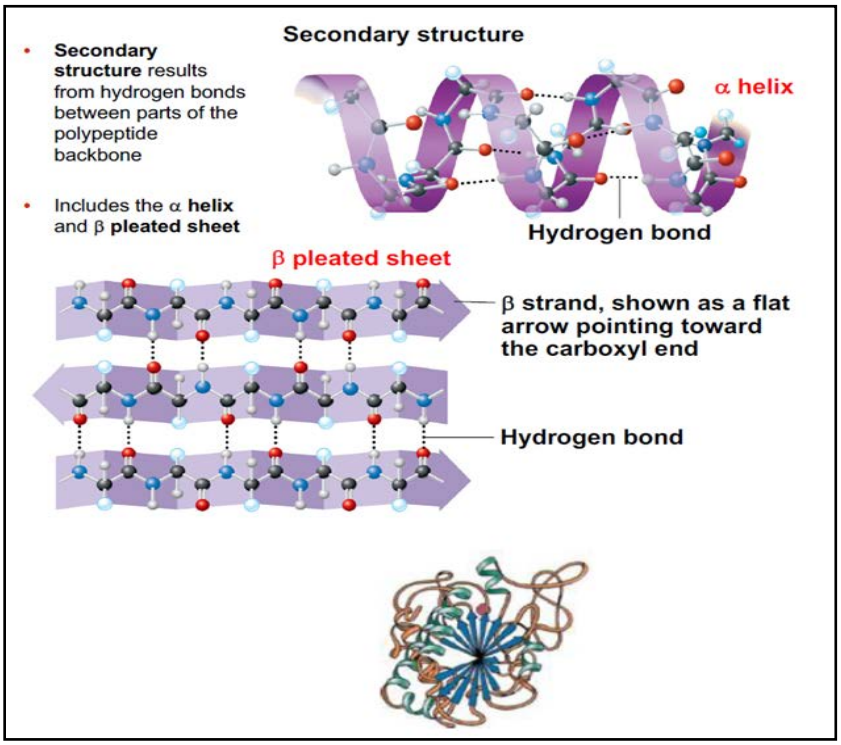


Figure 1.16 Enzyme Carboxypeptidase. The β-pleated sheet portions are shown in blue, the green structures are the a-helix portions, and the orange strings are the random coil areas (Source: http://thebiologs.blogspot.com/2016/09/cape-1-proteins.html)

C. **Tertiary structure**

It is a three-dimensional conformation that is formed due to the interaction between R-groups or side chains of the amino acids that make up the proteins. There are a variety of forces at work to stabilize the polypeptide chain in this final structure, including electrostatic attraction, disulfide bonds, hydrogen bonds, and ionic interactions (Salt bridges). The disulfide bond is the covalent bond that is most frequently engaged in stabilizing the tertiary structure of proteins. Tertiary structures are stabilized by hydrogen bonding between polar groups on side chains or between side chains and the peptide backbone; electrostatic attractions also called Salt bridges, occur between two amino acids with ionized side chains. The nonpolar groups prefer to interact with each other, excluding water from inward regions (Fig.1.17). When the polypeptides fold in spherical shape, they are called globular proteins while fibrous proteins have extended conformation. Globular proteins (Enzymes) and fibrous proteins are example of proteins with a tertiary structure.

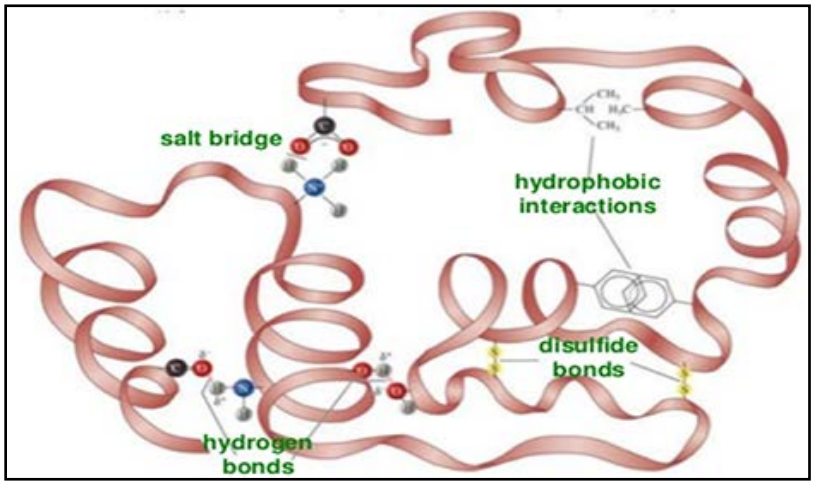


Figure 1.17 Tertiary structure of Protein (Source: creative-proteomics.com)

D. Quaternary structure

Quaternary structure forms between two or more polypeptide chains. Each polypeptide chain is called a subunit. The structures can exist between polypeptide chains that are similar or dissimilar. Hydrophobic, electrostatic, hydrogen, and covalent cross-links are some of the bonds that contribute to the formation of these structures. Hemoglobin, DNA polymerase, and ion channels are a few examples of proteins possessing quaternary structures.

Hemoglobin in adult humans is made of four chains (called globins): two identical α-chains of 141 amino acid residues each and two identical β-chains of 146 residues each. Hemoglobin, the oxygen carrier of blood, has a quaternary structure of 4 polypeptides: 2α and 2β and hence α2β2. α and β polypeptides resemble the myoglobin structure. In hemoglobin, each globin chain surrounds an iron-containing heme unit. Proteins that contain non-amino acid portions are called conjugated proteins. Prosthetic groups are the non-amino acids that make up a conjugated protein. The globins in hemoglobin are the sections of amino acids, while the heme units are the prosthetic groups (Fig. 1.18).

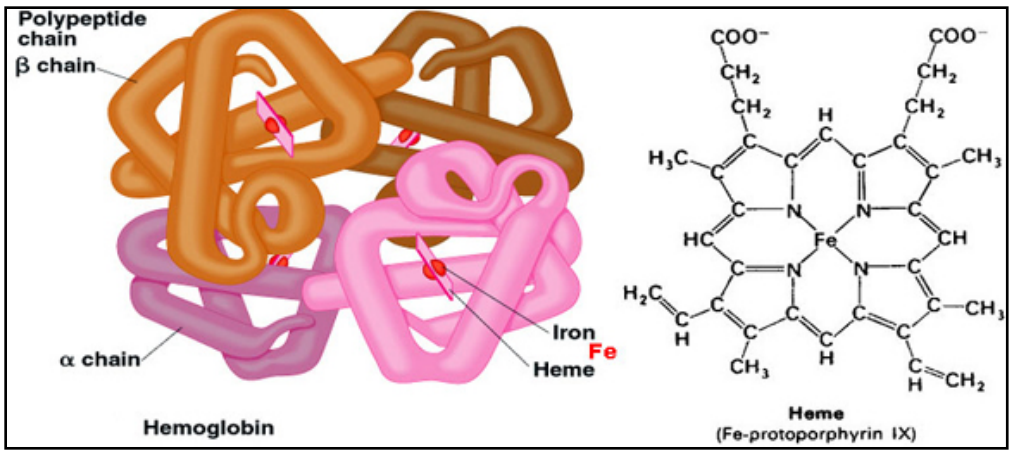


Figure 1.18 Quaternary structure of hemoglobin (Source: www.toppr.com)

1.7.2 Classification of Proteins

On the basis of their form, composition, and solubility, as well as their biological roles, proteins are divided into the following three categories.

1. Classification of Proteins Based on Shape

i) Globular or Corpuscular Proteins: The shape of globular proteins is primarily spherical or ovoid and they are compactly folded and coil-folded. They are usually soluble in water. Examples are insulin, plasma albumin, and globular enzymes.

ii) Fibrous or Fibrillar Proteins: These proteins have an axial ratio greater than 10, which gives them the appearance of long fibres or ribbons. They are primarily present in animals and aren't soluble in water or diluted acid solutions. Fibrous proteins provide structural support and protection. For instance, keratin, fibroin, collagen, and elastins.

Axial ratio, which is used to describe any structure or shape with two or more axes. It is the ratio of the lengths (or magnitudes) of the axes to one another, or the longer axis divided by the shorter axis.

2. Classification of Proteins Based on Composition and Solubility

i) Simple Proteins

These proteins are composed only one type of amino acid as a structural component, and when they are broken down by acids, the constituent amino acids are released. They are mostly globular type of proteins except for scleroproteins, which are fibrous in nature. In terms of solubility, simple proteins are further divided.

a) Protamines and histones: They have a low molecular weight, a simple structure, water soluble, and do not coagulate by heat. They are strongly basic in character due to the high content of lysine, arginine. They are fundamental proteins that are only found in animals. Example: Protamines- clupine, cyprinine, salmine; Histones - globin and nucleoshistones.

b) Albumins: They are distributed widely in nature and are mostly seen in seeds. They can dissolve in both water and diluted solutions of salts, acids, and bases. Example: Leucosine, legumeline, serum albumin.

c) Globulins: They can be divided into two categories: Pseudoglobulins which are soluble in water and euglobulins which are insoluble in water. They are coagulated by heat. Example: Pseudoglobulin, serum globulin, glycinine etc.

d) Scleroproteins or Albuminoids: These are typically found in animals and are also referred to as animal skeleton proteins. They cannot be dissolved in water or diluted solutions of acids, bases, or salts.

ii. Complex or Conjugated Proteins or Heteroproteins

These are proteins that are produced from amino acids and other chemicals. These chemical elements or non-protein compounds attached to simple proteins are referred to as prosthetic groups. The prosthetic group may be either a metal or a compound. A protein without its prosthetic groups is called as apoprotein while, a protein molecule combined with its prosthetic group is called holoprotein. Depending on the type of prosthetic group present, complex proteins are further classified.

a) Metalloproteins: These are proteins that are connected to various metals. Example: Casein, collagen, ceruloplasmin, etc.

b) Chromoproteins: These are proteins that are joined to a coloured pigment. Example: Myoglubin, hemocyanin, cytochromes, flavoproteins, etc.

c) Glycoproteins and Mucoproteins: These proteins have carbohydrates as the prosthetic group. Example: Glycoproteins- Egg Albumin, serum globulins, serum albumins; Mucoproteins - Ovomucoid, mucin etc.

d) Phosphoproteins: These proteins are linked with phosphoric acid. Example: Casein.

e) Lipoproteins: Proteins forming complexes with lipids are lipoproteins. Example: Lipovitellin, lipoproteins of blood.

f) Nucleoproteins: These are compounds containing nucleic acids and proteins. Example: Nucleoproteins, nucleohistones, nuclein.

g) Derived Proteins: These proteins are synthesised from simple proteins by the use of heat, enzymes, or chemical reagents; they are not naturally occurring proteins. There are two categories of derived proteins: primary derived proteins and secondary derived proteins.

- **Primary derived proteins:** These are protein derivatives in which the size of the protein molecule has not been significantly changed. Primary derived proteins are classified into three types - Proteans, Infraproteins and Coagulated proteins. Example: Edestan, coagulated egg-white.
- **Secondary derived proteins:** Secondary derived proteins are those that are created by excessively altering the structure and characteristics of protein molecules. The peptide bonds gradually break down through hydrolysis. They are further classified into 3 types - Proteoses, Peptones and Polypeptides

3. Classification of Proteins on Biological Function

i) Enzymic Proteins: They are the most diverse and highly specialised catalytic proteins. Example: Urease, catalase, cytochrome C, etc.
ii) Structural Proteins: These proteins support in strengthening or protecting biological structures. Example: Collagen, elastin, keratin, etc.
iii) Transport or Carrier Proteins: These proteins support the movement of ions and molecules throughout the body. Example: Myoglobin, hemoglobin, etc.
iv) Nutrient and Storage Proteins: These proteins provide nutrition to growing embryos and store ions.
v) Contractile or Motile Proteins: These proteins function in the contractile system. Example: Actin, myosin, tubulin, etc.
vi) Defense Proteins: These proteins defend against other organisms. **Example:** Antibodies, Fibrinogen, thrombin.
vii) Regulatory Proteins: They regulate cellular or metabolic activities. **Example:** Insulin, G proteins, etc.
viii) Toxic Proteins: These proteins hydrolyze or degrade enzymes. **Example:** Snake venom, Ricin.

1.7.3 Properties of *Proteins*

General properties

- The most significant biomolecules are proteins, which are also the building blocks of a cell's cytoplasm. They are organic substances made up of nitrogen and also, oxygen, carbon and hydrogen.
- Proteins are the structural elements of body tissues and are made up of amino acids.
- Proteins are referred to as the "bricks" of the body since they form the bones, muscles, hair, and other tissues.
- A protein's molecular weight ranges from 5 to 300 kilo-Daltons.
- Proteins help the body produce and repair tissues while also providing heat and energy.
- Proteins, like enzymes, are useful substances that participate in metabolic processes.
- Proteins are also used to create antibodies and blood haemoglobin.

Physical Properties

- The shapes of proteins can range from short fibrillar structures to simple crystalloid forms.
- Proteins can be arranged in two different ways: as globular proteins or fibrous or fibrillar proteins. The spherical proteins known as globular proteins are found in plants. Fibrillar proteins are thread-like, they occur generally in animals.
- Proteins are flavourless and colourless, and they have a very large size, which results in v arious colloidal features.
- Proteins often have high molecular weights between 5 X 103 and 1 X 106.
- They are homogeneous and crystalline.
- Proteins diffuse very slowly and exhibit the Tyndall effect.

- Proteins tend to change their properties like denaturation. Coagulation frequently occurs after denaturation. Physical or chemical agents may cause denaturation. Chemical agents are things like X-rays, radioactive radiation, and ultrasonic waves, while physical agents include things like shaking, freezing, heating, etc.
- Proteins, like amino acids, have an amphoteric characteristic, meaning they can behave as both acids and alkalies. Since the proteins are amphoteric by nature, depending on the net charge, they can form salts with both cations and anions.
- The solubility of proteins depends upon the pH. Lowest solubility is seen at isoelectric point, the solubility increases with increase in acidity or alkalinity.
- All the proteins show the plane of polarized light to the left, i.e., laevorotatory.

Chemical Properties

- Amino acids are produced as their hydrochlorides when proteins are hydrolyzed by acidic substances such as concentrated HCl.
- Proteins when are hydrolyzed with alkaline agents leads to hydrolysis of certain amino acids like arginine, cysteine, serine, etc., also the optical activity of the amino acids is lost.
- Proteins with reaction with alcohols give its corresponding esters. This process is known as esterification.
- Amino acid reacts with amines to form amides.
- When free amino acids or proteins are said to react with mineral acids like HCl, the acid salts are formed.
- Xanthoproteic test: When proteins are boiled with concentrated HNO3, a yellow colour appears because to the presence of the benzene ring.
- Folin's test is a particular test for tyrosine amino acid, where blue colour develops with phosphomolybdotungstic acid in alkaline solution due to presence of phenol group.

1.7.4 Function of Proteins

Proteins are vital parts of all living things. It takes part in practically all cellular processes. Cell signalling, metabolic reaction catalysis, cellular and tissue structure formation, DNA replication, and molecule transport are all activities in which it participates. Based on their functional characteristics, proteins are divided into the following classes.

1. **Enzymes:** The term "biological catalysts" refers to these globular conjugated proteins. Some proteins act enzymes, they catalyze metabolic reactions by reducing the activation energy that increases the rate of the reaction. Some examples of protein enzymes are DNA polymerase, Lysozyme, Nnitrogenase, Lipase, Pepsin and Tripsin.
2. **Hormones:** - Proteins can act as hormones and control a variety of bodily processes. These are long polypeptides made up of long chains of linked amino acids. They are important regulators of the body's physiological functions, such as those related to reproduction, growth and development, electrolyte balance, sleep, etc. Some examples of these hormones

are growth hormone (GH), follicle-stimulating hormone (FSH), Insulin hormone is a protein and it regulated the blood sugar level.

3. **Structural proteins:** These proteins are rigid and insoluble in water; they are fibrous proteins. They form the structural component of connective tissues, bones, tendons, cartilages, nails, hairs, and horns. Examples of structural proteins are Collagen, Elastin, and Keratin.
4. **Respiratory pigments:** These pigments are globular proteins, typically soluble in water. Examples include haemoglobin, which carries blood to all tissues and organs through the blood, and myoglobin, which delivers oxygen to the working muscles.
5. **Transport proteins:** Proteins transport different substances in blood of different tissues. Example: Haemoglobin is an oxygen transport protein. These are structural components of the cell membrane. They form channels in the plasma membrane to transfer selective molecules inside the cells. Some of them also form components of blood and lymph in animals. Examples of transport proteins are Serum Albumin, which transport hemin and fatty acids; Channel proteins; Carrier proteins and Fibrinogen (a glycoprotein helps in healing of wounds. It prevents blood loss and inhibits passage of germs).
6. **Motor proteins:** These proteins are involved in the contraction and relaxation of the muscle (muscle movement). It includes Actin, Myosin, Kinesin, and Dynein.
7. **Storage proteins:** These proteins are the storage reserve of amino acids and metal ions in cells. They are present in eggs, seeds, and pulses. Examples of storage proteins include Ferritin, Ovalbumin, and Casein.
8. **Toxins:** These proteins are generally produced by bacteria. They include Diphtheria toxin, Pseudomonas exotoxin, and ribosome-inactivating proteins. They help bacteria to attack and kill their host organism by creating cytotoxicity.
9. **Defense protein:** Some proteins act as antibodies; they protect the body from the effect of invading species or substances.

1.8 NUCLEIC ACIDS

The nucleic acid was first discovered by Friedrich Miesher, a Swiss physician in 1869 isolated and identified a macromolecular substance from the pus cells in the nuclei of leukocytes and from salmon sperm which he termed as nuclein. Meischer's student Richard Altmann in 1899 used the term nucleic acid for phosphorus containing nuclein, which was later proved to be the hereditary material in 1950s. Later, further studies showed that it's a mixture of basic proteins and phosphorus-containing organic acid (Kumar et al., 2016).

Nucleic acids are long-chain polymeric molecules. The monomer or the repeating unit is known as the nucleotides and hence sometimes nucleic acids are referred to as polynucleotides. Nucleic acids can be defined as organic molecules present in living cells. It plays a key factor in transferring genetic information from one generation to the next. Nucleic acids are composed of DNA-deoxyribonucleic acid and RNA-ribonucleic acid that form the polymers of nucleotides. You will study in detail about nucleic acids in Unit 02.

1.8.1 Structure of Nucleic acids

Structurally, nucleic acids (both DNA and RNA) are polymers of nucleotides (or polynucleotides) composed of pentose sugar, phosphate and nitrogenous bases. Pyrimidines and Purines are two types of nitrogenous bases. Pyrimidines are composed of cytosine and thymine. Purines are composed of guanine and adenine. Thymine is replaced by Uracil in ribonucleic acid whereas deoxyribonucleic acid comprises of all four bases. Sugar is deoxyribose in DNA (deoxyribonucleic acid) while ribose in RNA (ribonucleic acid). Deoxyribose means the 2' carbon of sugar lacks the oxygen atom which is present in ribose. Numerals with a prime (2' or 3') distinguish atoms of the sugar from those of the bases. Structure of Ribose and deoxyribose (pentose sugar) Sugars (Fig. 1.19) in the nucleic acid backbone are linked together by phosphodiester –bond/linkages/bridges. Phosphate group is attached to C-5 of pentose. During the formation of a phosphodiester bond, the C-5 of one sugar moiety is joined to the OH of C-3 with phosphate group (phosphate attached to C-5 of pentose). These phosphodiester bonds are strong covalent bonds and do not break even on heating the DNA molecule at high temperature.

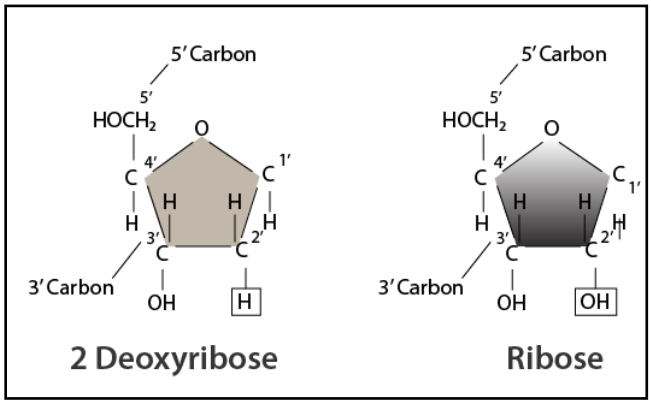


Figure 1.19 Structure of Ribose and Deoxyribose (pentose sugar) Sugars

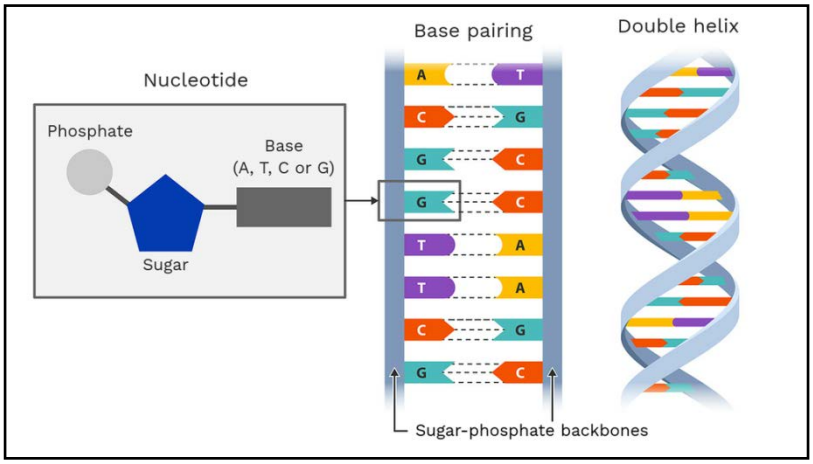
1.8.1.1 Types of Nucleic Acids and Their Functions

Based on nature, structure, and function, the nucleic acids are categorized into two groups: Deoxyribonucleic acids (DNA) and Ribonucleic acids (RNA).

A) Deoxyribo Nucleic acids (DNA)

DNAs are the hereditary material that resides inside the nucleus. In 1953, the first structure of DNA double helix (B-form of DNA) was discovered by Watson and Crick (Fig. 1.20). DNA has two other forms as well, A and Z forms. The conformation DNA will adopt depends on the hydration level, DNA sequence, and chemical modification of the bases, the type, and concentration of a metal ion in the solution. The double helix structure represents two polynucleotides DNA coiled around a central helix. The two strands are antiparallel and interact by hydrogen bonds between complementary base pairs. In some cases, like at low pH, the triple helix form of DNA also exists. It's formed by laying a third strand into the major groove of the DNA.

It is the genetic material that stores all the information required to be transferred to the progeny. It specifies the biological development of all living organisms and viruses.



Are you aware? Compared to DNA duplexes, RNA duplexes are more stable. RNA duplexes need a greater temperature to denaturate than DNA duplexes do at physiological pH. However, the physical cause of this distinction is still unknown (Kumar et al., 2016).

Figure 1.20 Double helical structure of DNA (B-form) (Source: theory.labster.com)

Do you know?It is believed that, around 4 billion years ago, RNA was the first genetic material! Scientists say it is largely because of its self-replicating ability and enzymatic activity. This hypothetical period is known as the RNA world. But when the protein-forming enzymes came into existence, DNA became the most dominating and stable form of genetic material. The DNA structure is more stable than RNA because of the absence of a 2' hydroxyl group. The other advantage DNA has is that its double-stranded structure allows for the correction of mutations as well (Kumar et al., 2016).

B) Ribonucleic acids (RNA)

RNA is present in all living cells. It has different roles to play in different organisms. It acts as genetic material in some viruses and has enzymatic activity in other organisms (where it is called *ribozyme*). Three types of RNA are present among organisms: rRNA, mRNA, and tRNA. All three have essential roles in the development and maintenance of life.

The importance of RNA and DNA is incomparable. DNA carrying the genetic information can't leave its home, the nucleus, and this is why RNA exists. They are involved in the transfer of genetic information for protein synthesis via the processes of transcription and translation (outside the nucleus), and they control gene

Do you know? It is estimated that approximately 4 billion years ago, RNA was the first genetic material! Scientists say it is largely because of its self-replicating ability and enzymatic activity. This hypothetical period is known as the RNA world. But when the protein-forming enzymes came into existence, DNA became the most dominating and stable form of genetic material. The DNA structure is more stable than RNA because of the absence of a 2' hydroxyl group. The other advantage DNA has is that its double-stranded structure allows for the correction of mutations as well (Kumar et al., 2016).

expression as well (Kumar et al., 2016). Structurally, RNA exists in both single-stranded (primary structure) and double-stranded (secondary structure) forms. The double-helical structure of RNA is present in the A form.

1.8.2 Functions of Nucleic Acids

- Nucleic acid is responsible for the transmission of inherent characters from parent to offspring.
- DNA fingerprinting is a method used by forensic experts to determine paternity. It is also used for the identification of criminals. It has also played a major role in studies regarding biological evolution and genetics.
- The major function of nucleic acids is to store the genetic code of living organisms. DNA reserves genetic information and is responsible for maintaining the identity of species over the centuries.
- They are responsible for the synthesis of protein, where RNA functions as an adapter molecule. RNA facilitates the translation of protein from DNA. In the process of protein synthesis, mRNA copies DNA and carries the information to rRNA, where rRNA decodes the information. tRNA takes amino acids to rRNA, where the protein is formed.
- Cellular metabolism is a function of DNA, where it integrates a complex set of biochemical pathways devoted to the maintenance of cell functions.

1.9 SUMMARY

- A Biomolecule, is a broad term for molecules found in organisms that are necessary for one or more distinctive biological processes, such as cell division, morphogenesis, or development. Large macromolecules i.e. proteins, carbohydrates, lipids, and nucleic acids and small molecules such as primary metabolites, secondary metabolites, and natural products.
- Based on molecular weight and solubility, biomolecules are divided into two categories: micromolecules and macromolecules.
- The carbohydrates are poly functional organic compounds, it comprises of only oxygen, carbon and hydrogen. Its contain functional group Alcoholic hydroxy group (-OH), Aldehyde group (-CHO) and Ketone (>C=O). Originally it referred to compounds of general formula Cn(H2O)n.
- The carbohydrates are divided into three major classes depending on the number of monomer units such as Monosaccharides, Oligosaccharides and Polysaccharides. Glucose is the main carbohydrate.
- The maltose, sucrose and lactose are common examples of naturally occurring disaccharides. Starch and glycogen are polysaccharides of glucose in plants and animals.
- Wedge-and-dash structures and Fischer projections are frequently used to illustrate the chemical structures of carbohydrates. Fischer's projection is used to distinguish between D and L carbohydrates.

- Glycosidic linkages combine monosaccharides or longer sugar chains with other carbohydrates to make disaccharides, oligosaccharides, and polysaccharides. It is a specific kind of covalent bond.
- Carbohydrates are the principal source of energy and are a quick supply of power. The Glycolysis and Kreb's cycle breaks down glucose to produce ATP.
- Lipids are a heterogeneous group of organic molecules that are hydrophobic and are structurally made up of a chain of hydrocarbons (–CH2–CH2–CH2–CH2–). Typically, these are fats, oils, steroids, waxes, or substances that are similar.
- On the basis of chemical composition lipids are mainly classified into three types: Simple, Complex, and Derived lipids.
- Simple lipids are esters of long chain fatty acids and alcohol. They include fats, oils and waxes. Compound lipids are made up by further modification of simple lipids such as addition of phosphate, sulfate and carbohydrate or protein group. This class of lipids includes phospholipids, glycolipids and lipoproteins.
- Derived lipids are derived from hydrolysis of simple and compound lipids. Steroids, cholesterol, various lipid soluble hormones as well as fatty acids belong to this class of lipids.
- The term protein refers to a class of macromolecules, made up of one or more long chains of amino acid residues and they are known as building blocks of life.
- There are only 20 distinct amino acids that can be polymerized to produce linear chains to form proteins. Proteins are the polymers of L-α-amino acids. They are divided into four categories based on how they are structurally organized such as primary, secondary, tertiary and quaternary.
- Amino acids are joined together by peptide bonds to form the basic structure of proteins. Proteins participate in almost every biochemical process in the body.
- Nucleic acids are one of the major biomolecules required for the proper functioning of the body. These are the molecules responsible for carrying the genetic information from parents to offspring, gene expression, and synthesis of proteins required for metabolic functions.

1.10 SELF ASSESSMENT QUESTIONS

1.10.1 Multiple Choice Questions

1. There are _____ major classes of biomolecules.
   a) Four b) Six
   c) Three d) Two
2. Biomolecules are very large molecules of many atoms, which are bound together from
   a) Ionicaly b) Non-covalently
   c) Covalenty d) None of the above
3. Lipids are insoluble in water because lipid molecules are
   a) Hydrophobic b) Hydrophilic

c) Neutral d) Both a & b

4. In double helix of DNA, the two strands are
   a) Coiled over base b) Coiled around each other
   c) Coiled around a common axis d) Coiled differently
5. Nucleotides are building blocks of nucleic acids, it is composition of
   a) Base, Sugar, and Phosphate b) Base and Phosphate
   c) Base, Phosphate, and carbon d) Sugar and phosphate
6. ATP is_____
   a) Types of nucleotide b) Types of nucleic acid
   c) Types of enzyme d) Both a & b
7. Amino acids are mostly synthesized from
   a) Fatty acids b) α-ketoacids
   c) Proteins d) None of the above
8. In which of the following groups are all polysaccharides?
   a) Glycogen, Cellulose and Starch
   b) Glycogen, Sucrose and Starch
   c) Glycogen, Cellulose and Fructose
   d) Glycogen, Cellulose and Glucose
9. Sucrose is also called as ____
   a) Milk sugar b) Invert sugar
   c) Laevose d) Dextrose
10. Linoleic acid and α-linolenic acid are types of
   a) Essential fatty acids b) Simple lipids
   c) Complex lipids d) Both a & b
11. DNA polymerase, and ion channels are example of_____protein structure.
   a) Tertiary b) Secondary
   c) Primary d) Quaternary
12. Which of the following are fibrous proteins?
   a) Insulin and Albumin b) Salmine and Clupine
   c) Fibroin and Elastins d) Casein and Myoglubin
13. Polysaccharides are formed by _____
   a) Vanderwaal forces b) Phosphodiester linkage
   c) Glycosidic linkages d) Peptide linkage
14. In RNA, thymine is replaced by
   a) Cytosine b) Adenine
   c) Uracil d) Guanine
15. Glycoside bond are absent in
   a) Monosaccharaides b) Oligisaccharides
   c) Polysaccharides d) Both b & c

1.10.2 Fill in the Blanks

a) The alpha helix and the beta sheet first proposed by …… in 1951.

b) A glucose molecule has four chiral carbons so that glucose molecule will have a total of ……stereoisomers.

c) Palmitic acid, Stearic Acid and Arachidic acid are examples of saturated fatty.

d) Serum albumin is the example of…… proteins.

e) The double helix structure represents..……..polynucleotides DNA coiled around a central helix.

f) The disulfide bond is the covalent bond that is most frequently engaged in …….. the tertiary structure of proteins.

g) A protein without its prosthetic groups is called as ……..

h) Waxes are long-chain of ……..and alcohol esters.

i) Triacylglycerols are tri-esters of…….

j) The α- and the β-forms are defined as …….....isomers of the cyclic carbohydrates.

Answer key

1.10.1: 1-(a); 2-(c); 3-(a); 4-(c); 5-(a); 6-(a); 7-(b); 8-(a); 9-(b); 10-(a); 11-(d); 12-(c); 13-(c); 14-(s); 15-(a)

1.10.2: a) Linus Pauling; b) sixteen; c) saturated fatty; d) transport; e) two; f) stabilizing; g) apoprotein; h) fatty acid; i) glycerol and fatty acids; j) *trans* or *cis*

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1.12 SUGGESTED READINGS

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1.13 TERMINAL QUESTIONS

1.13.1 Short Answer Type Questions

1. What is the glycosidic linkage or bond?
2. What do you understand about saturated and Unsaturated Fatty acids?
3. Write a note on the mutarotation.
4. Describe in brief about properties of lipids?
5. Write a short note on classifications of biomolecules.
6. Distinguish monosaccharides, oligosaccharides and polysaccharides.

1.13.2 Long Answer Type Questions

1. What are Biomolecules? Discuss about properties of carbohydrates, proteins and lipids.
2. What are Carbohydrateds? Explain its structure and functions.
3. Give a detailed account on classification, structure and functions of lipids.
4. Write an essay about structure and functions of proteins.

UNIT 2: NUCLEIC ACIDS, PROTEIN SORTING

Contents:

1.1 OBJECTIVES

After reading this unit you will be able to:

- Describe physical and chemical structure of DNA and Types of DNA.
- Understand Watson and Crick model of DNA.
- Explain RNA, its synthesis and types.
- Describe Post transcriptional changes in RNA.
- Know about Genetic Code.
- Understand Protein Sorting.

1.2 INTRODUCTION

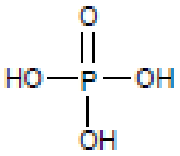
Cell contains four major classes of biomolecules, i.e. carbohydrates, lipids, proteins, and nucleic acids, making the majority of cell's mass. Each biomolecule is an important component of the cell that performs different type of functions. Among these biomolecules, **nucleic acids**, namely DNA or RNA, serve as the ultimate source or storehouse of biological information in every organism. Nucleic acids, due to its millions of monomeric units, are large and heavy with molecular weight from 30,000 to several millions. Johann Friedrich Miescher, in 1969, first isolated a substance from the pus cell, obtained from discarded surgical bandages. Miescher called this material as 'nuclein', which was later renamed as 'nucleic acid'. Nucleic acid carries all the hereditary information from parents to offspring. Being the linear polymer of nucleotides, they are also known as **polynucleotide**. Nucleotides are made by combining nucleosides, i.e. heterocyclic rings of nitrogenous bases and pentose sugars, and a phosphate groups. Each nitrogenous base is linked to a sugar to which one phosphate group is attached. Subsequent studies have shown that there are two types of nucleic acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

DNA or RNA is the principal genetic materials present in entire living organism. DNA is genetic material of most of the organisms, except some viruses, which have RNA as their genetic material. DNA is a macromolecule and is a double helical chain of polydeoxyribonucleotides. Eukaryotic DNA is present in membrane bound cell organelle, whereas, prokaryotic DNA, lying free in cytoplasm, is known as nucleoid. In prokaryotes it also occurs as plasmids, both plasmid and nucleoid are double stranded circular DNA. RNA is a generally single stranded, linear polymer of nucleotides consisting of a ribose sugar and phosphate backbone (rather than deoxyribose as in DNA). RNA also differs from DNA since the base, uracil, replaces thymine found in the DNA.

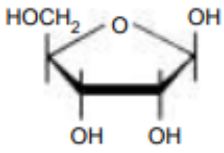
1.3 PHYSICAL AND CHEMICAL STRUCTURE OF DNA

Each nucleotide (a subunit of nucleic acid) comprise of three parts *viz.* (i) phosphate group, (ii) sugar component and (iii) heterocyclic nitrogenous bases.

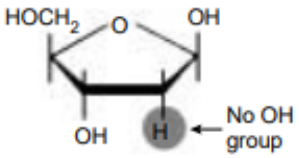
(i) Phosphate group: Phosphoric acid (H3PO4) has three reactive hydroxyl (-OH) groups of which two are involved in making bond with pentose sugar forming sugar-phosphate back bone of nucleic acid. In a nucleotide, the phosphate groups binds to C5' and C3' of the ribose or deoxyribose sugar through sugar-phosphate bonds known as **phosphodiester linkages.**



(ii) Sugar: The only two types of pentose sugar *viz.* ribose and deoxyribose are present in nucleic acid. The sugar component of RNA is ribose, whereas in DNA it is deoxyribose. The chemical structure of

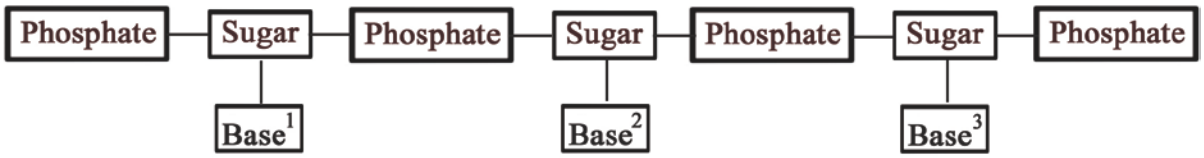


Ribose



Deoxyribose

(iii) Nitrogenous base: A nitrogenous base is covalently linked to the carbon of the sugar residue with the help of **glycosidic bond**. Each nucleic acid contains millions of nitrogenous bases, but only four different types of bases can occur in a nucleic acid. The nitrogenous bases found in nucleic acid are planar, aromatic and heterocyclic molecule which are related to either purine or pyrimidine. Purines (double ring system) include Adenine and Guanine whereas pyrimidines (single ring system) include Cytosine, Thymine and Uracil.



Purines

Purines are double ringed aromatic organic compound consisting of a six-membered pyrimidine ring fused to a five-membered ring i.e. imidazole ring. In purine a total of four nitrogen atoms are present. In first ring of purine, numbering of carbon atoms is done in anti-clock wise direction whereas in other carbon atoms are numbered in clockwise direction. It is water soluble and is especially found in high concentration internal organs such as liver and kidney. Both DNA and RNA contain two types of purines i.e. Adenine and Guanine.

Pyrimidines

Pyrimidines are six membered, single ringed, simple aromatic compounds composed of carbon and nitrogen atoms. In a pyrimidine ring two nitrogen atoms present at 1^{st} and 3^{rd} position and four carbon atoms present at 2^{nd}, 4^{th}, 5^{th} and 6^{th} position. The most common pyrimidine occurring naturally are cytosine, thymine and uracil. The first two, viz. cytosine and thymine, are present in DNA, whereas in case of RNA uracil is present in place of thymine.

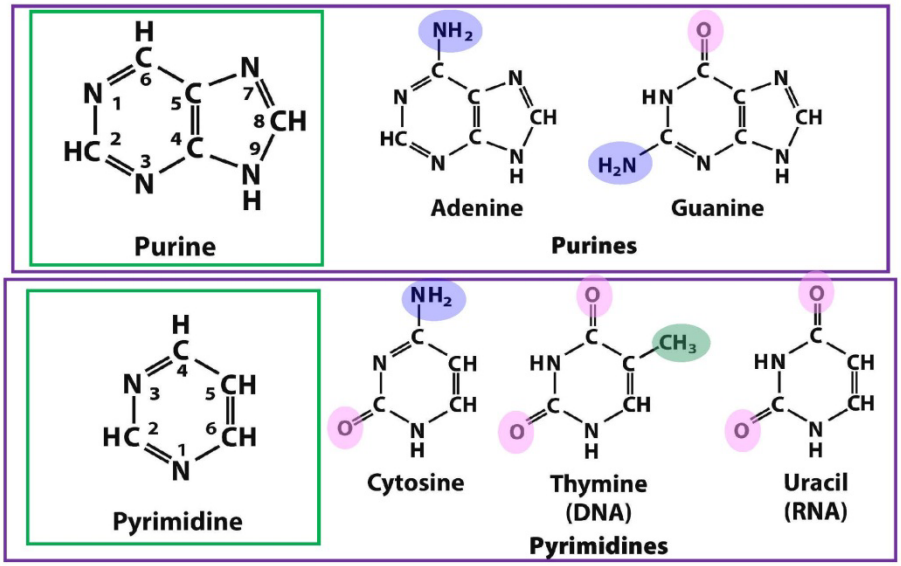


Figure: Purines and Pyrimidines

Chargaff's rule

Erwin Chargaff in 1950 studied the bases and other components of DNA and concluded that almost all DNA, irrespective of type of organism or tissue, maintain certain properties. He proposed that DNA of any cell organism should have pyrimidine and purines in 1:1 ratio i.e. the amount of adenine (A) is equal to the amount of thymine (T), and the amount of guanine (G) is equal to the amount of cytosine (C). The total amount of purines (A + G) is equal to the total amount of pyrimidines (C + T). He also proposed that adenine (A) always pairs with thymine (T) with two hydrogen bonds and cytosine (C) always pairs with guanine (G) with three hydrogen bonds.

Nucleoside and Nucleotide

N of a Nitrogenous bases (N9 in case purine whereas N1 in pyrimidine) attaches to C1 carbon of pentose sugar with the help of a glycosidic bond. The whole structure formed by this association is known as Nucleoside. Depending on the type of sugar nucleosides are of two types: a) Ribonucleoside (if pentose sugar is ribose) and b) Deoxyribonucleoside (if pentose sugar is deoxyribose). A phosphate group binds to pentose sugar of nucleoside with the help of phosphodiester bond to constitute a nucleotide. Phosphate group attaches to C5 of the ribose and C3 of deoxyribose sugar to form ribonucleotide and deoxyribonucleotide respectively**.**

William T. Astbury, an experienced crystallographer, studied calf thymus DNA with the help of X-ray diffraction technique. Astbury missed the actual structure of DNA due to the poor quality of the photos. During 1950's 3 separate groups or researchers were intensively working to discover the structure of DNA: 1) Maurice Wilkins and Rosalind Franklin, 2) Linus Pauling and 3) James Watson and Francis Crick. Wilkins studied nucleic acid and proteins via X-ray imaging and successfully isolated fibres of DNA. Rosalind Franklin, an expert in X-ray crystallography, joined King's College, London and took the high quality photographs of DNA, she discovered two forms of DNA: A form (crystalline form) and B

form (paracrystalline form). In 1953, Pauling along with Corey proposed a triple stranded helical model of nucleic acid in which phosphate forming the helical core and the bases placed outside. In 1953 James Watson visited King's College and was shown Franklin's image by Wilkins. Based on the x-ray diffraction image and Chargaff's rule Watson and Crick successfully deduced the double helical structure of DNA.

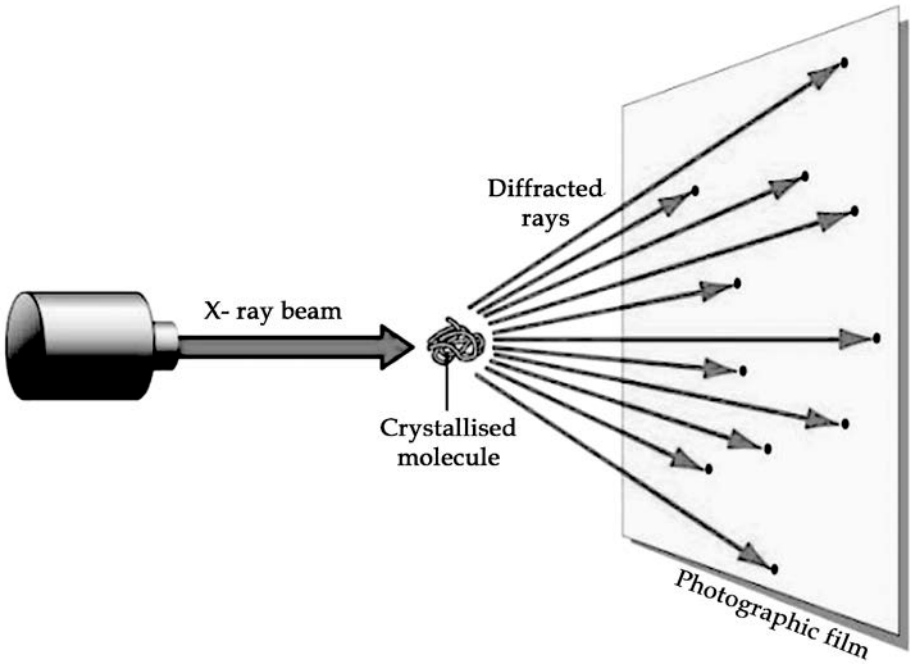


Figure: X-ray crystallography of DNA

1.4 WATSON AND CRICK MODEL OF DNA

J. D. Watson and F. H. C. Crick, in 1953, studied physical and chemical properties of DNA and deduced a three dimensional model of DNA as double helix. Watson and Crick, on the basis of Chargaff's rule and x-ray diffraction image of Wilkins and Franklin, proposed that DNA is composed of two complementary strands of polynucleotides twisted around each other in the form of double helix and attached together by hydrogen bonding between purines and pyrimidine bases. The two polynucleotide strands forming the double helix are anti-parallel, i.e., both the chains run in opposite direction. If a chain in a helix is aligned in 5'→3' direction, then the other chain in the helix will be aligned in 3'→5' direction. A free OH group is present at 3' end of a helix and a PO_4^{3-} at 5'. The phosphate group imparts negative charge to the DNA molecule. The sugar phosphate backbone of each polymerized nucleotides strand projects outward the helix and forms the backbone of the structure whereas the nitrogenous bases toward the center. A pyrimidine base in one polynucleotide chain is hydrogen bonded with purine base of other chain, i.e. adenine (A) and thymine (T) pair together, and cytosine (C) and guanine (G) pair together. When adenine occurs in one strand, thymine is found in the complementary one; similarly, when guanine occurs in one strand, cytosine is found in the other. The pairing between the bases held both the strands in a helix together and makes it a stable structure. X-ray diffraction images of DNA have determined that there are approximately 10 bases per turn in DNA (i.e. it make one complete turn after

every 10 residues). Two adjacent bases are separated by the distance 3.4 Å, hence the length of one complete turn of helix is 34 Å. The diameter of the helix is 2 nm. Two grooves of different width are formed due to asymmetrical spacing of the sugar-phosphate backbones between the adjacent turns. One wider is known as major groove (ca. 22 Å wide) and the other narrower is known as minor groove (ca. 12 Å wide). These grooves are locations where protein binds to DNA.

1.5 TYPES OF DNA

DNA is a flexible molecule. It exist in various three dimensional structure. Early x-ray diffraction studies by Franklin, Goslin, Wilkins and co-workers revealed the presence of two kinds, i.e. A form and B form, of DNA. The double helical model of DNA proposed on the basis of study of physical and chemical property by Watson & Crick is commonly referred as B form of DNA or B-DNA. In B- form the two polynucleotide strands are spirally coiled around each other to form right handed helix. A form contains 11 base pair per turn and similar to B form it also shows right handed coiling. Recent studies has shown the existence of left handed coiling in DNA, commonly referred as Z-DNA or Z form. The sugar phosphate backbone of this DNA follows a zigzag path containing 12 base pairs in one turn and is assumed to play role in gene regulation. Other than these (A, B, & Z form), there are other conformations of DNA which are found to occur in controlled or experimental condition.

FEATURES	A	B	Z
Helical sense	Right handed	Right handed	Left handed
Diameter	25.5 Å	20 Å	18.4 Å
Base-pairs per helical turn (n)	10.7	10.4	12
Helical twist (or Rotation) per bp (360/n)	33.6º	34.3º	60º / 2 bp
Rise per bp	2.3 Å	3.3 Å	3.8 Å
Base tilt to helix axis	20º	6º	7º
Major groove	Narrow and deep	Wide and deep	Flat
Minor groove	Wide and shallow	Narrow and deep	Narrow and deep
Helix pitch	24.6 Å	34.3 Å	45.6 Å
Mean propeller twist	+18º	+16º	0º
Glycosyl-bond conformation	anti	anti	anti at C, syn at G
Sugar pucker	C3'-endo form	C2'-endo form	C2'-endo for pyrimidine and C3'-endo for purines.

Table: Features of A, B and Z type of DNA

Viral DNA

The central core or genome of a virus may only have a single type of nucleic acid. They may either have ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) and are known as RNA virus or DNA virus respectively. Once the DNA is injected into the host cell, it will enter the nucleus of the host cell, where the viral DNA can integrate with the host cell genome. The

viral DNA utilises the host cell's polymerase enzyme to replicate the viral DNA and produces new viruses. Both DNA and RNA are never found together in a virus. Nucleic acid in virus can either single stranded or double stranded, with a circular, linear or segmented arrangement. DNA virus can be divided into two groups; i.e. double stranded DNA (dsDNA) viruses, and single stranded DNA (ssDNA) viruses. **Double stranded DNA (dsDNA) viruses** are considerably complex and its size varies from 5 kbp in polyomaviruses to 130–365 kbp in poxviruses. Viruses with genome more than 300 kbp are known as 'giant virus', although giant viruses had been known for a few years. Mimivirus is the largest virus ever discovered that infect amoebae. In dsDNA, during transcription a preinitiation complex binds with the positive strand and RNA polymerase uses negative strand as template. dsDNA viruses use different mechanism, i.e. Bidirectional replication mechanism, Rolling circle mechanism, Strand displacement mechanism and Replicative transposition mechanism, to replicate viral genome in a host cell. S**ingle stranded DNA (ssDNA) viruses** are comparatively small and simple DNA viruses and its size varies from 2-6 kb in length. These viruses infect diverse hosts from all three domain of life. ssDNA viruses depends on the host cell for much of their replication machinery. For transcription, ssDNA is made into dsDNA by DNA polymerase after entering a host cell, mRNA is then synthesized in same manner. The DNA in these viruses can be either circular or linear, the former ones are replicated by rolling circle replication (RCR) mechanism with the help of endonucleases, while the linear one 'hairpin' structures at the end of DNA play a part in DNA replication.

Bacterial DNA

The main distinguishing feature between prokaryotic cell and eukaryotic cell is that in prokaryotic cell nuclear membrane is absent. The DNA present in bacteria is of two types- Genomic DNA and Plasmids. Similar to eukaryotic DNA, bacterial DNA performs the function of encoding of all of the information needed to program the cell's activities including reproduction, metabolism, etc. Bacterial cells lacks true nucleus or a nuclear membrane and do not possess the complex chromosome of the kind as present in eukaryotes. Instead, the genes are encoded within DNA, which is packed and localised in dense zone of cytoplasm of the cell. In most of the bacteria the DNA is a single circular molecule, called the bacterial chromosome. The chromosome, along with several proteins and RNA molecules, forms an irregularly shaped structure known as the **nucleoid.** Most of the bacteria have a haploid genome. Usually, the bacterial genome is represented by long, single circular DNA attached to a cell membrane, sometimes a closed loop of DNA is also present. Not all bacteria have a single circular chromosome: some bacteria have multiple circular chromosomes. Sometimes linear chromosomes are also reported in some bacterial cell. A bacterial cell contains approximately 0.5–20 fg DNA. When the bacterial cells are immersed in a medium having high refractive index then with the application of phase-contrast microscopy nucleoid region can be observed. Nuclear bodies were first observed with the help of Giemsa and Feulgen stains using light microscope.

Plasmid DNA

Many bacterial cells, in addition to nucleoid, possess small, independently replicating circles of double stranded DNA called plasmids. They are best thought of as an excised portion of

the bacterial chromosome. Normally plasmid DNA occurs independently in cytoplasm of cells or it may also be found in associated with main chromosomal DNA, called **episome.** Plasmids contain only a few genes (apart from main genome) that are usually not vital for the cell's survival but they carry genes that encodes different proteins which aid the host cell to perform specific functions viz. drug resistance, mating ability, toxin production, etc.

MITOCHONDRIAL AND CHLOROPLAST DNA

Mitochondrial DNA

The mitochondria are a highly specialized organelle present almost in all the cells. The functions of mitochondria are well conserved across species. They generally encode proteins for oxidative phosphorylation (energy production), in addition to this, mitochondria are involved in many other activities, it plays a vital role in calcium signalling, it also play role in biosynthesis of heme, steroid, amino acids and regulates cellular metabolism, and apoptosis. Mitochondria, independent from their nucleus, they possess their own genetic material and the machinery to manufacture RNA and proteins.

The genetic material in mitochondria is small, circular DNA and known as mtDNA. In case of plants, mtDNA exists as a collection of linear DNA with combinations of small circular and branched molecules. A study performed by Lo *et al.* (2011) went into extensive detail concerning the structure of DNA in mung bean mitochondrial nucleoids. They observed plant mtDNA as supercoiled molecules, sub-genomic open circles of variable size, linear molecules, and other highly complex structures. mtDNA encodes for small number of polypeptides that are tightly integrated into the inner mitochondrial membrane along with the other polypeptides, forming a DNA-protein complexes in the mitochondrial matrix. In comparison to nuclear genome, the mitochondrial genome is relatively short with ca. 16,500 base pairs and 37 genes (28 on H-strand and 9 on L-strand). Plant mitochondrial genomes are relatively larger and more complex, than of animals. The plant mitochondrial genomes range between 200-2,000 kbp. Irrespective of the genome size of plant mitochondria, it codes for relatively few genes. With the increase in size of mitochondrial genome the genes does not increases. The increased size of mitochondrial DNA is due to the presence of repeated sequences, AT-rich non-coding regions, and large introns and non-coding sequences.

Both mitochondrial and nuclear DNA works in coordination to regulate energy production. mtDNA differs transcriptionally from the nuclear DNA of any eukaryotic cell. Nuclear DNA produces messenger RNA that is monocistronic. On the other hand, the mitochondrial DNA is polycistronic. According to Lynn Margulis, mitochondrial genome has been derived from a eubacterial ancestor through endosymbiosis. It means that genome of plant mtDNA is a remnant of its prokaryotic symbiotic ancestor. It is considered that this genome must have been much larger earlier but it evolved rapidly in structure because of its extensive recombination activities. Because of the size and complexity of plant mitochondrial genomes, the exact mechanism for plant mtDNA replication remains unclear. Multiple hypothesis viz. recombination-dependent replication, rolling circle mechanism, bidirectional replication method, etc. has been given by different researchers to explain the mtDNA replication.

Chloroplast DNA

The chloroplast is a well-known green coloured plastid found in all the photosynthetic cells of plant. It comprises of green-coloured pigments called chlorophyll and is a membrane bound organelle that converts light energy into chemical energy and provide plants with foods and energy in the form of sugar or starch by the process of photosynthesis. Apart from nuclear DNA, chloroplast has its own DNA, involved in the preservation, replication, and expression, known as chloroplast DNA (cpDNA). The DNA in chloroplast is maternally inherited, circular and is localized in specific regions of the plastid and sometimes is called plastidome. Chloroplast genome characteristically exhibits the presence of two identical inverted repeats in the DNA sequence. The first existence of chloroplast DNA was first suggested in 1951. Later, in 1962, Ris and Plaut cytologically provided the first convincing evidence that chloroplasts contain DNA.

cpDNA codes for proteins and RNAs that is crucial and plays a significant role in metabolic processes. It encodes for rRNA, tRNA, larger subunit of RUBISCO and many proteins participating in photosynthesis and other essential processes like gene expression and electron transport. Although vast majority of the proteins of chloroplast are encoded by nuclear DNA and are produced in cytosol of plant and transported to organelle by specialized transport machinery. In comparison to mitochondria, the amount of DNA per chloroplast is much greater. mtDNA and plastid DNA together amounts for about one-third of the nuclear DNA. Replication of chloroplast DNA is independent of nuclear DNA replication. The chloroplast genome ranges between 70-200 kbp and encodes for approximately 120 genes, having the contour length of 30-60 mm and mass of about 80-130 mDa. A single mesophyll chloroplast is able contain up to 300 chromosomes organized into nucleoids. These are complex structures each consisting of 10-20 copies of the plastid genome along with RNA and various proteins (Krupinska et al, 2013). The first complete study of chloroplast genome was done in tobacco (*Nicotiana tabacum*) in 1986. Chloroplast genome comprises of 04 genes for rRNA, 20 for ribosomal proteins, 30 for tRNAs, many of the photosynthetic proteins, 6-9 genes for *ATPase*, and chloroplast *RNA polymerase*.

It has been considered that chloroplasts are originated from photosynthetic prokaryotes that became part of a plant cell by endosymbiosis. In contrast to the evolution of the nuclear DNA in the plant cell, cpDNA has evolved at a more conservative pace and exhibits a more interpretable evolutionary pattern. Over time, there appears to have been some genetic exchange between the nuclear and chloroplast genomes. Both morphological and molecular studies on chloroplast can provide a clearer understanding of its evolutionary processes.

S. No.	Properties	Mitochondrial DNA	Chloroplast DNA
1	Present in	all eukaryotes	plant cell
2	Organelle	mitochondria of the cell	chloroplast of the cell
3	Shape	circular and linear	circular
4	DNA type	double stranded.	double stranded.
5	Abbreviation	mtDNA	cpDNA
6	No. of genes	37	200
7	Existence	as multiple copies	as multiple copies
8	Distribution	randomly distributed to daughter	randomly distributed to daughter cells

		cells	
9	No. of base pairs	16.5 kbp in humans 200-2000kbp in plants	70-200 kbp
10	Histone proteins	absent	absent
11	Introns	absent	absent
12	Functions	genes in the mitochondrial DNA take part in oxidative phosphorylation for the production of ATPs and in the synthesis of proteins.	chloroplast DNA participate in photosynthesis in plants.

Table: Properties of Mitochondrial DNA and Chloroplast DNA

1.7 RNA SYNTHESIS AND ITS TYPES

RIBONUCLEIC ACIDS (RNA)

After the discovery of double helical model of the DNA by Watson and Crick in 1953, scientists turned to RNA structure and said that it is usually a single stranded molecule consisting of a long chain of nucleotide units attached by phosphodiester bond. Each unit of RNA nucleotide consists of a nitrogenous base, a ribose sugar, and a molecule of phosphate. Similar to DNA, RNA has four nitrogenous bases, but unlike DNA, it has uracil (U) instead of thymine (T). Thus, the four RNA nucleotides are adenine (A), cytosine (C), guanine (G), and uracil (U). During complementary base pairing with DNA, G pairs with C, T in DNA pairs with A in RNA, A in DNA pairs with U in RNA. While during RNA-RNA base pairing, G and C pairs with each other and A and U pairs together.

RNA Synthesis

Francis Crick explains the flow of genetic information in biological system and proposed Central Dogma of Molecular Biology. He explained the directional flow of genetic information from DNA to RNA to protein. The process of by which molecules of RNA is synthesized from the genetic information encoded on DNA template with the help of enzyme RNA polymerases called transcription. DNA segment, that are transcribed into RNA molecule and synthesize protein, produces mRNA (also known as coding RNA), whereas other segments that are copied into RNA molecule forms non coding RNAs (ncRNAs). RNA polymerases use nucleotide triphosphate (ATP, UTP, GTP and CTP) and a template of DNA. During the process of RNA synthesis in prokaryotes one type of RNA polymerase is used while in eukaryotes 3 types of RNA polymerases are used. RNA polymerase I transcribes ribosomal RNA (rRNA), pol II transcribes mRNA and pol III tRNA.

During transcription three different types of RNAs are synthesized:
i. mRNA (or Messenger RNA): carries message for protein synthesis.
ii. tRNA (or transfer RNA): carries amino acid during protein synthesis.
iii. rRNA (or ribosomal RNA): forms the part of ribosomes.

Types of RNA

RNA is classified on the basis of their nature and function. On the basis of nature, RNA is of two types, namely genetic RNA and non-genetic RNA.

Genetic RNA: In some organisms viz. some viruses, RNA act as hereditary material. It carries genetic information from parent to offspring. It has self replicatory property. It can be double stranded or single stranded.

Non-genetic RNA: On the basis of functioning, RNA is classified into three types, namely mRNA, tRNA and rRNA. Non genetic RNA does not serve as hereditary material but is the one that is transcribed by DNA.

mRNA (Messenger RNA): The name messenger RNA (mRNA) was proposed by Francois Jacob and Jacques Monod in 1961, it carries the genetic information contained in DNA which control the cellular activities. It is synthesized in the nucleus of cell from DNA and then carried out of the cell to facilitate protein translation. The cell does not contain large quantities of mRNA, accounting for only 5% - 10% of the total RNA in the cell. This is because mRNA constantly undergoes breakdown into its constituent ribonucleotides by ribonucleases, unlike rRNA and tRNA. The main function of mRNA is to carry genetic codes copied from DNA in nucleus in the form of triplets, known as codons, during the process of transcription to the site of protein synthesis i.e. ribosome (present in cytoplasm).

The structure of mRNA

The molecule of messenger RNA (mRNA) consists of single strand having A, G, U and C nitrogenous bases. The main purpose of mRNA is that it functions as a template for protein synthesis, it carries genetic information from DNA (in nucleus) to a ribosome (in cytoplasm) and assemble amino acids properly. Each amino acid in a protein is coded by the sequence of three nucleotides which together form a unit of genetic code is called codons. The structure of mRNA molecule comprises of following segments:

5’ Cap : This region has a methylated structure at 5’ end and is associated with binding of m-RNA molecules to ribosomes.

Non-coding region: The 5’ cap is then followed by a non coding region with length of 10-100 nucleotides. This region is rich in A and U residues. As per its name it does not code for any protein.

Initiation codon: This is the triplet which marks the beginning of protein synthesis. It is AUG and codes for Methionine.

Coding region: This region is between initiation codon and termination codon and contains the sets of triplet codon which codes for different types of amino acids.

Termination codon: These are the codon that signals the termination of translation. These codons are also known as nonsense or stop codons. For these codons no molecule of tRNA exist. There are three nonsense codons are called UAG (Amber), UAA (Ochre) and UGA (Opal).

Another non coding region: This region consists of 50-150 nucleotides with a specific sequence AAUAAA.

3'Poly-A tail: This is the last segment of mRNA consisting of 200-250 nucleotides. It consist of large number of A residues.

rRNA (Ribosomal RNA): Ribosomal RNA or rRNA, as its name suggest, is present in ribosomes and constitutes about 80% of total cellular RNA. Among all the different types of RNA, rRNA is most stable and insoluble. It is a single stranded which might gets twisted and forms complementary base pair within the strand. It unfolds on heating and got refolded on cooling. The ribosomes are composed of two major nucleoprotein subunits, the eukaryotic 80S ribosome has 60S and 40S subunit whereas the prokaryotic 70S ribosome has 50S and 30S subunits. Each ribosomal subunit comprises of one or more rRNA and a variety of proteins. In eukaryotes 40S subunit contains of 18S rRNA and 50S subunit contains 28S rRNAs, 5.8S rRNAs and 5S rRNAs. In prokaryotes 30S subunit contains of 16S rRNA and 50S subunit contains 5S rRNAs and 23S rRNAs. Small subunit of ribosome reads the RNA and the larger one joins the amino acid to synthesize protein.

The functions of rRNA molecules are little known. It is the part of the protein-synthesizing organelle. The primary function of rRNA is in protein synthesis ensuring the proper alignment of the mRNA and the ribosomes. rRNAs combine with proteins in the cytoplasm and has the enzymes needed for the process. rRNA also act as enzymes, known as ribozymes, having role introns splicing.

tRNA (Transfer RNA): Transfer RNA (tRNA), also known as soluble RNA, is the smallest RNA among the 3 types of RNA with 71-80 nucleotides. Its molecular weight ranges from 25000 to 30000. Apart from usual nitrogenous bases tRNA contains unusual bases with some modifications, viz., methyl adenine, methyl cytosine, methylguanine, pseudouracil and others, which make it unique from other RNAs. Each tRNA molecule binds with specefic amino acid and transfers it to the site of protein synthesis and recognizes the codons of the mRNA. Hence, tRNA molecule acts as an adapter between mRNA and amino acid. Earlier it was considered that there are 20 tRNA molecule for 20 aminoacid i.e. one tRNA for each amino acid. Later it was reported that at least two types of tRNA exist for one amino acid.

Structure of tRNA

The primary structure of tRNA was first given by Holley et al. (1965) in yeast and presented the clover leaf model. It is stabilized by strong hydrogen bonds between the nucleotides. Clover leaf model of tRNA consists of 5 arms made up of single polynucleotide chain and three structural loops that are formed by hydrogen bonding. The 3' end serves as the site of amino acid attachment and three bases 5'-CCA-3' are added to the 3' end of every tRNA molecule.

Five arms or parts of cloverleaf model are as follows:

i. Acceptor arm: Amino acid attaches to this region of tRNA, hence also named as amino acid carrier arm. It is 3' terminal end of acceptor arm that attaches amino acid. Base pairing in 3' terminal end is absent. 3' end of every tRNA molecule is capped by the nucleotide sequence CCA-OH (5'-3').

ii. Anticodon arm: This arm of tRNA, consisting of seven unpaired bases out of these three nucleotide bases which is complementary to triplet codon of mRNA forms the anticodon, is responsible of recognising the codon. mRNA attaches to this site of tRNA and transport amino acid to protein synthesis site.

iii. D-arm or D-loop or DHU loop: Due to the presence of an unusual or modified pyrimidine base dihydrouridine it is named so. This arm helps in attachment of amino-acyl synthetase.

iv. TΨC arm: This region contains thymidine, pseudouridine and cytidine residues hence named TΨC arm or T loop. This region recognises and determines the ribosomal site where tRNA molecule has to attach during protein synthesis.

v. Variable arm: Also known as extra arm, this is the most variable region in tRNA molecule. Nucleotides composition is variable and is lacking entirely in some tRNA molecule. On the basis of variability it is classified into two classes, viz. Class I tRNAs (The dominant form, with 3-5 base pairs long) and Class II tRNAs: (with 13-20 base pair long arm)

Function of tRNA: tRNAs are an essential component of translation. The main function of transfer RNA is to bring or transfer amino acids to the ribosome that corresponds to each triplet codon of mRNA. The amino acids are then joined together and processed to form polypeptide chains and proteins.

S. No.	Features	mRNA	rRNA	tRNA
1.	Constitutes total percent of total cellular RNA	5-10 %	80%	10-15%
2.	Sedimentation cofficient	8S	28S, 18S, 5.8S, 5S, 23S, 16S, 5S	3.8S
3.	Molecular weight	50000	23S: 1.1 X 10^5 30S: .55 X 10^6	25000-30000
4.	Unusual bases	less	less	high
5.	Site of synthesis	Periphery of nucleolus	Centre of nucleolus	Outside nucleolus
6.	Base-DNA relation	Bases of mRNA are complementary to DNA bases	Forms from a small part of very less DNA	Forms from a small part of very less DNA
7.	Function	carry genetic codes copied from DNA in nucleus in the form of triplets, known as codons	ensures the proper alignment of the mRNA and the ribosomes during protein synthesis	transfer amino acids to the ribosome that corresponds to each triplet codon of mRNA

8.	Shape	linear	spherical	Clover leaf model
9.	Presence of codons or anticodon	Carries codons	codons or anticodons are absent	Carries anticodons

Table: A comparative study of different types of RNA

1.8 POST TRANSCRIPTIONAL MODIFICATION

During the process of transcription (or RNA synthesis) RNA is transcribed but before the translation it must be processed to generate a mature and functional RNA. If the RNA is not processed then no protein synthesis will take place. This process of generating a mature functional RNA is called Post-Transcriptional Modification (PTM). It includes phosphodiester bond cleavage, addition or alterations of existing bases or sugars, specific cleavage or modification of nucleotide or introns splicing. The primary transcript formed by RNA polymerase enzyme is called as pre RNA. In prokaryotes pre mRNA molecule undergo little or no modification, whereas in eukaryotic organisms it is significantly modified. In both prokaryotes and eukaryotes pre rRNA and pre tRNA undergo modification to become rRNA and tRNA.

Types of post transcriptional modifications

In post transcriptional modifications pre RNA, before being transported to cytoplasm, involves 5' end capping, 3'-OH polyadenylation and RNA splicing.

5' Capping: It is an enzyme catalysed process, in this 7-methylguanosine residue is added to 5' end of nascent RNA. When the mRNA transcript is 20-25 nucleotides in length, RNA triphosphate removes one of the three phosphates at 5' end. Guanosine Mono-Phosphate is added at 5' diphosphate forming Guanosine 5-5 triphosphate. Finally, enzyme methyltransferase methylates the newly attached guanine. The main function of 5' end capping is that it protects mRNA from degradation, it transports the mRNA to cytoplasm from nucleus and it also aid mRNA in binding with ribosomes.

3'-OH, polyadenylation: A poly A tail (consisting of about 50-250 nucleotides) is added to 3' end of nascent mRNA transcript without the involvement of DNA template. This process is catalysed by template independent RNA polymerase enzyme called as poly (A) polymerase. Polyadenylation process took place in two steps: (i.) Cleavage of nascent RNA at internal site (not at extreme 3' end) creates a new 3' end, (ii.) Addition of poly (A) tail with the help of polymerase and binding protein. The functions of 3'-OH Polyadenylation are that it export mature mRNA from nucleus, it affect the stability of mRNA and serves as recognition signal for the ribosomes.

RNA Splicing: In eukaryotic organism interrupted coding regions are present. In this process the non-coding sequences (i.e. introns), which are present in-between the nascent mRNA transcript, are removed and protein-coding sequences (i.e. exons) are ligated. Hence, it is also known as Introns splicing. The non-coding sequences of transcript are removed by two ways:

(i.) Self splicing or auto splicing: This occurs in some rare introns that help in the cleavage of phosphodiester bond and formation without the help of spliceosomes. In this process RNA itself act as enzyme to remove the non-coding regions from the mRNA transcript.
(ii.) Alternative splicing: In this process exons from the same gene are joined in varying combinations, by this several different functional mRNA transcripts are produced.

1.9 GENETIC CODE

Twenty different types of amino acids involved in the synthesis of polypeptides are present in nature. Single and double base coding will not cover all the 20 amino acids. A single genetic code involving each nucleotide, coding for single amino acid, can code only for one polypeptide with four amino acids, while the two bases, codes for 16 amino acids in the synthesis of protein. Crick suggested that each amino acid is a specified by a triplet of nucleotides i.e. codon. Marshall Nirenberg and Heinrich Mathaei used the tailor made artificial triplets of RNA in cells free systems to experimentally prove that a sequence of three nucleotide bases codes necessary for a single amino acid. The three nucleotide bases in mRNA constitute the genetic code or codon. This codon act as a code word for the synthesis of protein. The genetic code is regarded as a dictionary of nucleotide bases, viz. A, G, C and U, that determines the sequence of amino acid and proteins.

Second letter

First letter	U	C	A	G	Third letter
U	UUU, UUC: Phe UUA, UUG: Leu	UCU, UCC, UCA, UCG: Ser	UAU, UAC: Tyr **UAA Stop** **UAG Stop**	UGU, UGC: Cys **UGA Stop** UGG Trp	U C A G
C	CUU, CUC, CUA, CUG: Leu	CCU, CCC, CCA, CCG: Pro	CAU, CAC: His CAA, CAG: Gln	CGU, CGC, CGA, CGG: Arg	U C A G
A	AUU, AUC, AUA: Ile AUG: Met	ACU, ACC, ACA, ACG: Thr	AAU, AAC: Asn AAA, AAG: Lys	AGU, AGC: Ser AGA, AGG: Arg	U C A G
G	GUU, GUC, GUA, GUG: Val	GCU, GCC, GCA, GCG: Ala	GAU, GAC: Asp GAA, GAG: Glu	GGU, GGC, GGA, GGG: Gly	U C A G

Characteristics of Genetic Code

The code is degenerate: Particular amino acid can be specified by more than one triplet code on except for material and tryptophan

Code is triplet: Code is triplet of three nitrogenous bases constitute a triplet call this codes on which specify a particular amino acid on a polypeptide chain

Code is unambiguous: Each triplet code on specifies only one single amino acid
Code is non-overlapping: Change in single nitrogenous base could affect more than one codon Effecting more than one amino acid acid
Code is commaless: The second Amino acid is automatically coded coded by the next triplet. There is no space for punctuation in between every codon

1.10 PROTEIN SORTING OR PROTEIN SORTING

Majority of the proteins are synthesized in 80S ribosomes present in the cytosol of the cell, whereas the few proteins are synthesized on 70S ribosomes of mitochondria and chloroplast. In both eukaryotic and prokaryotic cell each of the newly synthesized proteins are sorted with the help of molecular labels (specific amino acids) and then transported to the specific location such as inner space of an organelle, intracellular membranes, plasma membrane, etc. of cell in which it functions. This process of transporting newly translated protein to a particular destination is known as protein targeting or protein sorting. Protein targeting or protein sorting is the biological mechanism by which proteins are identified and correctly transported to their appropriate positions in the cell or outside of it.

Targeting signals or Sorting signals
Synthesized polypeptide chains contain information that facilitates the cellular transport machinery to accurately target the protein from cytosol to a specific organelle (for eg. nucleus, endoplasmic reticulum, mitochondria, peroxisomes) inside or outside the cell. These pieces of information are known as targeting signals or sorting signals. Without targeting signals proteins cannot be transported to their specific destination. They remain permanently in the cytosol.

The delivery of newly synthesized proteins is done mainly by two different types of translocation processes:
(i) Co-translational transport
(ii) Post-translational transport

Co-translational Transport: This type of transport occurs in the proteins that are being translated in the ribosomes of RER. This protein transport occurs along with the process of translation. During the process of translation, proteins (along with the ribosomes) are initially brought to the endoplasmic reticulum. From ER, proteins enter endomembrane system i.e. it is transported to golgi apparatus in membrane vesicles. Here protein may undergo some modifications and then go to final destination. From the Golgi apparatus, protein may be transported to plasma membrane, lysosomes or plasma membrane. The proteins transported in this process may be soluble proteins or secretory proteins or integral proteins.

Signal sequence: A hydrophobic chain of 6-12 hydrophobic amino acids present on N terminal of nascent polypeptide chain.
Signal recognition protein (SRP): A ribonucleic protein, formed of 1 RNA and 6 proteins, recognize and binds with the signal sequence, smaller subunit of ribosome and SRP receptor on ER.

Translocon: A channel, made up of 3 subunits, is present on ER membrane which prevents the leakage of calcium from ER lumen to cytoplasm.

Proteins of SRP will recognize and binds with the signal sequence of protein and smaller subunit of ribosomes forming a protein-ribosome-SRP complex. This blocks the N terminal of polypeptide chain and smaller subunit of ribosome, therefore preventing the addition of amino acids and the translation is paused. The complex is transported to SRP receptor in ER. SRP of complex binds with SRP receptor of ER with GTP and larger subunit (with N terminal) attaches to translocon. GTP will be hydrolysed and translocon will open. SRP will be released from the complex, nascent protein will enter the translocon channel and translation will restart. Signal peptidase enzyme present in ER will cut the signal sequence. When translation is completed Ribosomal Releasing Factor (RRF) will remove the attached ribosome. The newly formed protein is covered by Chaperone protein which aids in its folding. Folded protein is then transferred to golgi apparatus and then to targeted organelles.

Post-translational transport: Some of the proteins are translated on free ribosomes i.e. those present in cytosol. **In prokaryotes** a closed membrane protein (Sec Y) is present on plasma membrane. A enzyme Sec A (ATPase enzyme), continuously breaks ATP to ADP, will bind SecY and facilitate the entry of protein inside the extracellular fluid. **In eukaryotes** chaperon proteins binds with the translated protein. The N terminal of signal sequence of the complex interacts with the translocon (Sec 61) of ER. The translocon opens and facilitate the entry of protein inside the lumen. Signal peptidase, enzyme present inside the lumen, will cleave the signal sequences present in the protein. A BiP (Binding immunoglobulin protein) protein present in the lumen binds with the protein (without N terminus), hydrolyses ATP to ADP with the help of Sec 63 complex. Energy due to ATP hydrolysis helps in pulling of protein little by little. This hydrolysis and pulling will continuously take place until whole protein is pulled inside the lumen. BiP is removed from the completely pulled protein is released into the ER lumen.

1.11 SUMMARY

DNA or RNA is the principal genetic materials made up of linear polymer of nucleotides. Each nucleotide comprise of three parts *viz.* phosphate group, sugar (either ribose or deoxyribose) and heterocyclic nitrogenous bases (related to either purine or pyrimidine). Based on the x-ray diffraction image and Chargaff's rule Watson and Crick successfully deduced a three dimensional structure of DNA. They reported that DNA is formed of two complementary strands of polynucleotides twisted around each other in the form of double helix running in opposite direction. There are approximately 10 bases per turn in DNA. Two adjacent bases are separated by the distance 3.4 Å, the diameter of the helix is 2 nm. Two grooves (major & minor) of different width are formed due to asymmetrical spacing of the sugar-phosphate backbones between the adjacent turns. There are three main types of DNA, A, B and Z type. Experiments conducted by **Griffith** in 1929, **Hershey and Chase in 1952 and Meselson and Stahl in 1958** demonstrated that DNA is the genetic material, rather than protein.

In some living organism, other than DNA, RNA is genetic material. Similar to DNA, RNA has four nitrogenous bases, but unlike DNA, it has uracil (U) instead of thymine (T) and the sugar present in RNA is ribose. RNA is synthesized from the genetic information encoded on DNA template with the help of enzyme RNA polymerases called transcription. There are three different types of RNAs mRNA (or Messenger RNA), tRNA (or transfer RNA) and rRNA (or ribosomal RNA). The mature RNA is later translated to synthesize the polypeptide chain encoded by the original gene. But before the process translation, the RNA must be processed to generate a mature and functional RNA. Twenty different types of amino acids involved in the synthesis of polypeptides are present in nature. Each amino acid is a specified by a triplet of nucleotides i.e. codon.

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1.13 SUGGESTED READINGS

- A Textbook of Biotechnology – R. C. Dubey
- Lehninger Principles of Biochemistry- Nelson and Cox
- Elements of Biotechnology – P. K Gupta
- Biotechnology Fundamentals and Applications – S. S. Purohit

1.14 TERMINAL QUESTIONS

Q1. Explain physical and chemical structure of DNA. Discuss Watson and Crick model of DNA.
Q2. What are different types of DNA? Discuss.
Q3. Describe about different experimental approaches which established DNA to be the universal genetic material?
Q4. What are different types of RNA? Discuss.
Q5. Explain Post-transcriptional modification of RNA.
Q6. What do you understand by Protein sorting?
Q7. What is Genetic Code?

UNIT-3- PROKARYOTIC AND EUKARYOTIC REGULATION OF GENE EXPRESSION

3.1 OBJECTIVE

After reading this unit you will be able to:

This topic about control of gene expression focuses on various aspects of gene regulation and the mechanisms.

1. Know about the basic facts and mechanisms of gene expression.
2. Study this gene regulation or gene expression by considering suitable examples.
3. Understand how genes regulating the expression and the role of RNAs and its various forms.
4. Understand the difference between prokaryotic and eukaryotic gene regulation.

3.2 INTRODUCTION

As we know the central dogma, a major theme of molecular biology states that the genetic information flows from DNA to RNA and RNA to proteins. This provides a molecular basis for the connection between genotype and phenotype but failed to answer that how this flow of information along the molecular pathway regulated.

Now in this chapter we consider one of the most fundamental questions: How is genetic expression regulated? Because it's clear that not all genes are expressed at all times in all conditions or situation, their expression shows variations depending upon different condition in a unit time. The basics cellular function of an organism is influenced by cellular environment and the adaptation to specific environments is achieved by regulating the expression of genes that encode the enzymes and proteins needed for survival in a particular environment.

The level of gene expression may differ from one cell type to the next or according to stage in the cell cycle. The expression of genes varies according to the need and specialization of the cell. For examples an animal contains numerous cells of define functions and with the minor exceptions, all having same genetic composition, but differ in function only due to the expression of genes. In multicellular plants and animals, gene expression is under elaborate control whereas in the single – celled bacterium it can be as simple as switching on and off of gene to make the enzymes needed to digest whatever food sources are available. For example, the β cells of the pancreas makes the insulin and the α cells of the pancreas make glucagon, in another example the genes for hemoglobin are expressed at high levels only in precursors of the red blood cells. The difference between all these cells is because of the precise control of gene expression. Factors that may influence gene expression include

nutrients, temperature, light, toxins, metals, chemicals, and signals from other cells. Malfunctions in the regulation of gene expression can cause various human disorders and diseases.

The gene expression system or regulatory systems of prokaryotes and eukaryotes are somewhat different from each other. The process is essential for prokaryotes and eukaryotes as it increases the versatility and adaptability of an organism by allowing the cell to express protein when needed. In both, prokaryote and eukaryotes gene expression can be regulated at a number of different points. In prokaryotes, they generally adapted to free-living, unicellular and simple structure symmetry of life, in which they grow and divide indefinitely as long as environmental conditions are suitable and the supply of nutrients is adequate. Their systems are provided to utilize the growth condition or unit conditions in unit time to attain the maximum growth rate. While the most eukaryotes are not only adapted to utilize the growth condition or unit conditions but the progeny cells must also undergo considerable changes in morphology and biochemistry and then each maintain its altered state. In eukaryotes the first, regulation can be through the alteration of DNA or chromatin structure. A second point at which a gene can be regulated is at the level of transcription. A third potential point of gene regulation is mRNA processing. A fourth point for the control of gene expression is the regulation of RNA stability. A fifth point of gene regulation is at the level of translation, a complex process requiring a large number of enzymes, protein factors, and RNA molecules. Finally, many proteins are modified after translation and these modifications affect whether the proteins become active; so genes can be regulated through processes that affect posttranslational modification.

Further, on the basis of assigned function some structural genes, particularly those that encode essential cellular functions, are expressed continually and are said to be constitutive and therefore not regulated by any regulatory gene or their products. While few DNA sequences that are not transcribed at all but still play a role in regulating genes and other DNA sequences. These regulatory elements affect the expression of sequences to which they are physically linked. In this way, prokaryotes and eukaryotes use regulatory genes to control the expression of many of their structural genes. The regulation of gene expression can be through processes that stimulate gene expression, termed positive control, or through processes that inhibit gene expression, termed negative control.

In this chapter, we discuss and explore regulation of gene expression in prokaryotes and eukaryotes. Before considering gene regulation in both bacteria and eukaryotes, we must distinguish between the DNA sequences that are transcribed and the DNA sequences that regulate the expression of other sequences. According to one of the basic concept, genes include DNA sequences that encode proteins and as well as sequences that encode rRNA, tRNA, snRNA, and other types of RNA required to stabilize the integrity of cell and organism. On the basis of allotted function within the cells group of genes tends to work together to foster the functioning of the cells. Group of genes or structural genes encode

proteins that are used in metabolism or biosynthesis or that play a structural role in the cell. Similarly regulatory genes are genes whose products, either RNA or proteins interact with other DNA sequences and affect the process of transcription or translation.

3.3 CONTROL OF GENE EXPRESSION IN PROKARYOTES AND EUKARYOTES

3.3.1 Gene regulation in prokaryotes

The idea that microorganism regulated their gene product to different conditions is not new. Two French microbiologists, Jacob and Monad (1961) found that the genetic material possesses group of regulatory gene units called operons in prokaryotes for which they received Nobel Prize in 1965. Jacob and Monad proposed that the transcription of a set of contiguous structural genes is regulated by two controlling elements. The regulation of gene expression has been extensively studied in many prokaryotes, particularly in *E. coli*. In bacteria the genes that have related functions are clustered and under the control of a single promoter. These genes are often transcribed together into a single mRNA. A group of bacterial structural genes that are transcribed together is called an operon. In prokaryotic cells, there are three types of regulatory molecules that can affect the expression of operons: repressors, activators, and inducers.

Repressors and activators are proteins produced in the cell. Both repressors and activators regulate gene expression by binding to specific DNA sites adjacent to the genes they control. In general, activators bind to the promoter site, while repressors bind to operator regions. Repressors prevent transcription of a gene in response to an external stimulus, whereas activators increase the transcription of a gene in response to an external stimulus. Geneticists have developed that idea, that efficient genetic mechanisms have evolved in these organisms to turn transcription of specific genes on and off, depending on the environmental conditions and cell's metabolic need for the respective gene products.

A general structure of an Operon

A typical operon Figure 3.1. In general organization of Operon, a set of structural genes lies at the end of the operon, these structural genes are transcribed into a single mRNA, which than translated to produce enzyme and then translated enzyme produce product of interest. For example, structural genes (as gene a, gene b, and gene c) at one end of the operon These structural genes are transcribed into a single mRNA, which than translated to produce specific enzymes A, B, and C and these enzymes further carried out a series of biochemical reactions that convert precursor molecule O into product P. The transcription of structural genes a, b, and c is under the control of a promoter, which lies upstream of the first structural

gene. RNA polymerase binds to the promoter and then moves downstream, transcribing the structural genes.

Control of operons

In general the above said mechanism is controlled by a regulator gene. A regulator gene has its own promoter and is transcribed into a short mRNA, which is translated into a small protein. This regulator protein can bind to a region of the operon called the operator and affect whether transcription can take place. There are two types of transcriptional control:

(i) Negative control, in which a regulatory protein is a repressor, binding to DNA and inhibiting transcription.
(ii) Positive control, in which a regulatory protein is an activator, stimulating transcription.

Operons can also be either inducible or repressible.

(i) Inducible operons are those in which transcription is normally off and something must happen to induce the transcription or to turn it on.
(ii) Repressible operons are those in which transcription is normally on and something must happen to repress the transcription or turn it off.

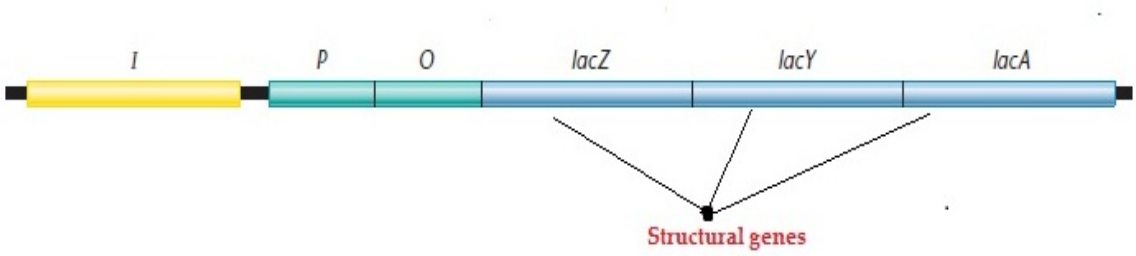

Figure 3.1: General overview of Operon (I=regulatory region, P=promoter region and O= operator region)

Negative inducible operons: In a negative inducible operon, the regulator gene encodes an active repressor that readily binds to the operator. Because the operator site overlaps the promoter site, the binding of this protein to the operator physically blocks the binding of RNA polymerase to the promoter and prevents transcription. For transcription to take place, something must happen to prevent the binding of the repressor at the operator site. This type of system is said to be inducible, because transcription is normally off (inhibited) and must be turned on (induced). Transcription is turned on when a small molecule, an inducer, binds to

the repressor. Regulatory protein being an allosteric protein has two binding sites one to bind DNA and another to bind small molecule (inducer). In general regulatory protein binds to DNA and stop transcription, but when inducer is present it compete and get itself bind to regulatory protein and thus altering the structure of regulatory protein so prevent its binding to DNA.

Lac structural genes

Lactose is **β**-galactoside that use as a carbon source by *E. coli*.

lacZ: encodes for β-galactosidase : enzyme responsible for breakdown of lactose into glucose and galactose. This enzyme also converts lactose to allolactose.

lacY: encodes for β-galactoside permease: responsible for facilitating the transport of lactose across the bacterial cell wall.

lacA: encodes β-galactoside transacetylase: the actual function of this gene is not known, but appears to have role in the detoxification of compounds by transferring acetyl-CoA to β-galactosides

All three of these genes are transcribed as a single, polycistronic mRNA. Polycistronic RNA contains multiple genetic messages each with its own translational initiation and termination signals.

The lac Operon of *E. coli*

Lac Operon in *E. coli* : Negative Inducible Control :

E. coli can use lactose as its sole carbon source. Lac operon is an operon required for the transport and metabolism of lactose in *E. coli* and some other enteric bacteria.

The lac operon includes the 3 structural genes –

• β galactosidase (lacZ gene)

• Galactoside permease (lacY gene)

• Thiogalactoside transacetylase (lacA gene)

β galactosidase cleaves lactose to galactose and glucose. Galactoside permease transports lactose into the cell. Thiogalactoside transacetylase appears to modify toxic galactosides to facilitate their removal from the cell. When *E. coli* is grown in medium where lactose is added, β galactosidase, lac permease and transacetylase appear at the same time in cells.

After lactose is transported into the cell, some of it is converted to allolactose by the few molecules of β galactosidase. Allolactose is a rearranged lactose molecule and an inducer of the lac operon. lacZ, lacY and lacA are coordinately expressed from one promoter called lacP that directs expression of a polycistronic mRNA. lacP is located upstream of lacZ. lacI encodes the lactose repressor protein and I located upstream of lacP. lacI has its own promoter and is constitutively expressed. Lac repressor or lacI binds to the DNA at the lac operator site called lacO. lacO is located between lacI and lacZ. In the absence of inducer, lac repressor is bound to lacO, preventing expression of the operon. In the presence of inducer, inducer binds to lac repressor and prevents repressor from binding to lacO, leading to transcription of the operon.

Lac Operon in *E. coli*: Catabolite Repression:

E. coli and many other bacteria metabolize glucose in preference to lactose and other sugar as a carbon source when they are offered a choice. They generally do this because directely enters in the Glycolysis and requires less energy to metabolize than the other sugars. So, in culture medium when *E. coli* find both sugars, it metabolize glucose first and genes that participate in the metabolism of other sugars are repressed, this phenomenon is called as glucose effect or Catabolite repression. Monod further suggested the diauxic growth of E coli to this glucose effect and this catabolite repression results in positive control in response to glucose.

This positive control is results due to the binding of a dimeric protein called the catabolite activator protein (CAP) to a site that located 22 nucleotides upstream to the promoter of lac genes (figure 3.2). But, RNA polymerase does not bind efficiently to many promoters unless CAP is first bound to the DNA. Before CAP can bind to DNA, it must form a complex with a modified nucleotide CAMP. Since, high level of glucose in the medium results in low level of cAMP, low levels of cAMP mean very few cAMP-CAP complexes. Subsequently, RNA polymerase has poor affinity for the lac promoter, and little transcription of the lac operon takes place. When intracellular levels of glucose drop and other sugars must be metabolized, levels of cAMP increase. Increased cAMP levels means there are more cAMP-CAP complexes. cAMP-CAP binds to a specific site on the DNA that is located adjacent to the promoter for the lac genes and adjacent to promoters controlling the expression of other sugar metabolizing operons. This increase enhances the binding of RNA polymerase to the promoter and increases transcription of the lac genes.

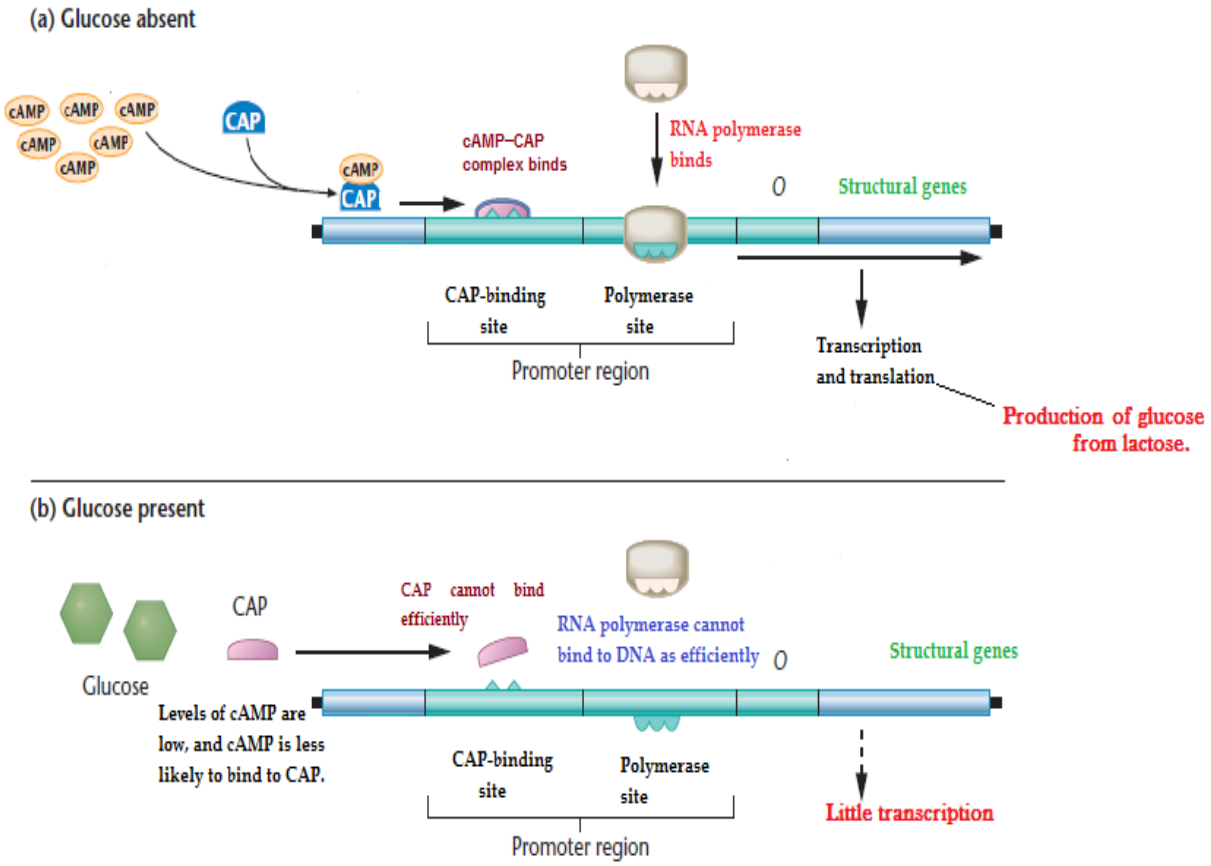

Figure 3.2: Catabolic repression: (a) Absence of glucose, in the absence of glucose cAMP levels increase, resulting in the formation of a cAMP–CAP complex, which facilitates transcription. (b) Presence of glucose, in the presence of glucose cAMP levels decrease, cAMP–CAP complexes are not formed and thus there is no transcription.

Tryptophan Operon – Negative Repressible Control:

Tryptophan is an important amino acid in living system and in bacteria a complex mechanism is needed in its regulation. *E. coli* are capable for producing enzymes necessary for the biosynthesis of amino acids. Monod worked on an amino acid tryptophan and enzyme tryptophan synthetase and discovered that if tryptophan is present in sufficient quantity in the culture medium for *E. coli*, the enzymes necessary for its synthesis are not produced. It is energetically advantageous for bacteria to repress expression of genes involved in tryptophan synthesis when tryptophan is present in the culture medium (figure 3.3 a &b). Thus, Trp operon is an operon in bacteria which promotes the production of tryptophan when tryptophan is not present in the growth medium. If the amino acid is present, then the operon is repressed and biosynthetic enzymes are not produced.

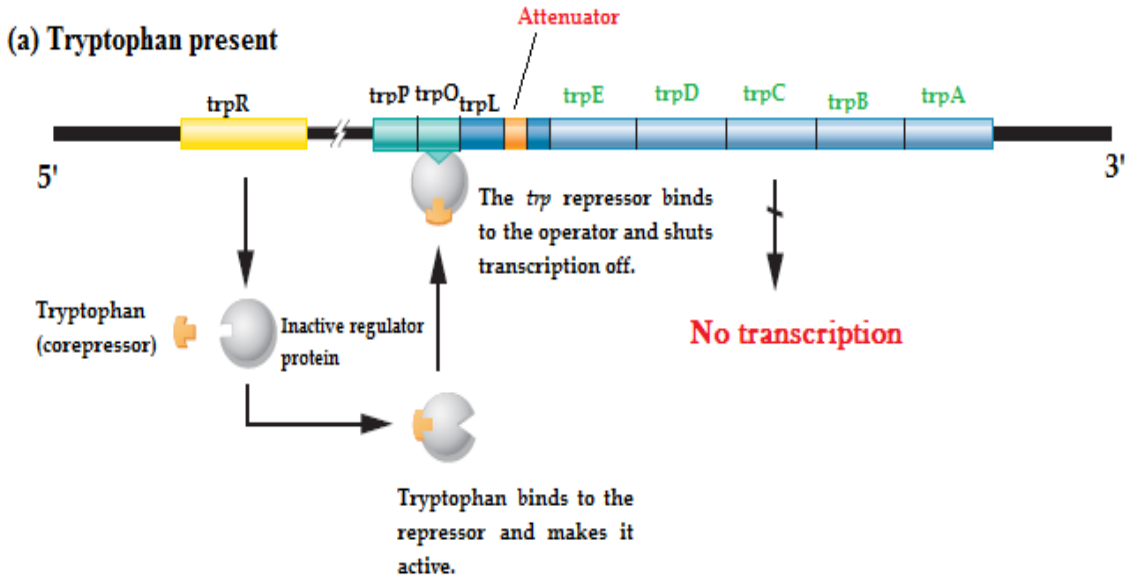

Figure 3.3 a: Tryptophan operon (when tryptophan present in the medium, it binds with the repressor causing allosteric reaction and formed product binds to operator and thus repressing transcription)

Further, investigation showed that a series of enzymes encoded by five contiguous genes (trpE, trpD, trpC, trpB, and trpA, and encode tryptophan synthetase) on the *E. coli* chromosome are involved in the conversion of chorismic acid to tryptophan. It also contains a promoter where RNA polymerase binds and a repressor gene which synthesizes a specific protein. This protein binds to the operator which then causes the transcription to be blocked.

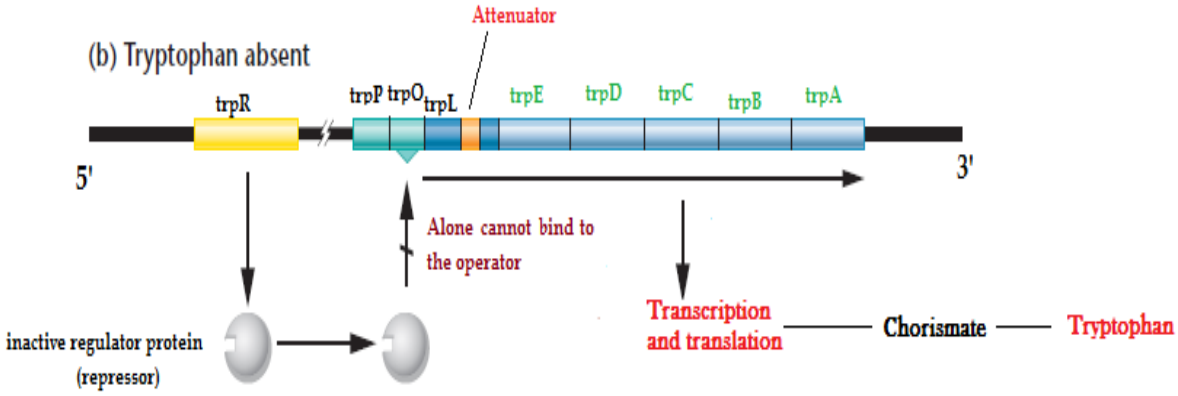

Figure 3.3 b: Tryptophan operon (in the absence of Tryptophan, inactive regulator protein cannot binds to operator, thus allowing transcription)

When tryptophan is abundant it binds to the trp repressor, causing a conformation change that permits the repressor to bind to the trp operator and inhibit expression of the trp operon. The

trp operator site overlaps the promoter, so binding of the repressor blocks binding of RNA polymerase.

Riboswitches:

Till know we have understands that operons of bacteria contain DNA sequences (promoters and operator sites) where the binding of small molecules induces or represses transcription. Besides these, some mRNA molecules may contain regulatory sequences and the sequences are called as riboswitches. They were first discovered in 2002 and now appear common in bacterium, In *Bacillus subtilis*, for example, approximately 5 percent of this bacterium's genes are regulated by riboswitches. They are also found in archaea, fungi, and plants, and may prove to be present in animals as well. These molecules may bind and affect gene expression by the formation of secondary structures in the mRNA. The two important domains within a riboswitch are the ligand-binding site, called the aptamer, and the expression platform, which is capable of forming the terminator structure.

3.3.2 Control of gene expression in Eukaryotes

In eukaryotic organism, gene expression is under tight control of series of reaction and checks in order to avoid any malfunction or over-function. So the genes are under very tight control to express the required level of proteins, because minor changes in the expression leads to lethal effects to the organism. To achieve the fine degree of control, eukaryotes employed wide range of mechanism for altering the expression of genes. Thus,control of gene expression in eukaryotes is controlled by a variety of mechanisms as followed –

1. Control at DNA level by Histone modifications and Chromatin Remodeling:

DNA is compacted into chromatin as part of its packaging strategy, this compacted form can affect the ability of transcriptional regulatory proteins and RNA polymerase to find access to specific genes for activation of transcription. Modification of the histones and of CpG methylation most affect accessibility of the chromatin to RNA polymerases and transcription factors.

a. **Acetylation of Histones**: One type of histone modification that affect chromatin structure. These modifications can bring about either the activation or the repression of transcription. Acetylation is catalyzed by histone acetyltransferase enzymes (HATs). A histone is wrapped twice around the nucleosome. They have positive lysine residues on their N-termini and are strongly attracted to DNA because of its highly negative phosphate backbone. If acetyl groups are added to the lysine 16 in the tail of the H4 histone prevents the formation of the 30-nm chromatin fiber causing the chromatin to be in an open configuration and available for transcription. The charge is neutralized which makes it possible for chromatin remodeling protein to nudge the

nucleosomes so that the TATA box is exposed. With TATA box exposed, TATA binding factor and transcription factor can bind to it, allowing RNA polymerase to bind to the pre-initiation complex and start transcription; other enzymes called deacetylases (histone deacetylases or HDACs) strip acetyl groups from histones and restore chromatin structure, which represses transcription because the deacetylase will remove acetyl groups from lysine on the N-termini of histones. This would cause the positive charge of the lysine and the negative charge on the DNA to become strongly attracted to each other and the histones would bind tightly.

b. **Methylation of Histones**: A common modification is the addition of three methyl groups to lysine 4 in the tail of the H3 histone protein, abbreviated H3K4me3. This modification is frequently found in the transcriptionally active genes in eukaryotes. Recent studies suggested that NUFR and certain proteins binds to the H3 histone tail and allowing the transcription to take place.
c. **Methylation of DNA:** Another type of change in chromatin that plays a role in gene regulation is the methylation of cytosine bases, which yields 5-methylcytosine. Methylation occurs most often in symmetrical CG sequences (CpG, where p represents the phosphate group in the DNA backbone, called CpG island) in small percentage of newly synthesized DNA. DNA methylation prevents the binding of the transcriptional machinery and it's associated with transcriptional silencing. This methylation have inverse relationship with the gene expression, therefore transcriptionally inactive genes possess higher levels of methylated DNA than transcriptionally active genes. There are number of evidence to support the observation i.e., inactivated X chromosome in mammalian female cells are often heavily methylated and another example is that the methylation patterns are tissue specific and once established, are heritable for all cells of that tissue.

2. Control at Transcription Initiation:

The regulation of gene expression at the level of transcription is most important and primary mode of control of eukaryotic gene expression. Transcription of the different classes of RNAs in eukaryotes is carried out by three different polymerase. Such as RNA pol I synthesizes the rRNA. RNA pol II synthesizes the mRNA and some snRNA. RNA pol III synthesizes the 5S rRNA and the tRNA. Transcription regulation in a eukaryotic cell is mediated by transcription factor, other than the general transcription factors, which recognized and bind to short regulatory DNA sequences associated with the gene. These sequences are also called cis-acting elements and the protein transcription factors that bind to these elements are also known as transacting factors. These factors which regulate specific gene transcription do so by interacting with the proteins of the transcription initiation complex and may either increase or decrease the rate of transcription of the target gene. Many transcription factors bind to control elements located upstream within a few hundred base parirs of the protein

coding gene. The SPI box (the SPI box has the core sequence GGGCGG, and binds to transcription factor SP1 which then interacts with one of the TAF_{II} proteins that bind to TBP to form TFIID) and CAAT box are examples of such regulatory elements found upstream of most protein coding genes, but some upstream regulatory elements are associated with only a few genes and are responsible for gene specific transcriptional regulation. Besides this, many repressor protein that inhibits the transcription of specific gene in eukaryotes may bind either to control elements near to the target gene or to silencers that may be located a long distance away. The repressor protein may inhibit transcription of the target genes directly (example in the mammalian thyroid hormone receptor which, in the absence of thyroid hormone, represses transcription by blocking activation). However, other repressors inhibit transcription by blocking activation or by interfering with the function of an activator protein required for efficient gene transcription.

3. Control at mRNA Splicing – Alternate Splicing:

Most eukaryotic pre – mRNA contain multiple introns (non coding DNA). The process of excising the sequences in RNA that corresponds to introns and joining of sequences corresponding to exon is called RNA. Splicing is done by spliceosomes, ribonucleoprotein complexes that can recognize the two ends of the intron, cut the transcript at those two points, and bring the exons together for ligation. Cajal bodies within the nucleus are centers of post-transcriptional modification of snRNAs and snRNPs assembly. Alternative RNA splicing is a mechanism that allows different protein products to be produced from one gene when different combinations of exons are combined to form the mRNA. Use of alternative splice sites in the same pre – mRNA allows the synthesis of different proteins from the same gene or can function as an on – off switch to make or not make a functional protein. Drosophila sex determination, T-antigen gene of the mammalian virus SV40 and human genome provides the best example of a regulated alternative splicing. Further, in trans-splicing (found in few eukaryotic organisms), exons from two separate RNA transcripts are spliced together to form a mature mRNA molecule.

4. Control at mRNA Stability:

Once mRNAs are produced, they get exported from the nucleus to the cytoplasm. They are given two protective "caps" that prevent the ends of the strand from degrading during its journey. The 5' cap, which is placed on the 5' end of the mRNA, is usually composed of a methylated guanosine triphosphate molecule (GTP). The poly-A tail, which is attached to the 3' end, is usually composed of a long chain of adenine nucleotides. These changes protect the two ends of the RNA from exonuclease attack. Within the cytoplasm they are translated until degradation. Long – lived mRNA give rise to more polypeptides than short – lived mRNAs. Therefore mRNA life time also can be targeted as an important point for regulation of gene expression. The longevity of mRNA is influenced by some factors such as: The sequence of the 3’ UTR preceding the poly – A tail also affects mRNA stability. Some short – lived

mRNAs have many repeated sequences AUUUA in this region, referred to as an AU rich element. Further, the concentration of some metabolites, such as hormones, can also influence mRNAs longevity.

5. Control at Initiation of Translation:

Regulation of gene expression may also occur at the level of translation, but the regulation is less efficient in comparison to the transcriptional regulations. Control mechanism at initiation of translation either prevent the synthesis of protein or controls the amount of protein produced from a given mRNA. An example of translational control is proteins involved in iron metabolism. The level of ferritin is regulated in response to the iron supply. When iron concentration is low, translation of ferritin mRNA is reduced, while when iron concentration is high, ferritin synthesis increases.

6. Post – Translational Control:

Post translational control of gene expression can be defined as a regulation process in which protein structure is modified and as a result protein function is modified. These modifications involves chemical modification of amino acids or alteration of the order of amino acids in the polypeptide backbone. The most common chemical modification of amino acids include phosphorylation, glycosylation and ubiquitiation.

7. RNA Editing:

RNA editing can be best define as a process in which the RNA sequence is modified, so the mature RAN differ from that encoded by the genomic sequence or in other words RNA editing is the name given to several reactions whereby the nucleotide sequence on an mRNA molecule may be changed by mechanism other than the RNA splicing. An example of RNA editing in human is apolipoprotein B mRNA. Liver cells produce apolipoprotein B100 without any RNA editing, but in the cells of intestine, RNA editing causes the conversion of single C residue to U and this changes the codon for glutamine (CAA) to a termination codon (UAA), which further results in shorter apolipoprotein B48(48% of the size of apolipoprotein B100). There are two RNA editing mechanisms which include: a site specific base modification and insertion-deletion editing.

a. Site specific editing (substitution editing): In site specific editing (include transversion and transition in all classes of RNA), a cytosine residue within the RNA is changed into uridine by deamination. For example, deamination of adenosine to produce inosine. Since inosine (inosine behave like guanosine) can base pair with cytosine, this induces point mutation which alter the protein sequence.

b. Insertion-Deletion editing: This type of editing reported in the mitochondrial RNA of some Protozoans. In this mechanism uridines are inserted into the transcripts as found in the mitochondrial mRNA of trypanosomes. These insertions completely shift the reading frame and give rise to totally different proteins.

8. Regulation by miRNA:

MicroRNAs (miRNAs) or short RNA molecule that are about 20-22 nucleotide long. The miRNAs are made in the nucleus as longer pre-miRNAs. These pre-miRNAs are chopped into mature miRNAs by a protein called Dicer. There are hundreds of genes encoding miRNA in eukaryotes and most of them are involved in developmental regulation. These microRNAs regulate gene expression through at least four distinct mechanisms: (1) cleavage of mRNA, (2) inhibition of translation, (3) transcriptional silencing, or (4) degradation of mRNA.

3.4 SUMMARY

In this unit, we learnt various mechanisms for control of gene expression in prokaryotes as well eukaryotes. Jacob and Monod 1961, proposed a scheme for induction and repression for which they awarded nobel prize in 1965. Genes in bacterial cells are typically clustered into an operons group of functionally related structural genes and the sequences that control their transcription. Structural genes in an operon are transcribed together as a single mRNA molecule. In negative control, a repressor protein binds to DNA and inhibits transcription and in positive control, an activator protein binds to DNA and stimulates transcription. Further, In inducible operons, transcription is normally off and must be turned on; in repressible operons, transcription is normally on and must be turned off. The lac operon of *E. coli* is a negative inducible operon. While the, trp operon of *E. coli* is a negative repressible operon that controls the biosynthesis of tryptophan. Attenuation causes premature termination of transcription. riboswitches in mRNA molecules induce changes in the secondary structure of the mRNA, which affects gene expression.

But this process is somewhat different in eukaryotic. Eukaryotic cells differ from bacteria in several ways that affect gene regulation, including, in eukaryotes, the absence of operons and the presence of chromatin & nuclear membrane. Chromatin play an important role in the gene expression and during transcription, chromatin structure may be altered by the modification of histone proteins, including acetylation, phosphorylation, and methylation. Besides these, the repositioning of nucleosomes and the methylation of DNA also affect transcription or may contribute in gene regulation. The Epigenetic effects are resulted due to the DNA methylation and alterations to chromatin structure. Further, the initiation of eukaryotic transcription is controlled by general transcription factors that assemble into the basal transcription apparatus and by transcriptional activator proteins that stimulate normal levels

of transcription by binding to regulatory promoters and enhancers. The expression of gene in eukaryotic cells may also influenced by RNA processing. The 5′ cap, the coding sequence, the 3′ UTR, and the poly(A) tail are important in controlling the stability of eukaryotic mRNAs. Proteins binding to the 5′ and 3′ ends of eukaryotic mRNA can affect its translation. . Posttranscriptional gene regulation includes alternative splicing of nascent RNA, RNA transport, or changes in mRNA stability. Alternative splicing increases the number of gene products encoded by a single gene. Posttranscriptional gene regulation at the levels of translation and protein stability also affect the levels of active gene product. RNA-induced gene silencing is a posttranscriptional mechanism of gene regulation that affects the translatability or stability of mRNA as well as transcription.

Thus from this chapter we come to understand that, the aim of regulation is to control the gene expression to only when they are needed. For example, in prokaryotes the catabolic machinery needed to metabolize lactose only needs to be available when lactose is present. We also saw that regulation of gene expression in eukaryotes is crucial for an essential multicellular organism to develop harmoniously according to a pre – determined genetic program. The eukaryotic cell structure provides possible control for gene expression at many levels such as chromatin structure, transcription initiation, at RNA level and post – translation level.

3.5 GLOSSARY

3' UTR: 3' untranslated region; region just downstream of the protein-coding region in an RNA molecule that is not translated.

5' cap: a methylated guanosine triphosphate (GTP) molecule that is attached to the 5' end of a messenger RNA to protect the end from degradation.

5' UTR: 5' untranslated region; region just upstream of the protein-coding region in an RNA molecule that is not translated.

Activator: protein that binds to prokaryotic operators to increase transcription.

Catabolite activator protein (CAP): protein that complexes with cAMP to bind to the promoter sequences of operons which control sugar processing when glucose is not available.

Cis-acting element: transcription factor binding sites within the promoter that regulate the transcription of a gene adjacent to it.

Cistron: a section of a DNA or RNA molecule that codes for a specific polypeptide in protein Synthesis.

Codon: Sequence of three nucleotides in DNA or mRNA that specifies a particular amino acid during protein synthesis; also called triplet.

Consensus sequence: The nucleotides or amino acids most commonly found at each position in the sequences of related DNAs, RNAs or proteins.

Dicer: enzyme that chops the pre-miRNA into the mature form of the miRNA.

DNA methylation: epigenetic modification that leads to gene silencing; a process involving adding a methyl group to the DNA molecule.

Enhancer: segment of DNA that is upstream, downstream, perhaps thousands of nucleotides away, or on another chromosome that influence the transcription of a specific gene.

Epigenetic: heritable changes that do not involve changes in the DNA sequence.

Eukaryotic initiation factor-2 (eIF-2): protein that binds first to an mRNA to initiate translation.

Exon: Segment of a eukaryotic gene that reaches the cytoplasm as part of a mature mRNA, rRNA or tRNA.

Gene expression: Overall process by which the information encoded in a gene is converted into an observable phenotype.

Gene: Physical or functional unit of heredity, which carries information from one generation to the next.

Genetic code: The set of rules whereby nucleotide triplets or codon in DNA or RNA specify amino acid in proteins.

Genome: Total genetic information carried by a cell or organism.

Glycosyl transferase: An enzyme that forms a glycosidic bond between a sugar residue and an amino acid side chain of a protein or a residue in an existing carbohydrate chian.

Histone acetylation: epigenetic modification that leads to gene silencing; a process involving adding or removing an acetyl functional group.

Inducible operon: operon that can be activated or repressed depending on cellular needs and the surrounding environment.

Initiation complex: protein complex containing eIF-2 that starts translation.

Intron: Part of a primary transcript that is removed by splicing during RNA processing and is not included in the mature, functional mRNA, rRNA or tRNA.

microRNA (miRNA): small RNA molecules (approximately 21 nucleotides in length) that bind to RNA molecules to degrade them.

Monocistronic: Describing a type of messenger RNA that can encode only one polypeptide per RNA molecule. In eukaryotic cells virtually all messenger RNAs are monocistronic.

Negative regulator: protein that prevents transcription.

Operator: region of DNA outside of the promoter region that binds activators or repressors that control gene expression in prokaryotic cells.

Operon: In bacterial DNA, a cluster of contiguous genes transcribed from one promoter that gives rise to a polycistronic mRNA.

Poly-A tail: a series of adenine nucleotides that are attached to the 3' end of an mRNA to protect the end from.

3.6 SELF ASSESSMENT QUESTION

3.6.1 Objective type questions

Q1. To which region of a gene does an RNA polymerase bind to initiate transcription?

(i) 5’ UTR (ii) 3’ UTR

(iii) CDS (iv) Promoter

Q2. Which of the following processes generates multiple transcripts from the same gene?

(i) Riboswitching (ii) Alternative splicing

(iii) Short-peptide translation (iv) All of these

Q 3. Which of the following processes involves metabolite-sensing in non-coding portions of mRNAs to control gene expression?

(i) Alternative polyadenylation (ii) Riboswitching

(iii) Adenosine methylation translation

(iv) Short-peptide

Q4. A genomic DNA possesses functioning units, a group of genes under the influence of promoters known as

(i) genes

(ii) operons

(iii) anticodon

(iv) codon

Q5. There are these many histones in the core of a nucleosome

(i) 8

(ii) 6

(iii) 4

(iv) 2

Q6. In eukaryotes and bacteria, the most common form of regulation is

(i) promoter control

(ii) translation control

(iii) repressor control

(iv) transcriptional control

Q7. In a bacterial operon, which is located downstream of the structural genes?

(i) operator

(ii) inducer

(iii) promoter

(iv) regulatory gene

Q8. The lac operon consists of ____ structural genes.

(i) 1

(ii) 2

(iii) 3

(iv) 4

Q9. Which of the following acts as an inducer in the lac operon?

(i) glucose

(ii) tryptophan

(iii) lactose

(iv) galactose

In bacteria, mRNAs bound to small metabolites are called

(i) euchromatin

(ii) riboswitches

(iii) heterochromatin (iv) nucleosome

3.6.1 Answers: 1-(iv), 2- (ii), 3- (ii), 4- (ii), 5-(i), 6- (iv), 7-(iv), 8- (ii), 9-(iii), 10- (ii).

3.6.2 Short notes

Q1. What are promoters and operators?

Q2. Mention the functions of lac operon genes.

Q3. What is RNA interference?

Q4. What are inducible genes and repressible genes?

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3.9 TERMINAL QUESTIONS

Q1. What is the difference between positive and negative control? What is the difference between inducible and repressible operons?

Q2. Briefly describe the lac operon and how it controls the metabolism of lactose.

Q3. How does bacterial gene regulation differ from eukaryotic gene regulation? How are they similar?

Q4. Outline the role of alternative splicing in the control of sex differentiation in Drosophila.

Q5. What role does RNA stability play in gene regulation?

Q6. What is catabolite repression? How does it allow a bacterial cell to use glucose in preference to other sugars?

Q7. Write an essay on regulation of gene activity in eukaryotes.

BLOCK-2

BIOTECHNOLOGY

UNIT-4: BASIC CONCEPTS, PRINCIPLES AND SCOPE OF BIOTECHNOLOGY

Contents:

4.1. OBJECTIVES

- After reading this unit you would be able to:
    - Describe the basic concepts of biotechnology;
    - Define and enumerate the principles of plant biotechnology;
    - Discuss the scope of Biotechnology

4.2. INTRODUCTION

The use of living organisms to create technologies and goods for human improvement and sustainable development is known as biotechnology. It is a branch of applied biology and a synthesis of biology and technology. The idea of biotechnology first emerged in the 19th century when plant and animal cells, along with their component parts, were cultivated in vitro to produce desired goods. Since then, microbes have been employed in the mass manufacture of biochemicals, processing of food, and environmental cleanup.

The main applications of biotechnology are in the areas of therapeutics, diagnostics, food processing, bioremediation, waste treatment, energy production, technology, medicine and other fields that require bio-products. It is also used to improve agriculture through the use of genetically modified crops. Recombinant DNA technology has made it feasible to produce microbes, plants, and animals that possess superior and novel capabilities. Genes from one organism have been added to another to generate genetically modified organisms, or GMOs. For tolerance to high temperatures, salt stress, and resistance to disease and insect attack, plants have been genetically engineered. These approaches have shown to be very effective in raising crop plant production and lowering post-harvest losses. The use of biotechnology has enabled the development of food plants with increased nutritional value and a decreased dependency on chemical pesticides (pest-resistant crops). The current Unit introduces you to the idea of biotechnology and informs you about its scope, applications and several plant biotechnology techniques and their uses.

4.3. BASIC CONCEPT OF BIOTECHNOLOGY

Biotechnology is NOT brand-new. Man has been utilizing living things to better his life ever since the beginning of time. Man has used plants and animals for various purposes since the beginning of time (about 8000–4000 B.C.). It is important to understand the basic concept of biotechnology in order to understand its principles and processes.

Biotechnology is the use of living systems and organisms to produce or develop useful products. It can also refer to "any technological" application that uses biological systems, live organisms,

or their derivatives to produce, alter, or produce goods for a specific purpose. The application of biotechnology in agriculture, food production, and medicine has been practiced by humans for thousands of years.

The development of pharmacological treatments and diagnostic procedures, as well as new and diversified sciences like genomics, recombinant gene technologies, applied immunology, and others, have all been included into the field of biotechnology in the late 20th and early 21st centuries. Over time, there were several developments made in the field of biotechnology.

4.3.1. Various stages of biotechnology development

The advancement of biotechnology falls under the following categories:

- **Ancient biotechnology (8000–4000 BC):** Early biotechnology history is associated with the domestication of animals and the cultivation of plants.
- **Classical biotechnology (2000 BC; 1800–1900 AD)**: This period of biotechnology was founded on the use of microbes in fermentation, food production, and medicine. Genetics developed during this time (1900–1953), and DNA study started (1953–1976).
- **Modern biotechnology (1977):** During this time, genetic engineering was used to alter the genetic material of living things.

Ancient biotechnology

Before the year 1800, activities like the domestication of animals for milk or meat and the cultivation of plants like rice, barley, and wheat as the primary source of sustenance were considered as biotechnological advances. Animals and plants have been bred in an effort to better them by introducing desirable traits.

Classical biotechnology

Microorganisms were employed to produce foods like bread, cheese, and beverages after carefully breeding plants and animals. Leguminous crop rotation, vaccines, and animal-drawn machinery all became common in the late eighteenth and early nineteenth centuries. A turning point in biology occurred around the end of the nineteenth century. Mendel's genetic research was completed, microorganisms were identified, and Koch, Pasteur, and Lister founded research centers to study fermentation and other microbial activities. Edward Jenner, who is regarded as the father of vaccination science, established the effectiveness of vaccination in preventing smallpox in 1796. Louis Pasteur gave the first description of the scientific evidence for fermentation in the late 1800s. He put out the germ theory, which holds that microbes are crucial to the fermentation process. Since ancient times, honey and soybean curds have been utilized as natural antibiotics and have been proven to be successful in treating wounds. Proteins and enzymes were first identified in 1830.

The term "Biotechnology" was first used by Hungarian engineer **Karl Ereky** in 1919. He defines biotechnology as the use of living organisms to the production of valuable resources or products. In other words, it is the technology that uses biological mechanisms, systems, or processes to produce products that enhance human life.

Karl Ereky

Beginning in the early 20th century, biotechnology started to connect business and agriculture. For the rapidly expanding vehicle industry, fermentation techniques were created during World War-I, in 1920, Chaim Weizman produce valuable chemical compounds using biological methods. Utilizing a bacterium called *Clostridium acetobutylicum*, he produced butanol from starch. Later, acetone from starch and biotechnology techniques like fermentation was developed for use in the automotive paint industry. The production of penicillin began with the outbreak of World War-II. In 1929, Alexander Fleming produced antibiotic penicillin from cultures of *Penicillium notatum*. The penicillin was helpful in treating soldiers who were injured World War-II. After that the focus of biotechnology shifted to drugs such as development of antibiotics, fermentation techniques, and work with microorganisms etc.

Modern biotechnology

Currently, modern biotechnology is applied in numerous industries, such as agriculture, bioremediation, food processing, and energy production. In forensics, the use of DNA fingerprinting is increasingly widespread. Cloning vectors with the chosen gene already present allows for the production of insulin and other medications. Cellular and molecular technologies started to develop later in the 1960s and 1970s. Research on DNA was undertaken between 1953 and 1976.To produce valuable products, cells and biological substances like DNA, RNA, and proteins were used. The real biotechnology was initiated with the production of Insulin in 1970. A similar discovery was made in 1970 regarding the first restriction enzyme. By making this finding, the researchers were able to introduce foreign genes into bacteria. The development of recombinant DNA technology was based on this, and it is regarded as the beginning of modern biotechnology. Living things' DNA was manipulated, and substances produced from GMOs were applied to the alteration of molecules. Dolly, the sheep was cloned in 1977 by scientists in Scotland; later, Polly the sheep was cloned using nuclear transfer technique since it possessed a human gene expressing Factor IX, a protein important in preventing hemophilia. In the middle to late 1970s, saw the founding of businesses like Genentech, Amgen, Biogen, Cetus, and Genex. They produced genetically modified materials mainly for environmental and medical purposes. Stem cell research has attracted the attention of molecular biologists. Antibody analysis was discovered to be another useful tool in 1999. Modern biotechnology is used in therapeutics, primarily for the discovery, development, and production of novel medications, and in diagnostics, for protein- and nucleic acid-based tests. It has mostly been employed in the environmental field to treat waste and prevent pollution. To produce plants that are resistant to pests, weeds, and plant diseases, genetic engineering is being applied in agriculture. Thus modern biotechnology makes significant contribution in improving the lives of humans. A brief overview of the biotechnology's past occurrences has been provided in Table 4.1.

4.4. DEFINITIONS

Biotechnology is the combination of the two words "bio" for life and "technology," which refers to the application of knowledge for practical use, i.e., the use of living things to make or improve products.

A "Janus-faced" description of biotechnology has been used. By implication, two sides are present. Techniques enable the transfer of genes from one organism to another by manipulating DNA on the one hand. On the other hand, it makes use of recent technologies whose effects have not yet been thoroughly investigated and should be approached with caution.

- "All area of work by which product are produced from raw materials with the aid of living things". **-Karl Ereky**
- Biotechnology is defined as the "integrated use of biochemistry microbiology and engineering science in order to achieve technological application of the capabilities of microorganism. Cultured tissue cell and part's thereof".

  -European Federation of Biotechnology
- "A technology using biological phenomenon for copying and manufacturing various kind of useful substances". **-Japanese biotechnology**
- "The controlled used of biological agent such as micro-organism or cellular components for beneficial use." **-U.S. National science foundation**

Table 4.1 An overview of biotechnology's past occurrences.

Period	Time/ Year	Historical Events
Pre-1800	6000 BC	Yeast was used to prepare beer
	4000 BC	In Egypt, yeast was used to prepare bread.
	420 BC	Greek philosopher Socrates found that similar characteristics are found between parents and their offspring.
	320 BC	Greek philosopher Aristotle gave the concept that characters get inherited from father.
	1000 AD	Theory of abiogenesis, or spontaneous generation was proposed.
	1630	William Harvey discovered the circulation of blood in the body.
	1660–1675	Marcello Malpighi found blood circulation in capillaries using a microscope and found that the brain is connected to the spinal cord by bundles of fibers which form the nervous system.

	1673	Antonie van Leeuwenhoek identified microorganisms such as protozoa and bacteria, and suggested that micro-organisms play an active role in fermentation.
	1701	Giacomo Pylarini found that the administration of smallpox prevents its occurrence later in life. The procedure was termed 'vaccination'.
1800–1900	1809	Nicolas Appert invents a technique using heat to sterilize food.
	1856	Justus von Liebig, Pasteur (1822–1895) suggested that microbes are responsible for fermentation.
	1859	Charles Darwin (1809–1882) speculated 'Natural Selection'.
	1863	The method of pasteurization was discovered by Pasteur. Heinrich Anton de Bary found that fungus was responsible for potato blight.
	1865	Mendel suggested ‘the Laws of Heredity’.
	1868	Johannes Friedrich Miescher separated Nuclein (a compound made of nucleic acid) from pus cells.
	1870	Walther Flemming discovered Mitosis.
	1871	Koch investigated anthrax and explored techniques to identify culture and stain micro-organisms.
	1880	Louis Pasteur explored weakened (attenuated) strains of micro-organisms that might not be virulent.
	1881	Koch explained techniques for harvesting bacterial colonies on potato slices, gelatin and agar medium.
	1884	Gram described the differential staining technique for cellular peptidoglycan-containing bacteria now known as Gram staining.
1900-1953	1900	Mendel's work was revived by de Vries, von Tschermak and Correns.
	1902	Sutton found that chromosomes (paired) contain certain elements which are transferred from one generation to another.
	1905	Edmund Beecher Wilson and Nettie Stevens demonstrated that a single Y chromosome determines maleness, while two copies of the X chromosome decide femaleness.
	1905-1908	William Bateson and R C Punnett found that several genes alter or modify the action of other genes.
	1906	Paul Erlich also investigated atoxyl compounds and discovered the important features of Salvarsan (the first chemotherapeutic agent).
	1907	Thomas Hunt Morgan revealed that chromosomes have a defined role in heredity. He discovered mutation theory into fruit flies.
	1909	Wilhelm Johannsen used the word 'gene' for the carrier/transporter of heredity. He also coined the terms 'genotype' and 'phenotype'.

	1910	Morgan demonstrated that genes are present on chromosomes.
	1911	Morgan established the separation of certain inherited features that are generally linked to the separation/breaking of chromosomes during the process of cell division.
	1912	William Lawrence Bragg discovered the application of X-rays in the determination of the molecular structure of crystalline substances (Crystallography).
	1918	Herbert M Evans found that human genetic material is made up of 48 chromosomes.
	1926	Hermann Joseph Muller discovered that X-rays are responsible for genetic mutations in fruit flies.
	1928	Frederick Griffiths observed the 'transforming principle' by which a rough type of bacterium is transformed to a smooth type.
	1938	Proteins and DNA were studied by means of X-rays. The term 'molecular biology' was coined.
	1941	George Wells Beadle and Edward L Tatum proposed 'one gene, one enzyme' theory.
	1943	Canadian scientist Oswald Theordore Avery discovered that DNA is the carrier of genes.
	1943-53	Cortisone, a steroid hormone was considered as the first biotech product.
	1944	Selman Abraham Waksman explored streptomycin, an antibiotic active against TB.
	1945-1950	Animal cell cultures were harvested for the first time in laboratories, giving birth to the field of animal tissue culture.
	1947	Barbara McClintock first demonstrated 'transposable elements' known as 'jumping genes' with the capability to move (or jump) from one site on the genome to another site.
	1950	Erwin Chargaff discovered that the same levels of adenine and thymine are present in DNA, as are the same levels of guanine and cytosine. These associations were later named 'Chargaff's rules'.
	1952	George Otto Gey created a cell line known as HeLa from a human cervical carcinoma.
1953-1976	1953	Watson and Crick's article based on unfolding the double-helix structure of DNA was published in the journal, Nature.
	1953–1976	The discovery of the structure of DNA revolutionized molecular biology and genetics.
	1957	Crick and Gamov studied 'central dogma', demonstrating how DNA functions to construct protein.

	1958	Arthur Kornberg created DNA in a test tube for the first time. The mechanical protein sequencer, the Moore–Stein amino acid analyzer, was developed.
	1959	François Jacob and Jacques Lucien Monod documented concept of genetic regulation. They explained the concept of 'repressor' and 'operon'.
	1960	A French researcher discovered messenger RNA (mRNA).
	1961	Francois Jacob and Jacques Monad gave the concept of Operon.
	1962	Watson and Crick were awarded the Nobel Prize in Physiology or Medicine with Maurice Wilkins.
	1963	Samuel Katz and John F Enders developed the first vaccine for measles.
	1964	Enzyme reverse transcriptase was discovered.
	1966	Marshall Warren Nirenberg, J Heinrich Matthaei and S Ochoa reported that a genetic sequence of three nucleotide bases (called codons) decides each of 20 amino acids.
	1967	Arthur Kornberg reported a study using single stranded natural viral DNA.
	1969	An enzyme is synthesized in vitro conditions.
	1970	Virologists Peter H Duesberg and Peter K Vogt identified the first oncogene in a virus.
	1967-1971	Maurice Hilleman made the first American vaccine for mumps. The first vaccine for rubella was developed.
	1972	Paul Berg, a biochemist, utilized a restriction enzyme to cut DNA into fragments. He employed a ligase enzyme to join two DNA strands concurrently to form a hybrid circular molecule.
	1973	Bruce Nathan Ames, a biochemist developed an investigation to distinguish chemicals that damage DNA. Later, the Ames test became extensively used to identify cancer-causing substances.
	1974	The first vaccine for chicken pox was developed.
	1975	Colony hybridization and Southern blotting were explored for identifying specific DNA sequences. The first monoclonal antibodies were prepared.
	1976	J Michael Bishop and Harold Varmus established the presence of cancer-causing genes called oncogenes on animal chromosomes. The NIH published the first guidelines for rDNA research.
1977– (Modern biotechnology)	1977	With the advent of genetic engineering it was possible to produce human protein in bacteria for the first time. R Austrian and his coworkers developed the first vaccine for pneumonia.

1978	Herbert W Boyer synthesized synthetic human insulin by introducing the insulin gene into the bacterium *Escherichia coli*. Louise Brown, the first test-tube baby, was born in the UK. The first vaccine for meningococcal meningitis was developed.
1980	Kary Mullis and coworkers established a tool for multiplying DNA sequences in vitro using the polymerase chain reaction (PCR).
1982	US Food and Drug Administration (FDA) allowed the first genetically engineered drug in the form of human insulin produced by bacteria. Michael Smith at the University of British Columbia, Vancouver, established a procedure for producing precise amino acid changes anywhere in a protein.
1983	The first genetically modified (transgenic) plant is formed.
1984	The DNA fingerprinting technique was discovered. The first genetically engineered vaccine was discovered for hepatitis B.
1985	The NIH published guidelines for performing experiments in gene therapy on humans. Genetically engineered plants resistant to viruses, insects and bacteria were field-tested for the first time.
1986	The NIH sanctioned guidelines for executing trials of gene therapy on humans. The automated DNA sequence was discovered in California.
1987	Calgene Inc. obtained a patent for the tomato polygalacturonase DNA sequence, which was later used to synthesize an antisense RNA sequence that can further extend the shelf life of fruit. Maynard Olson and colleagues at Washington University discovered yeast artificial chromosomes, which are expression vectors for large proteins.
1989	A gene responsible for cystic fibrosis was explored.
1990	Human Genome Project was launched.
1993	Kary Mullis won the Nobel Prize in Chemistry for inventing the tool of PCR. FDA approved a recombinant protein to treat multiple sclerosis.
1994	A number of genes, human and others were identified and their functions explained.
1995	The first vaccine for hepatitis A was explored. Researchers at the Institute for Genomic Research completed the first full gene sequence of a living organism for the bacterium *Haemophilus influenzae*.

1997	Researchers at the Roslin Institute in Scotland cloned a sheep called Dolly from the cell of an adult ewe. Dolly was the first mammal cloned by a technique called nuclear transfer technology. The first human artificial chromosome was discovered.
1998	A group of researchers succeeded in culturing embryonic stem cells. A draft of the human genome map is created that locates more than 30,000 genes. Embryonic stem cells were employed to regenerate tissue and produce disorders mimicking diseases.
2000	Sir Hargobind Khorana synthesized DNA in a test tube. He demonstrated the role of nucleotides in protein synthesis and helped in finding the genetic code.
2001	The journals Science and Nature reported the human genome sequence.
2002	A very rapid shotgun sequencing of major genomes was completed.
2003	Celera and the NIH successfully finished the sequencing of the human genome.
2004	The FDA supported the first monoclonal antibody for cancer therapy.
2006	The FDA sanctioned a recombinant vaccine against human papilloma virus. Researchers established the three-dimensional (3D) structure of HIV.
2008	Japanese chemists developed the first DNA molecule made nearly entirely of artificial parts.
2009	Scientists identified three new genes connected with Alzheimer's disease, paving the way for possible new diagnostics and therapeutics.
2013	Researchers established the three-dimensional (3D) structure of HIV, which causes AIDS.
2010	Researchers from the J.Craig Ventere Institute create the first synthetic cell.
2011	Trachea derived from stem cells transplanted into human recipient. Advances in 3-D printing technology lead to “skin-printing.”

2012	Synthesis of a polymer- XNA, third information-storing molecule by molecular biologists Vitor Pinheiro and Philipp Holliger of the Medical Research Council in the United Kingdom. Just like DNA, XNA is capable of storing genetic information and then evolving through natural selection.
2013	Researchers in Japan developed functional human liver tissue from reprogrammed skin cells.
2014	Created new DNA bases in the lab, expanding life's genetic code and opening the door to creating new kinds of microbes.
2015	Researchers in Sweden developed a blood test that can detect cancer at an early stage from a single drop of blood.
2016	For the first time, bioengineers created a completely 3D-printed 'heart on a chip.'
2017	Discovered a new molecular mechanism that might be the cause of severe premenstrual syndrome known as PMDD. Scientists engineer disease-resistant rice without sacrificing yield. Blood stem cells grown in lab for the first time.

4.5 TYPES OF BIOTECHNOLOGY

The application of biotechnology has grown in recent years. In accordance with the field in which it is used, it has been categorized into various types.

1. **Agricultural Biotechnology**

Biotechnology has been used in developing genetically modified plants to increase crop production or introduce characteristics that impart tolerance against biotic and abiotic stress, provide resistance against pest and pathogen attack. The contribution of biotechnology in the field of agriculture includes organic agriculture, agrochemical based agriculture, and genetically engineered crop based agriculture etc. Examples- development of crops that express anti-pest characteristics; the genes of *Bacillus thuringiensis* have been transferred to crops; a protein (Bt) produced by *Bacillus thuringiensisis* found to be very effective against pests such as the European corn borer.

2. **Medical Biotechnology**

It deals with the application of living cells and other substances to improve human and plant health. It is utilized as a research tool for studying human and plant health, identifying pathogens, and human cell biology, as well as for the treatment of diseases or the prevention of diseases. There is a huge variety of biotechnology products available for medicinal application, as outlined in Table 4.2. Some products are designed to resemble their human counterparts, while others are made to be different from them. These products can be analogues, ones that have undergone chemical modification (such being pegylated), or they can be completely new

(e.g., single chain or fragment antibody products, gene transfer vectors, tissue-engineered products). The procedure is employed to produce pharmaceutical medications and other compounds that combat diseases. The field is used for development of new drugs and treatments. An anti-lymphoma vaccination has been created using genetically modified tobacco plants that exhibit RNA (a chemical similar to DNA) from malignant (actively cancerous) B-cells.

Table 4.2 Biotechnological products in medicine

Class	Products
Hormones	Growth Hormone, Follicle stimulating hormone, Insulin, Insulin analogues
Growth factors	Platelet-derived growth factor, Nerve growth factor, Insulin growth factor-I
Cytokines	Interferones, Interleukins, Colony stimulating factor, Erythropoietin
Vaccines	Conventional, Recombinant protein antigen, Modified bacteria or viruses
Nucleic acid based products	Gene therapy, DNA vaccines, Ribozymes
Cell, tissue and organs	Autologous, Xenoxenix
Others	Clotting factors, Enzymes

3. Industrial Biotechnology

Biotechnology is also used in the industrial sector. It comprises the utilization of microorganisms or parts of cells, like enzymes, to produce products that are valuable for the industrial sector, such as food and feed, chemicals, detergents, textiles, paper and pulp, biofuels, and biogas. Examples: Industrial biotechnology has enabled the development of biocatalysts for the synthesis of chemicals.

4. **Environmental Biotechnology**

Environmental biotechnology is a branch of biotechnology that uses biological processes to address environmental issues like the genetic rescue of a species, the elimination of pollutants, the manufacture of renewable energy sources, or the production of biomass. Examples: In bioremediation, the potential of living organisms is used to remove and treat waste. The development of enzyme bioreactors allows for the efficient removal of industrial and food waste components via sewage systems. Recently, a new classification scheme for biotechnology was adopted. Different biotechnology branches have been assigned different colours in this form of classification (Table 4.3 and Fig. 4.1.).

Table 4.3 Classification of different biotechnology fields based on colour.

Color designation	Description

Gold biotechnology	Bioinformatics is connected to gold biotechnology. It can be focused into two main functions: creation and maintenance of databases of biological information & designing and manipulating of biological materials It is related to areas of life science, computer science, statistics, and specialised biological fields like genomics, proteomics, waste generation, agriculture, and drug development.
Red Biotechnology	Red or medical biotechnology is related to the application of biological techniques that can be used for the diagnosis, prevention, and treatment of diseases. It encompasses the creation of novel pharmaceuticals, vaccines, and antibiotics, as well as regenerative therapies, molecular diagnostics, and genetic engineering methods to treat diseases by genetic manipulation.
White Biotechnology	White biotechnology is connected to industrial processes. It is a modern application that uses limited resources than conventional methods to produce industrial items while utilising living organisms like yeast, fungi, bacteria, plants, and their enzymatic systems. Such processes are employed globally in industries such as chemicals, pharmaceuticals, food and feed, detergents, pulp and paper, textiles, materials, and polymers, as well as the energy sector. It includes designing of energy-efficient, less polluting, and low resource-consuming processes and products.
Yellow Biotechnology	Applies to the use of biotechnology in the production of food, such as the fermentation of wine, cheese, and beer. The main purpose of yellow biotechnology is improvement of certain food to obtain the most nourishing one.
Grey Biotechnology	Environmental applications are connected to grey or environmental biotechnology. It can be separated into two categories: preserving biodiversity and using microbes to remove contaminants (bioremediation). Through the use of biological systems in waste treatment and management, as well as for the protection and restoration of the environment, grey biotechnology plays a critical role in resolving environmental issues.
Green Biotechnology	It is connected to the agricultural process with the goal of enhancing nutrient quality, producing superior disease-resistant plants, increasing productivity, and lowering production costs. Genetic alteration is used in this area to produce transgenics. The development of genetically modified organisms, the production of synthetic seeds for commercial use, and tissue culturing and micropropagation are some of this field's major applications.
Blue Biotechnology	Blue or marine biotechnology is based on the use of marine and

	aquatic organisms for requirement of new substances that are used in various industrial sectors and also for the conservation of the environment. It deals with the law, ethical and philosophical issues related to biotechnology.
Violet Biotechnology	Aspects of law, ethics, and philosophy are connected to violet biotechnology. Significant advancements in biotechnology have been made in a number of fields, including the industrial, agricultural, and medical sectors. There are also some religious, moral, and societal difficulties in this area.
Brown biotechnology	Brown biotechnology is related to management of desert and dry regions which makes larger part of the earth. Main purpose of this arid zone biotechnology is improved seeds and high-quality disease-free plants.
Dark biotechnology	It is connected to bioterrorism and biological weapons. This industry produces a large variety of disease-causing biological agents with minimal production costs and simple transportation from one location to another. In this field, germs and toxins, particularly those of plant and animal origin, are employed as raw materials to cause disease and death in people, crops, and other living things.

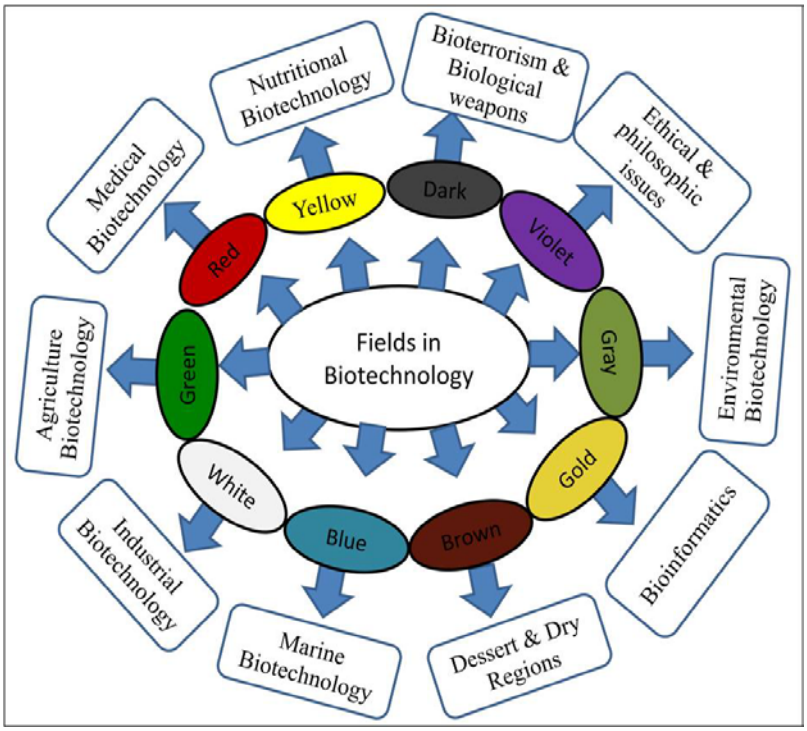

Figure 4.1: Classification of biotechnology based on color. (Source: Padhy et al., 2020)

4.6 APPLICATIONS OF BIOTECHNOLOGY

Biotechnology has been successfully used in many fields, including the environment, health care, agriculture, business, and many more. The applications of biotechnology in different areas are given in table 4.4

1. Agriculture

The Green Revolution began as a result of the introduction of biotechnology in agriculture. Organic farming, agrochemical-based farming, and genetically modified crop-based farming are few examples of how biotechnology has benefited agriculture. The introduction of pest-resistant plants and genetically modified crops has helped to enhance food production and fulfil the needs of the growing human population. It has also helped to triple food production.

Biotechnology's role in combating the Covid 19 pandemic

In the campaign against COVID-19, biotechnology has taken the lead. Using biotechnological methods, vaccines have been manufactured to treat corona virus infections. For COVID-19, Pfizer-BioNTech and Moderna created high-efficiency mRNA vaccines. It has been demonstrated that the mRNA vaccine created by BioNTech/Pfizer is 90% effective in preventing COVID-19 infection. Other vaccines have also been created using other methods, including interference RNA that stops the virus in nebulizers, recombinant ACE2 proteins that deceive the virus into not binding to human cells, and antibodies that fight the virus. Astra Zeneca developed the Sputnik V and AZD1222 viral vector vaccines. Moderna (Cambridge, MA, and USA) and Pfizer developed the mRNA vaccines mRNA-1273 and BNT162b2 (New York, USA)

The development of crops with higher yields, improved nutritional value, and resistance to a variety of biotic and abiotic challenges has proven to be a very fruitful use of biotechnological approaches. With the help of genetic engineering, food, horticultural crops, and tree species have all been successfully altered. One example is the modification of oil seeds to produce fatty acids for petrochemicals and fuels. There have been initiatives to create plant-based vaccinations. There have been produced crops that exhibit tolerance to a wide variety of insects and pests.

2. Medicine

The development of vaccinations, medicines, diagnostics, and human genome research has all made use of biotechnological methods. One of the main uses of biotechnology in medicine is the production of insulin and vaccines like the hepatitis B. By allowing the mass production of effective therapeutics, recombinant DNA technology has made a significant impact on the healthcare industry. Transgenic animals have been used to study how genes influence the onset of human diseases such cancer, cystic fibrosis, rheumatoid arthritis, and Alzheimer's. For instance, genetically engineered insulin called *Humulin* is used to treat diabetes. The insertion of genes into a person's cells and tissues during gene therapy has made the treatment of genetic

disorders feasible. PCR and ELISA are two further examples of how biotechnology is used in medicine.

3. Environment

Environmental issues have been identified, monitored, and remedied using biotechnological approaches. Waste management, bioremediation (the use of live organisms to treat environmental toxins), and phytoremediation method (use of plants to remove pollutants from the environment) have all benefited from the application of this. In addition to these renewable resources, biotechnology has also been used to produce biodegradable goods and alternative energy sources. By removing a wide range of pollutants from diverse areas of the environment, biotechnology is used to produce bacteria with tremendous potential for environmental cleanup.

4. Industries

For a variety of purposes, including the development of new materials for the construction industry, the production of cellular structures or the production of biological components, biotechnology has been used. Other applications include the production of beer and wine, washing detergents, and personal care products. For the finishing of fabrics and apparel, biotechnology is employed in the textile industry. Along with additional qualities like greater dye uptake and retention, increased absorbency, and wrinkle- and shrink-resistant fabric, it aids in the manufacturing of warmer, stronger, warmer, and wrinkle- and shrink-resistant clothing. In the food processing business, biotechnology has been successfully implemented.

5. Biofuels

In the area of energy production, biotechnology has been applied. Biofuel can be made from the waste from bioremediation and used to power generators. Methane generation may be aided by bacteria that break down sulphur liquor, a waste product of the paper manufacturing sector.

6. Evolutionary studies

Genes associated with ecological traits and evolutionary diversification can easily be traced using biotechnological tools.

7. Other Uses

- Biotechnology has used fermentation for centuries. Microorganisms like yeast have long been used in the production of alcohol and bread. In the current scenario, cultures have been purified and genetically improved to generate superior quality food items.
- Crop enhancement through the breeding of plants with desirable features is another way that biotechnology is used in agriculture.
- Transgenic plants are created through genetic engineering to have certain traits.

- Tissue culture is another application of biotechnology to produce a large number of plants with an explants. It also helps in increasing the number of endangered plant species.
- Enzymes needed to convert plant and vegetable sources into the components of biodegradable polymers can be produced by microbes under the control of certain conditions.
- It is also helpful in forensics for the identification of criminals, or in paternal disputes.

Table 4.4 The applications of biotechnology in different areas.

Area	Applications
Plant biotechnology	Transgenic plants, production of secondary metabolite, production of pathogen-free plants or crop improvement, production of herbicide-resistant crops, pest-resistant ('Bt concept' pest-resistant transgenic) plants, drought resistance, flood resistance, salt tolerance, high-yielding GM crops, nitrogen fixing ability, acidity and salinity tolerance, *in-vitro* germplasm conservation, genetic variability, *in-vitro* pollination, induction of haploidy, somatic hybridization, genetic transformation, somatic embryogenesis, organogenesis, phytoremediation, *in-vitro* plant germplasm conservation, mutant selection, somaclonal variation, plant genome analysis, hybrid seeds, artificial seeds.
Animal biotechnology	Biopharmaceuticals: Production of hormones, growth factors, interferons, enzymes, recombinant proteins, vaccines, blood components, oligonucleotides, transcription factor-based drugs oligonucleotides. Antibiotics, Diagnostics: antibodies, biosensors, PCR, therapeutics, vaccines, medical research tools, human genome research, development of biosensors. Gene therapy, Stem cell therapy, Animal tissue culture: Cell, tissue and organ culture, Gene cloning: rDNA technology, genetic engineering, transgenic animals, antibiotics, DNA markers, animal husbandry, xenotransplantation, medical biotechnology. Biopharmaceuticals (drug or vaccine developed through biotechnology), therapeutants, i.e. products used to maintain health or prevent disease, biopharming, i.e. production of pharmaceuticals in cultured organisms. Biopolymers, Designer drugs.

Agricultural biotechnology	crop biotechnology, horticultural biotechnology, tree biotechnology, food processing, plant biotechnology (photosynthesis improvers, bio-fertilizers, stress-resistant crops and plants, bio-insecticides and biopesticides) Food: Increased milk production Pharmaceuticals: Animals engineered to produce human proteins for drugs, including insulin and vaccines
Industrial biotechnology	Metabolite production (antibiotics, acetone, alcohol, enzymes, vitamins, organic acids), anaerobic digestion (for methane production), waste treatment (both organic and industrial), production of bio-control agents, fermentation of food products, bio-based fuel and energy, recovery of metals and minerals, bioethanol, pulp and paper, food, textiles and leather, pharmaceuticals, an enzymatic process for producing antibiotics.
Environmental biotechnology	Environmental monitoring, Waste management, Pollution prevention.
Fuel and fodder	Tissue culture technique for rapid afforestation of degraded forests, Renewable fuels and regeneration of green cover
Aquatic biotechnology	Aquaculture, environmental remediation, marine byproducts for human health, biomaterial and bioprocessing, restoring and protecting marine ecosystems, improving seafood quality, marine molecular biotechnology.

4.7 VARIOUS TECHNIQUES IN BIOTECHNOLOGY

In biotechnology, a wide range of techniques have been used. Table 4.5 lists some of the most significant ones.

Table 4.5 Various techniques used in biotechnology.

Techniques	Description
Genetic Engineering Technology	The procedure involves altering an organism's DNA by introducing foreign DNA.

Enzyme engineering	With this method, an enzyme's effectiveness can be increased or its activity can be generated by changing the amino acid sequence. Genetic engineering techniques are widely used to improve enzyme efficiency.
Protein Engineering Technology	The process involves altering current proteins or developing new proteins to produce useful products.
Cell and Tissue Culture Technology	In this technique, cells/tissues are grown under laboratory conditions to produce a organism or new products.
Bioinformatics Technology	Computational analysis of biological data, e.g., sequence analysis macromolecular structures, high-throughput profiling data analysis.
Antisense or RNAi Technology	A technique known as RNA interference (RNAi) controls the expression of genes in eukaryotic organisms by using short RNAs (RNAs with less than 30 bases). Research on gene function and therapies for the treatment of disease have both benefited from an RNAi-based method.
Protein separation and identification techniques	Procedures for separating and identifying desired DNA, including electrophoresis, microarrays, and blotting.
Genomics	The genome approaches are used to determine the biological function of genes and their products. Transcriptomics (profiling of microarray expression), proteomics (structures/ modifications/ interactions of proteins), metabolomics (e.g. metabolite profiling, chemical fingerprinting, flux analysis) are some of its diversifications

4.8 PLANT BIOTECHNOLOGY

New plant traits and varieties can be developed with the help of plant biotechnology. Plant biotechnology refers to procedures or scientific methods used to produce useful plants, their products, and the development of novel plant features. Utilizing biotechnological technologies accelerates crop improvement and makes it easier to transfer genes from unrelated species.

The two processes involved in plant biotechnology are tissue culture and genetic engineering. The most common approach in plant biotechnology is plant tissue culture. In 1939, Gautheret att empted to grow isolated cells and root tips on a structured medium, which was the beginning of p lant tissue culture. The tissue culture techniques provide the platform for the rapid multiplication

of plant species. The majority of plant tissue culture enterprises take advantage of the basic ability of plant cells to regenerate in order to produce elite varieties with superior genotypes quickly and on a big scale in a comparatively short time frame. The plants are genetically modified plants by transferring genes from one organism to plant to get the desired characteristics. Plants have been genetically modified to induce herbicide tolerance, salinity tolerance, drought tolerance, pest resistance, enhanced nitrogen-fixing ability, improved nutritional value and food quality.

Today's plant biotechnology represents a new era in science and technology, one that prioritizes the production of secondary metabolites, significant genetic advancements for plants, the conservation of germplasm, and the mass production of disease-free and novel kinds. Production of artificial seeds, biopharmaceuticals, plant-made pharmaceuticals, recombinant or other therapeutic proteins, transgenic plants, and plant-made vaccines or antibodies is part of the modern research work in plant tissue culture science.

4.9 METHODS IN PLANT BIOTECHNOLOGY

The following techniques are used in plant biotechnology.

- Plant tissue culture
- Genetic engineering/ recombinant DNA technology
- Molecular Assisted Breeding

4.9.1 Plant Tissue Culture

The plants are raised in a laboratory environment using cells, anthers, pollen grains, or other tissues. Then they are multiplied by a huge number. Explants from plants or tissues that produce calluses (undifferentiated masses of cells) can directly give rise to somatic embryos under certain cultural settings. Due to the rapid plant multiplication facilitated by plant tissue culture, breeders are able to introduce new cultivars considerably more quickly.

The steps in the tissue culture procedure include placing the tissue on a nutrient medium, allowing the cells to multiply to form a callus or the embryos, moving them to a medium containing plant growth regulators, and then allowing the embryos to produce roots (Fig. 10.6). The seedlings are moved into greenhouses. Through the use of plant tissue culture, a wide range of food and medicinal plants have been cultivated. Cell culture enables the production of single-cell clones, embryo rescue, the introduction of traits like disease resistance into elite breeding lines, as well as the generation of haploid plants allowing the development of homozygous breeding lines. This technique is now widely used for the improvement of important crops including major cereals, legumes and trees.

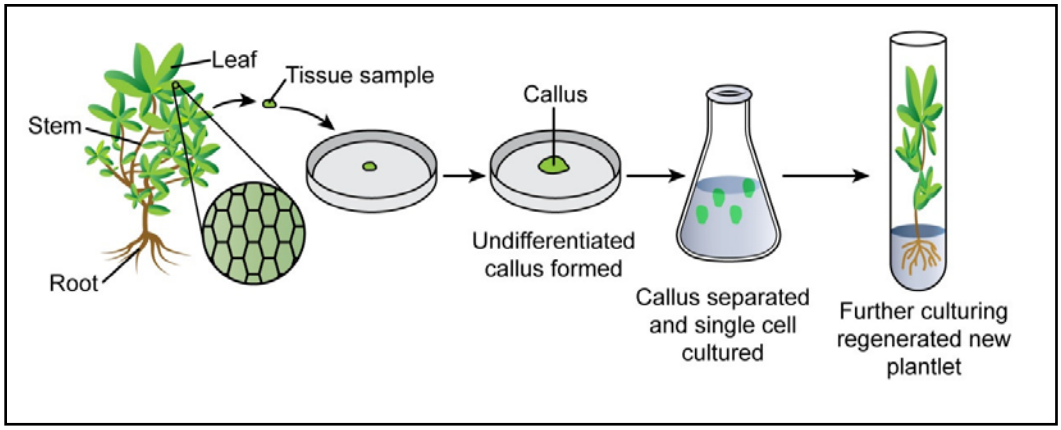

Figure 4.2: Sketch out of plant tissue culture technique (Source: sanjeetbiotech.blogspot).

4.9.2 Genetic engineering

Genetic engineering is the process of changing an organism's genetic (hereditary) material. The removal of a gene or genes from one organism and their transfer to another organism is known as genetic engineering or recombinant DNA technology. A transgenic plant or genetically modified organism is produced when additional DNA is incorporated into the native DNA of a plant (GMO). Restriction enzymes, cloning carriers (vectors) to carry the desired genes, and detection and selection of cloned genes are the fundamental elements for successful genetic engineering.

In genetic engineering, various gene transfer strategies are employed, such as:

- *Agrobacterium*-mediated gene transfer: The desired trait is extracted from an organism's DNA, inserted to an *Agrobacterium*, and then transferred to the target plant. The DNA is accepted by cells, which develop into plants with the new trait.
- Gene gun: DNA that codes for the desired trait is coated onto tiny particles of tungsten and fired into a group of plant cells. The desired feature is displayed in cells that receive the DNA.
- Polyethylene glycol (PEG) is a chemical that enables the quick uptake of DNA by plant protoplasts and integration into the chromosomal DNA of the plant.
- Another method is electroporation, in which short, high-voltage electrical pulses cause the formation of transient micropores in cell membranes, allowing DNA to enter the cell and then the nucleus.
- In addition to these, other techniques for transferring DNA include lipofection, microinjection, and calcium phosphate transfection.

Agrobacterium vectors are commonly used for the transfer of transgenes into plants The DNA sequences may be cut at various points, and the resultant fragments may then be placed into various locations throughout the genome (Fig. 4.3). Transgenes inserted into the genome contain regulatory elements and a selectable marker, often an antibiotic- or herbicide-resistant gene.

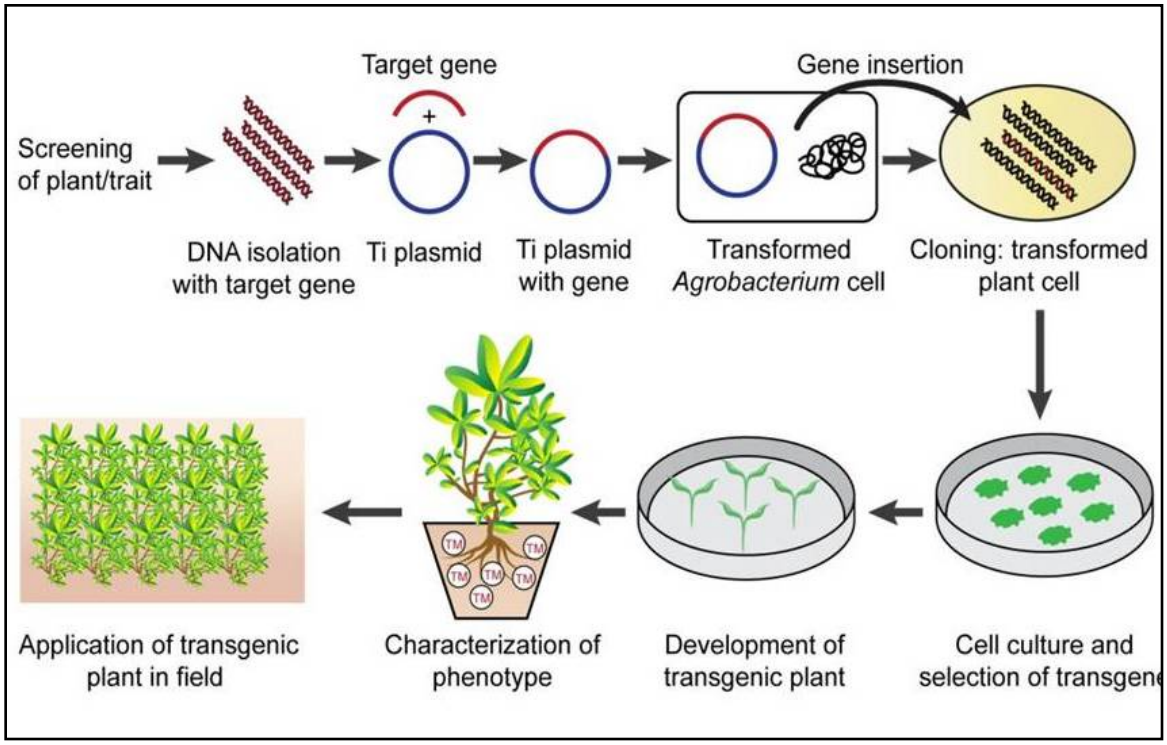

Figure 4.3: Diagrammatic representation of plant genetic engineering (Source: Kumar & Prasad, 2019)

4.9.3 Molecular Assisted Breeding (MAB)/Marker Assisted Selection (MAS)

Marker-assisted breeding, commonly referred to as molecular breeding, is a modern breeding method where the selection of desired traits is based on particular molecular markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) or molecular breeding is the process of selecting qualities by using DNA markers that are closely linked to phenotypic traits. Genetic markers such as microsatellites, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphism (AFLP) are important components of molecular breeding. Plant genetic improvement is facilitated by the data produced by molecular markers, transcriptome profiling, and genome sequencing. Molecular markers have been developed for many crops including trees. The markers can be used to track the presence of valuable characters in large segregating populations as part of a crop-breeding program

A new molecular breeding technique for crop development is genomic selection. It helps to segregate breeding populations and identify superior genotypes with higher breeding values (Fig. 4.4). It is based on genome-wide marker profile data. MAS have proved as an efficient breeding method for drought and salt-tolerant oilseed crops such as *Brassica*. It has provided valuable contributions in the designing and development of oilseed crop ideotype(s).

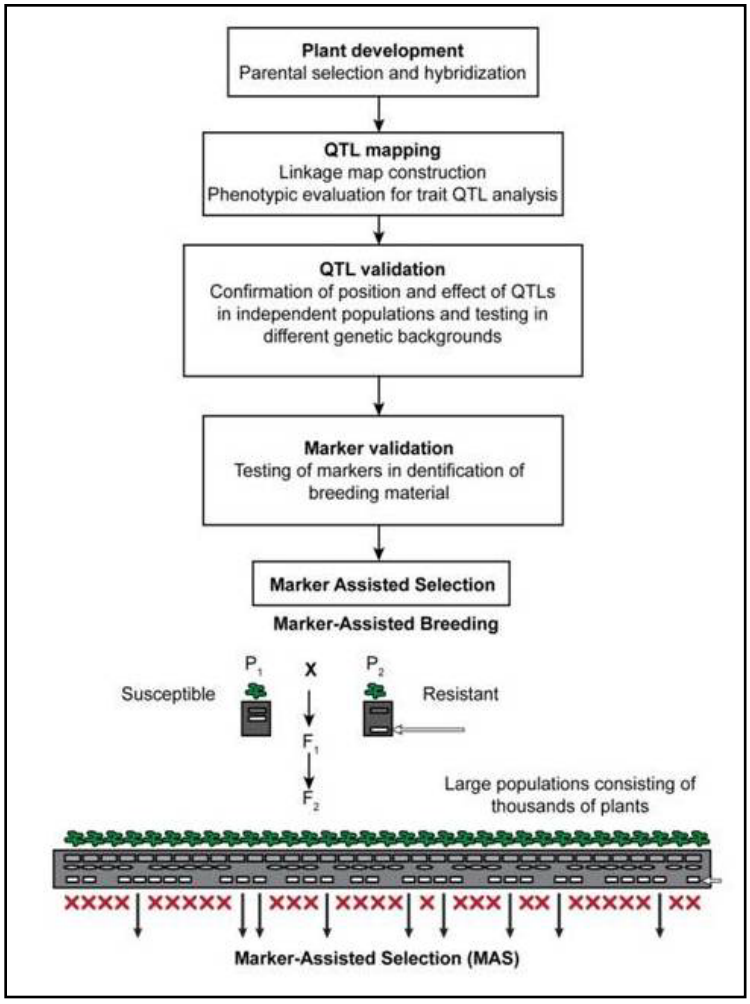

Figure 4.4: Marker-assisted breeding method process (Source: Collard Bertrand & Mackill David, 2008)

4.10 APPLICATIONS OF PLANT BIOTECHNOLOGY

Plant biotechnology has several, wide-ranging applications. Basic research and industrial applications are some of these. Understanding ideas like molecular pathways in plant cells is the fundamental application of plant biotechnology. Plant biotechnology techniques such as tissue culture and genetic engineering have been applied to plants for modification and improvement. These include growing pathogen-free plants, conserving genetic material, clonal propagation, producing secondary metabolites, and studying cell behavior. Biotechnology has got its application in crop improvement programs. There are many areas where plant biotechnology is used, including:

1. Improvement of varieties for various agronomic traits such as:
    - Crop improvement (resistance to biotic stress: pests, viruses, pathogens; abiotic stress: tolerance to drought, salinity).
    - Increasing the shelf life to delay the fruit ripening.

- Value addition: enrichment of crops with vitamins.
- Ornamental plants for characters such as size, color, smell
- Food processing.

2. Phytoremediation: removal of contaminants.
3. Biofuels: bioenergy crops.
4. Commercial products such as biopolymers, therapeutic proteins, biodegradable plastics, etc.
5. Protection of natural biodiversity.

4.10.1 Crop Improvement

Plant tissue culture technique

Plant clonal multiplication and propagation are facilitated by tissue culture. This is called micropropagation. The offspring produced by vegetative propagation i.e. asexual reproduction of a single plant or individual is called a clone. To develop plantlets, shoot buds produced on explants are separated and rooted. The technique proves useful in eliminating viruses and other pathogens. Useful chemical compounds are produced on a big scale using plant cell suspension culture and immobilized cells. The preservation of vegetative tissues and the conservation of endangered genotypes are both facilitated by cell and organ culture. The plants that do not produce seeds (sterile plants) or have 'recalcitrant' seeds can be preserved via in vitro techniques. Cryopreservation involves the storage of cells or tissues in liquid nitrogen at an extremely low temperature (−196°C) under in vitro conditions. This technique helps in the long-term conservation of important biological material and genetic resources. The embryonic tissues are generally cryopreserved for future use.

4.10.2 Genetic Transformation

Transgenic plants are produced using the genetic transformation approach by transferring the desired trait's genes into the host plants. In plants, genetic transformation is completed by vector-mediated i.e. *Agrobacterium*-mediated genetic transformation, or vector less i.e. direct gene transfer methods. The method presents a significant opportunity for genetic improvement in different crop species. Plant biotechnology and breeding programmes are integrated using this method. Plants can be genetically modified to have traits like higher yield and better quality. For instance: The development of transgenic "golden rice." Transgenic rice contains three genes encoding the enzymes responsible for the conversion of geranylgeranyl diphosphate to β-carotene (provitamin A). The transgenic rice produced is enrich in iron content. The plants are obtained by expressing ferritin or metallothionein transgenes, or making the existing iron more available by reducing levels of the iron-sequestering protein, *phytase*.

Transgenes can be used to create cells with a high concentration of the desired substances, which can have industrial applications. The modification of exogenous compounds by plant cells is called biotransformation. The enzymes found in the plant cell catalyze the bioconversion

reactions. Plants that exhibit resistance to or protection against numerous pests, illnesses, and viruses have been developed using the genetic engineering technique.

4.10.3 Pharmaceuticals

Using both conventional and novel methods, including genetic engineering and plant cell culture, it is possible to create medicines, secondary metabolites, proteins, medications, and vaccines from plants.

In addition of this, a new branch of biotechnology i.e. nanobiotechnology which involves the use of nanomaterials has proven useful in the development of crops mainly by manipulating the plant nutrient content, imparting disease and pesticide resistance in plants. In order to create nano insecticides, metallic nanoparticles with comparatively superior anti-pathogenic, anti-fungal, and anti-bacterial properties are utilised. Iron and zinc oxide nanoparticles function as nanofertilizers by facilitating the plant's efficient uptake of nutrients. Their effectiveness is dependent on a variety of variables, including plant susceptibility, size, content, and chemical characteristics of nanomaterials.

4.10.4 Industrial

Plants produce a variety of products that are used commercially, including proteins, antibodies (plantibodies), and fuels. For instance avidin and β-glucuronidase (GUS) are proteins produced in transgenic maize. Avidin is utilised as a biopesticide as well as a biochemical reagent for research and diagnostics. Oleochemicals are made from plant oils, which are employed as a feedstock. The soybean crop has been transformed to get high oleic acid content.

Starch produced by plants has also got uses in industry. Interest is also developed in manipulating complex carbohydrates in cereal crops such as rice, wheat, maize and barley. The starches are used for the development of packaging materials or even biodegradable plastics. Many of the enzymes involved in starch biosynthesis have been characterized and their genes cloned and attempts are been made to develop plants with enhanced capacity for the production of starch.

Polyhydroxyalkanoates (PHAs), which are non-toxic biodegradable polymers made by plants, can be produced more abundantly through agricultural practices. The polymer can be extracted in large quantities from seeds or leaves. To increase the production of these copolymers in the plant, efforts are being made to acquire transgenic plants (including one in oil palm).

4.11 SCOPE OF BIOTECHNOLOGY

The field of biotechnology is enormous in and of itself, and it encompasses many areas of biology. This comprises plant tissue culture, production of transgenic in animal and plants, development of monoclonal antibodies, uses in medicine as tools and therapeutics, production of new enzymes and their immobilization for industrial use, and control of pollutions, etc.

As we can say the 20th century was the era of electronics, similarly 21st century can be designated as the era of biotechnology. Biotechnology has rapidly become a field of activities with notable effects on all facets of human welfare, including food processing, environmental protection, human health, and overall quality of life. Biotechnology is making significant contributions in a number of fields, such as environment, medicine, agriculture, horticulture, floriculture, dairy, forestry, human health, animal health, fisheries, aquaculture, food processing, mining, animal husbandry, renewable energy, crime detection, parental dispute etc. Fundamentally, the goal of biotechnology is to enhance human life quality and safeguard against harmful diseases. The scope of biotechnology can be summarized below:

1. Developing immobilized cell and enzyme systems for chemical process industries.
2. For increasing disease-resistant, high-yielding varieties of crops,
3. Production of biocides in agriculture,
4. To introduce harmless bio fertilizers instead of harmful chemical fertilizers,
5. Genetical improvement of microorganisms for production of pharmaceutical products.
6. To preserve germplasm of plants, animals and microbes,
7. To develop automated bio-screening for therapeutic agents
8. To produce pharmaceutical products to treat severe diseases in man and animals,
9. Production of Bio-processing alkenes to valuable oxides and glycols
10. To produce biofuels for reducing the felling of forest trees for fuel wood,
11. Engineering of a series of organisms for specific industrial use.
12. To make use of various microorganisms in food making and preservation of food,
13. To employ microorganisms in the extraction of minerals from their poor quality ores,
14. To minimize pollution hazards.
15. In Human gene therapy.
16. For improved production of Vitamin B12.
17. Manufacturing ethanol by continuous fermentation.
18. Microbiological based production of human insulin and interferon’s,
19. Production of monoclonal antibodies for organ transplant tissue typing.
20. Production of photo-synthetically efficient plants.
21. Production of transgenic plants and animals.
22. Production of xanthan gum in oil fields for recovery of crude mineral oils.

4.12 SUMMARY

The use of living organisms to create technologies and goods for human improvement and sustainable development is known as biotechnology.

Biotechnology is the use of living systems and organisms to produce or develop useful products. It can also refer to "any technological" application that uses biological systems, live organisms,

or their derivatives to produce, alter, or produce goods for a specific purpose. The application of biotechnology in agriculture, food production, and medicine has been practiced by humans for thousands of years.

The term "Biotechnology" was first used by Hungarian engineer Karl Ereky in 1919. He defines biotechnology as the use of living organisms to the production of valuable resources or products. In other words, it is the technology that uses biological mechanisms, systems, or processes to produce products that enhance human life.

Modern biotechnology has got its applications in areas such as medicine, healthcare, therapeutics, diagnostics for development and production of novel drugs, agriculture for development of improved crop varieties, environment for treatment of waste and prevents pollution.

Plant biotechnology is referred to the use of plant cells, tissues, organs for the generation of useful products. The plant parts (explants) are cultured under in vitro conditions to get plantlets. Plant biotechnology plays a promising role in the field of agriculture, industry and pharmaceutical sciences.

Plant biotechnology has many applications such as large scale production of biochemicals, rapid clonal multiplication, development of homozygous lines via production of haploids, conservation of germplasm etc. The main methods in plant biotechnology are plant tissue culture, genetic engineering and marker assisted breeding. Clonal multiplication, raising of virus-free plants can be done via plant tissue culture. The development of plants with tolerance against abiotic and biotic stresses, better nutritional quality, and better storage can be achieved via manipulation of DNA by genetic engineering.

Biotechnology is making significant contributions in a number of fields, such as environment, medicine, agriculture, horticulture, floriculture, dairy, forestry, human health, animal health, fisheries, aquaculture, food processing, mining, animal husbandry, renewable energy, crime detection, parental dispute etc. Fundamentally, the goal of biotechnology is to enhance human life quality and safeguard against harmful diseases.

4.13 SELF ASSESSMENT QUESTIONS

4.13.1 Multiple Choice Questions

1. Who coined the term ‘biotechnology’?

a) Karl Ereky b) Arthur Kornberg

c) Both a & b d) Chaim Weizman

2. ----------is a product of biotechnology.

a) Skin b) Virus

c) Vaccine d) Fungi

3. The Golden Rice variety is rich in**:**

a) Biotin b) β-carotene and Ferritin

c) Vitamin C d) Thiamine

4. In which the following industrial areas, biotechnology is applicable?

a) Environment b) Health care

c) Agriculture d) All of the above

5. Tissue culture technique has been biotechnologically successful in production of

a) Beverages b) Plantlet

c) Insulin d) Cheese

6. Transgenic plants are produced by using Ti plasmid from the

a) E. coli b) *Agrobacterium tumefaciens*

c) Bacteriophage d) *Bacillus thuringiensis*

7. The biotechnology has contributed to field of

a) Pharmacy b) Industry

c) Agriculture d) All above

8. Bt stands for

a) Biotechnology b) *Bacillus thuringiensis*

c) Bollworm toxin d) None of the above

9. Which of the following techniques use genes and DNA molecules for diagnoses of diseases?

a) PCR b) Recombinant gene technology

c) Gene therapy d) All of the above

10. GMO stands for

a) Genetically modern organism

b) Genetically transferred organism

c) Genetically modified organism

d) Genetically mutant organism

11. What is the ‘part’ of plant used for culture called?

a) Explant b) Callus

c) Both a & b d) None of the above

12. Name the technique used for computational analysis of biologic data.

a) Python b) DNA fingerprinting

c) Bioinformatics d) SPSS

13. Name the antibiotic produced by Alexander Fleming.

a) Cephalexin b) Azithromycin

c) Amoxicillin d) Penicillin

14. Name of the sheep cloned by researchers in Scotland

a) Dolly b) Morag

c) Megan d) Both b & c

15. Grey field of biotechnology related to:

a) Environmental biotechnology b) Agriculture biotechnology

c) Industrial biotechnology d) Medical biotechnology

4.13.2 Fill in the Blanks

a) Biotechnology is the use of ……. to produce or develop useful products.

b) The plants formed after incorporation of genes for desired characters are called …….. .

c) Undifferentiated mass of cells produced via tissue culture is called …….

d) Genetically engineered insulin called ……. is used to treat diabetes.

e) Polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) are examples of ……..

Answer key

4.14.1: 1-(a); 2-(c); 3-(b); 4-(d); 5-(b); 6-(b); 7-(d); 8-(b); 9-(d); 10-(c); 11-(a); 12-(c); 13-(d); 14-(a); 15-(a)

4.14.2: a) living systems and organisms; b) transgenic; c) callus; d) Humulin; e) genetic markers

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4.16 TERMINAL QUESTIONS

4.17.1 Short Answer Type Questions

1. What is the basic concept of Biotechnology?

2. What do you understand about green biotechnology?
3. What do you mean by white biotechnology?
4. What is the use of biotechnology in the field of medicine?
5. Write a short note on Genetic engineering
6. How does Plant Biotechnology play a role in germplasm conservation?

4.17.2 Long Answer Type Questions

1. Discuss various techniques of Biotechnology.
2. Describe in brief various applications of Biotechnology.
3. How does Biotechnology be used for the improvement of crop plants?
4. Describe the methods of Plant Biotechnology.
5. Enumerate various applications of Plant Biotechnology.
6. Give an account on various stages of biotechnology development.

UNIT 5: PLANT CELL, TISSUE AND ORGAN CULTURE AND APPLICATION OF PLANT TISSUE CULTURE

Contents:

5.1. OBJECTIVES

After reading this unit you will be able to

- Describe plant cell, tissue and organ culture.
- Understand the concept of cellular differentiation.
- Know about various application of plant tissue culture.
- Understand about clonal propagation and artificial seed.
- Describe the production of hybrids, somaclones and secondary metabolites.
- Know about the process of Cryopreservation and germplasm preservation.

5.2. INTRODUCTION

You have already learned in the previous unit (unit 4) about biotechnology, basic concepts, its principle and scope. Through previous unit you must have understood that biotechnology involves the technology that utilizes biological system to obtain useful products for mankind. One of the important aspects of biotechnology is tissue culture techniques that have led to the novel possibilities in the field of agriculture, horticulture, forestry, medicines etc. In this unit we will discuss about plant cell, tissue and organ culture and its history and scope. We will also discuss the concept of totipotency, cellular differentiation and application of plant tissue culture in agriculture, forestry, medicines and industry. A study of this unit will help you to understand the concepts of clonal propagation, artificial seed, somatic hybrids, somaclones, secondary metabolites production and cryopreservation etc.

5.3. PLANT CELL, TISSUE AND ORGAN CULTURE

Plant tissue culture is a collection of various techniques used in maintaining and growing plant cells, tissues or organs under sterile conditions on a nutrient medium. Plant cell, tissue and organ culture have developed very rapidly in past few decades and it has been proved a major biotechnological tool in the field of agriculture, forestry, horticulture etc. Through techniques like micropropagation/ clonal propagation, artificial seeds, somaclonal variation etc., many problems now can be solved which were not feasible through traditional techniques.

The introduction of cell and tissue culture is linked to the discovery of cell and propounding of cell theory by Schleiden and Schwann (1839). However, Henri-Louis Duhamel du Monceau (1700-1782) executed the experiments on wound healing in plants through callus formation on decorticated region of elm plants (Gautheret, 1985). Similarly, Trecul (1853) observed callus formation in number of plants and Vochting (1878) suggested the presence of polarity as a key feature guiding the development of plant fragments (Gautheret, 1985). In 1898 and 1902, a German botanist, Gottlieb Haberlandt done intensive effort and developed the concept of *in vitro* cell culture (Krikorian & Berquam, 1969). His experiment failed to induce cell division as till that time auxin was not discovered. But he lent a foundation to plant physiology. Haberlandt is thus regarded as father of tissue culture. During 19^{th} century, the concept of callus culture from different plant parts came into existence and many attempts were made for organ culture. During

1940-1970, suitable nutrient media were developed for culture of plant cells, tissues, protoplasts, anthers and embryos. In 1950s, through several important achievements in the field of plant physiology the understanding of plant growth hormone was increased and this helped in rapid multiplication of totipotent cell during culture process. In 1960, G. M. Morel laid the foundation of commercial plant tissue culture through the million fold increase in clonal multiplication of an orchid (*Cymbidium*). F. Skoog with C. O. Miller (1957) found out the role of cytokinins in tissue culture and showed that the ratio of auxin and cytokinin in the growth medium influences the morphogenesis of shoots and roots during tissue culture. In 1960s, with the identification of the role of enzymes (cellulase and pectinase) in dissolution of cell wall at suitable pH, isolation and culture of protoplast was developed. In India, tissue culture work was started during mid 1950s and was pioneered by Panchanan Maheshwari. Sipra Guha Mukherjeee and S. C. Maheshwari (1964-67), first time developed the haploid through anther and pollen culture and this discovery was a landmark in the development of plant tissue culture.

Basic techniques and requirements of Plant Tissue Culture:

The important aspects of plant tissue culture are nutrient medium, maintenance of aseptic conditions and aeration of the tissue. Plants can synthesize their own food in nature through photosynthesis but while growing in vitro condition they are heterotrophic and require all essential minerals, carbohydrate, growth regulators etc. For this, several media have been developed time to time. Important components of media for plant tissue cultures are inorganic salts of major and minor elements, iron and carbon source, vitamins and plant growth regulators. Murashige and Skoog (1962) formulated MS medium suitable for many applications and most widely used in plant tissue culture. Other media used in plant tissue culture are proposed by Gamborg *et al* (1968), White (1963), Heller (1953) and Smith (1967).

The general techniques used in plant tissue culture are cleaning of glassware, sterilization of glassware and media, sterilization of plant material and culture. For cleaning glassware used in culture process, it is boiled for two hours in 10% sodium carbonate then after rinsing with tap water it is soaked in 30% nitric acid overnight. After this it is rinsed with distill water and then drained, dried and stored in clean place. Nowadays disposable sterile culture vessels are also available commercially. The cleaned glassware is sterilized using an autoclave or an oven. Sterilization of culture media involves autoclaving and filter sterilization. Culture media kept in glass containers sealed with cotton plugs or plastic closures are autoclaved at 15 psi and 121^{o}C for 15-40 min (exposure time depends upon the volume of nutrient medium). Solution containing thermolabile compounds like vitamins, plant extracts, amino acids, hormones etc. requires filter sterilization where these solutions are sterilized using millipore or seitz filter paper (0.2 µm pore). Surface sterilization of plant material requires greater care as microbes on surface have to be killed without killing plant tissues. Firstly, plant material is washed under running water and then treated with detergent solution to remove oils and dirt from the surface. After this, explant is treated with 70% alcohol for about 1 minute and then washed thoroughly with distill water. The chemical sterilizing agent mainly used is 20% chlorine water, 1.0% bleaching powder, 0.01% or 0.1% mercuric chloride. After this, the entire sterilized setup i.e. flask containing explants in

sterilizing solution is transferred to sterile laminar airflow cabinet. Here explants are transferred to sterile petridish with the help of sterile forceps and then specific portion of explants is inoculated on sterilized medium. The isolated single cell from cell suspension cultures is capable of division. They could proliferate and divide to form callus which in appropriate nutrient and hormonal conditions could regenerate into new plants.

Types of Plant Tissue Culture:

The plant tissue cultures are generally classified on the basis of the type of *in vitro* growth like callus or suspension culture, embryo culture, anther culture etc. (**Table – 1**).

Table – 1: Types of Plant Tissue Culture

PLANT TISSUE CULTURE TYPE	DESCRIPTION
Callus culture	Cell division in explants forms a callus. Callus is irregular, unorganized and undifferentiated mass of actively dividing cells. Callus is obtained within 2-3 weeks in culture medium containing growth regulators like auxins (e.g. 2,4-D) and cytokinins (e.g. BAP). Callus formation involves three stages – induction, cell division and differentiation.
Suspension culture	Suspension culture grows faster than callus culture. In this type of culture, single cells or group of cells are suspended in a liquid medium containing growth regulator like auxins (e.g. 2,4-D).
Shoot and root culture	Shoot and root cultures are controlled by auxin-cytokinin ratio. High auxin promotes root culture whereas high cytokinin promotes shoot culture.
Embryo culture	Embryo culture involves the process of extracting young immature embryos from developing seed and growing them *in vitro* on culture medium to form seedlings. This is helpful in embryo rescue e.g. jute, tomato, bean etc.
Endosperm culture	This involves culture of excised endosperm along with embryos from the immature seeds on suitable cultural medium. Triploid plants which produce seedless fruits (e.g. apple, banana etc.) are developed from endosperm culture.
Anther culture	Anther culture involves culture of pollens or anthers containing haploid microspores on suitable medium. It is important method in production of haploid which are very useful in plant breeding.
Ovule culture	Culturing of fertilized ovule on the usual basal medium. It is required in those cases where embryo abort early and is difficult to excise from ovule for embryo culture.

The ultimate objective in plant protoplast, cell and tissue culture is the reconstruction of plants from the totipotent cell. On the other hand, the totipotentiallity of somatic cells has been exploited in vegetative propagation of many economical, medicinal as well as agriculturally important plant species.

5.4. TOTIPOTENCY AND CONCEPT OF CELLULAR DIFFERENTIATION

The basis of plant cell and tissue culture is 'totipotency'. The term 'totipotency' is coined by T. H. Morgan (1901) which indicates the capacity of cell to develop into an organism by regeneration. The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular **totipotency** (L. *totus* = entire, *potentia* = power). In plants, mature and differentiated cells can retain the ability to regenerate to a meristematic state as long as they are viable. In plant tissue culture, we generally use explants (differentiated tissue) to initiate their growth in culture or for callus culture. Since explant is a portion of mature tissue, so its non-dividing quiescent cells undergo certain changes to regain its meristematic activity. This reversion of mature differentiated cells to the meristematic state leading to the formation of undifferentiated callus tissue is called '**dedifferentiation**'. The phenomenon of conversion of component cells of callus to whole plant or plant organ is described as '**redifferentiation**'. These two phenomenon of dedifferentiation and redifferentiation are described as '**cellular totipotency**' which is found only in plant cells and not in animal cells. The cells obtained from stem, root or other plant parts are allowed to grow in culture medium containing mineral nutrients, vitamins and hormones which produces an unorganized mass of cells which is known as callus tissue with totipotent cells. Here, totipotency of the cell is manifested through the process of differentiation where hormones play the major role than any other factors.

The basic event in the development of higher organisms is the specialization of cells, which is termed as **cytodifferentiation**. In tissue culture, undifferentiated callus cells are all parenchymatous in nature, so the differentiation of callus cells into a variety of cells is required during redifferntiation of these cells. The efficient way for the study of cytodifferentiation in vitro is the study of differentiation of callus parenchyma cells into vascular tissue e. g. xylogenesis. Xylogenesis is the differentiation of parenchyma cells into cells with secondary wall thickening as in xylem of vascular plants. It is shown that 'sucrose' and phytohormones like auxins, cytokinins and gibberellins play important role in vascular differentiation. Several report indicated that auxin is essential for cytodifferentiation and it effect vascular differentiation quantitatively as well as qualitatively. Auxins at low concentration (0.5 mg per litre) is known to stimulate xylem formation and its concentration show inverse relationship with the degree of xylem differentiation. Auxins effect on vascular differentiation is also dependent on the presence of sugar (sucrose) and with the variation in sugar concentration relative amount of xylem and phloem formation can be altered. For eg., cell suspension culture of french bean when treated with increased sugar concentration and cytokinin ratio to auxin, exhibits significant metabolic changes (Robertson *et al*., 1995).

For the development of whole plant from callus totipotent cell, apart from cytodifferentiation, there should be organogenesis i.e. formation of plant organs like stem, root, leaves and flower etc. This can be accomplished either through '**shoot bud differentiation**' or through '**somatic embryogenesis**'. Former involves formation of shoot buds and latter involves somatic embryo development and through both whole plant can be regenerated.

Shoot bud differentiation is initiated by both chemical and physical factors and these factors vary for different plant materials. In cultured tissue, besides genotypic influence shoot bud differentiation is also dependent on the auxin/cytokinin ratio in the medium. The ratio of cytokinin (kinetin) to auxin (indole acetic acid, IAA) plays major role in organogenesis of tobacoo pith tissue. High kinetin level causes bud differentiation whereas high level of auxin favours root differentiation. Some studies suggest that organ differentiation (bud or root differentiation) was determined by the concentration of auxin (NAA) and not by the ratio of cytokinin to auxin. Besides growth regulators, physical factors like light, temperature etc also plays important role in organogenesis. High light intensity has been found to have inhibitory effect on shoot bud differentiation in tobacco. Blue light promotes shoot bud differentiation whereas red light stimulates rooting in tobacco callus (Bhojwani and Razdan, 1983). The size and source of the explants also affects the shoot bud differentiation process. Larger the plant material with parenchyma, cambium and vascular tissue, then the probability of shoot bud formation is greater.

Somatic embryogenesis is an artificial process in which an embryo or a complete plant development is initiated from a single somatic cell or a group of cells. It is an in vitro method of plant regeneration which is used as biotechnological tool for sustained clonal propagation (Park *et al.*, 1998). In nature, embryo do not develop outside the ovules however instead of egg cell, it may develop from nucellar cells or integuments of the ovule but it mature only in a special environment of embryo sac and the same conditions should be maintained in culture for inducing somatic embryogenesis. Somatic embryogenesis was studied in *Daucus carota* (carrots) by Steward *et al.* (1958) where bipolar embryos developed from single cells. Later, this phenomenon has been studied on other plants like *Citrus* (Kochba and Spiegel-Roy, 1977; Gavish *et al.*, 1991, 1992), *Coffea* sp. (Nakamura *et al*., 1992) and *Zea mays* (Emons and Kieft, 1991) etc. In vitro somatic embryogenesis is influenced by explant, growth regulators and physical culture environment. Somatic embryogenesis either can be initiated directly from the explants or indirectly by the establishment of callus (Suman and Kumar, 2016). Generally, for somatic embryogenesis immature and less differentiated plant parts are used and in many cases zygotic embryo in its early stage of development is used to initiate cultures as they possess embryogenic competence and are termed as pre-embryogenic determined cells (PEDCs). Embryogenesis is easy in suspension cultures, where presence of auxin in the medium is generally essential for initiation of embryo. Mostly 2,4-D has been used to induce somatic embryogenesis but in some cases, other auxins like NAA and IBA have also been used for this purpose. On the other hand, phytohormones like cytokinins and gibberellins are known to have inhibitory effect on potential embryonic cells. It has also been shown that a substantial amount of nitrogen supplied in reduced form such as ammonium salts stimulate somatic embryogenesis (Merkle *et al.*, 1995). The source of nitrogen can be coconut milk, casein hydrolysate, amino acids and ammonium ions (NH_4Cl, NH_4NO_3).

5.5. APPLICATION OF PLANT TISSUE CULTURE

Plant protoplast, cell and tissue cultures have wide application in several areas like agriculture, horticulture forestry, floriculture, medicines and industries. It is an important tool for crop improvement, micropropagation, production of disease resistant plant, somaclonal variation, hybridization, genetic transformation, commercial production of natural compounds, preservation of germplasm etc. (**Fig. 1**)

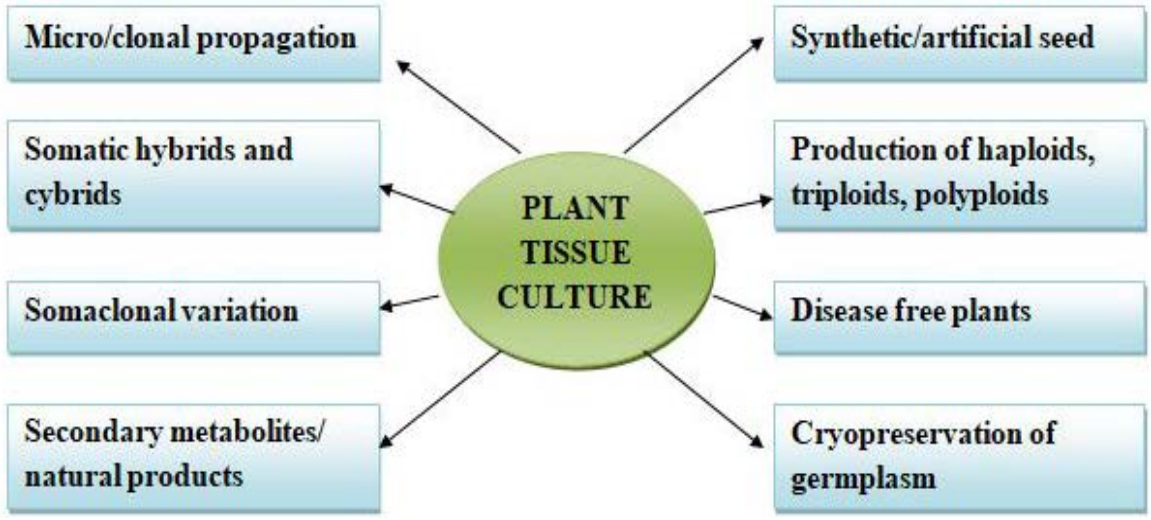

Fig. 1: Some of the application of plant tissue culture technology

Agriculture:

Now a day's plant tissue culture is widely used in agriculture as it is the most efficient technology in plant breeding for crop improvement. It has been used to create genetic variability among crop plants to improve their health and to incorporate specific desirable traits through gene transfer. Plant tissue culture is an important tool involved in micropropagation and this technology has a wide application in crop improvement. The micropropagation technology has a potential to producing superior quality plants, isolation of useful high yielding variants with better disease resistance and stress tolerance capacities (Brown *et al*., 1995). Increase in yield up to 150% of virus-free potatoes was obtained in controlled conditions (Singh *et al*., 1992). Clones are produced through callus culture having inheritable characteristics different from those of parent plants due to the occurrence of somaclonal variability (George *et al.*, 1993). Haploid and triploid production has an important use in plant breeding and improvement of crop plants. Haploid is an easier system for induction of mutation and can be used for rapid selection of mutants with desirable traits. Similarly, triploid plants raised through tissue culture are self sterile and seedless. This characteristic increases the edibility of commercially important edible fruits like apple, banana, grape, mulberry, watermelon etc.

Forestry:

Since time immemorial man had been utilizing forest resources to fulfil his basic requirements. Today, rapid industrialization, urbanization and over-exploitation have led to depletion of forest

resources. Now, it has become the major concern to increase the forest product to meet the need of growing global population. Here, tissue culture techniques play the important role for the production of quality planting stock. Micropropagation method is most extensively used in forestry for rapid multiplication of important forest trees yielding high fuel, pulp, fruits or oil at a large scale. Triploids of popular (*Populus tremuloides*) have better quality pulpwood. Therefore it is important to the forest industry.

Medicine:

Today, with the advances in tissue culture techniques, molecular farming of various pharmaceutical compounds like edible vaccines, antibiotics, diagnostic or therapeutic proteins, secondary metabolites etc. has been started. For eg., there are some proteins that are used for diagnosis of human disease (diagnostic proteins) whereas some proteins are used to cure diseases (therapeutic proteins). By using tissue culture method, transgenic plant with foreign genes of required proteins is produced in bioreactor on large scale at low cost. Similarly, many secondary metabolites are harvested from plants for pharmaceutical medicines, for eg. anticancerous compound taxol is obtained from *Taxus* sp. But these metabolites are naturally produced in plant at low amount, here, micropropagation techniques are used for large scale production of these plants in short period of time preventing over exploitation of plant species.

Industry:

In recent years plant tissue culture has become a major tool in production of compounds valuable to the industry. Cultural practices are used in growth and maintenance of microorganisms involved in industrial production of breweries, drugs, milk products etc. Higher plants are also very important source of industrially important natural products like flavours, essential oils, pigments, sweeteners etc. Genetic manipulation of cell cultures has great potential for altering the metabolic profile of plants making them profitable to the industry. Transgenic plant cell culture hold promise for industrial production of useful compounds like food additives, colours etc. Plant cells can be cultured in fermenters for the industrial production of secondary metabolites using cell culture. The success of plant cell or tissue culture has led to the many novel possibilities and much advancement have been achieved in the area of micropropagation, production of secondary metabolites, somatic hybridization, induction of haploid and production of transgenic plants etc. Some of the above mentioned biotechnological techniques involving tissue culture methods are discussed below.

5.6. CLONAL PROPAGATION OR MICROPROPAGATION

From the ancient time men have been vegetatively propagating variety of plant species for fulfilling his need. Only during past few decades, this can be easily achieved through the techniques of cell, tissues or organ culture and this process is known as **clonal propagation** or **micropropagation**. Micropropagation is an alternative means of asexual propagation of important plants through tissue culture or cell culture techniques. It offers many advantages over traditional methods of plant propagation. Some of the major advantages are as follows:

(i) Millions of clonal plants can be regenerated from small portion of tissue (explants).
(ii) Large number of plants can be produced in a short time and space.
(iii) Useful in developing disease free plants or developing resistance in many plant species.
(iv) Plants develop through this process are of same physiological age and hence they show uniformity in growth and other traits.
(v) Continuous production of plants or its products round the year as it is independent of seasonal changes.
(vi) Long time storage of important germplasm.
(vii) Provides disease free plant materials for international exchange.

Mostly micropropagation or clonal propagation is achieved by culturing sterilized shoot tips, axillary buds, adventitious roots, somatic embryogenesis etc. This technique involves following steps: Selection of explants and its surface sterilization, media selection and in vitro establishment of tissue, shoots proliferation, rooting, conditioning of propagules, acclimatization of plantlets in green house and finally transferring of plants to the fields (**Fig. 2**). Before transferring to the field, plantlets should be transferred first to green house for acclimatization so that they can easily survive sudden change in the environment and invasion by soil microbes in natural field condition. This acclimatization requires many weeks and is followed by potting plantlets into sterile peat or soil.

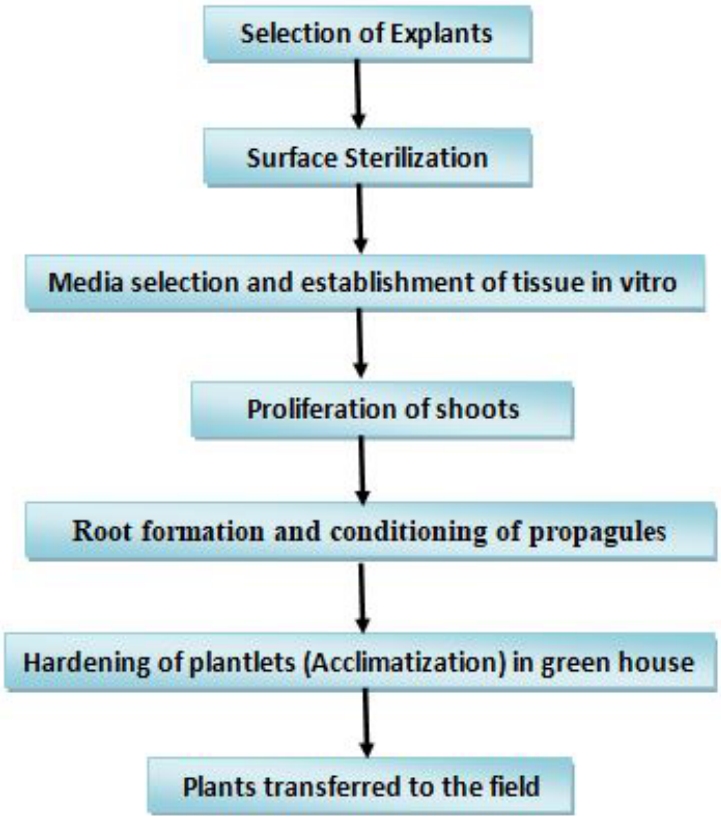

Fig. 2: Stages involved in Clonal propagation or Micropropagation

Regeneration of plantlets through cell and tissue culture techniques has been achieved in wide range of trees of high economic value. In recent years, many studies are aimed at large scale micropropagation of important trees yielding fuel, pulp, timber, oils or fruits and through this large number of valuable plant species have been successfully grown worldwide basis (**Table – 2**) (Gupta, 2019; Purohit, 2005).

Table – 2: List of some plant species grown in large scale through micropropagation

S. NO.	CATEGORY	PLANT SPECIES
1	**Agro-forestry plant species**	*Bambusa bambos, Dendrochalamus asper, Dendroclamus strictus, Eucalyptus camaldulensis, Eucalyptus citriodora, Eucalyptus terecticornis, Leucaena spp., Populus deltoids, Populus euphratica*
2	**Cash crops**	*Curcuma longa, Solanum tuberosum, zinziber officinale*
3	**Ornamentals**	*Alpinia, Bougainvillea, Callistemon, Chrysanthemum Clerodendron inerme, Dahlia, Dianthus, Dracaena, Duranta spp., Ficus benjamine, Ficus spp., Gardenia, Geranium, Hibiscus, Hydrangea, Iresine, Miscanthus, Nandina, Peperomia, Petunia, Plumbago, Philodendron scandens, Pogonantherum, Pothos, Rosa spp., Thalictrum, Viola, Yucca*.
4	**Medicinal & Aromatic plant species**	*Aloe vera, Artemisia spp., Bacopa monnieri, Centella asiatica, Chlorophytum borivilianum, Pelargonium spp., Pogostemon cablin, Swertia chirata, Vanilla planifolia.*
5	**Fruit & vegetables**	*Allium spp., Asparagus spp., Carica papaya, Fragaria ananassa, Musa* spp.

In India, the **Department of Biotechnology (DBT)**, Government of India constituted a "**Conservation on Micropropagation Research and Technology Development (CMRTD)**", which is involved in micropropagation related activities of various plant species, where it may prove useful.

Advantages of Micropropagation:

1. Rapid multiplication of plants,
2. Large number of plantlets is obtained within a short period and from a small space.
3. Plants are obtained throughout the year, independent of seasons.
4. Since genetically similar plants or clones are produced by this method, therefore desirable characters of superior variety are kept constant for many generations.
5. The rare and endangered species are saved by this method.

5.7. ARTIFICIAL SEED

Through micropropagation, large number of propagules can be produced in limited time and space but their storage and transportation for transplantation is a major problem. To solve this problem, the concept of synthetic or artificial seeds has become popular. The concept of artificial seed was first time given by T. Murashige of the U.S.A. at a Symposium in Belgium, in 1977. **Artificial or synthetic seeds** are the somatic embryos encapsulated in a suitable matrix (e.g. sodium alginate), along with substances like mycorrhizae, insecticides, fungicides and herbicides. In these seeds, the protecting gel acts as seed coat and artificial endosperm providing nutrient as in true seeds (**Fig. 3**). In India, this technique of synthetic seeds was standardized and practiced for sandalwood and mulberry at BARC (Bombay).

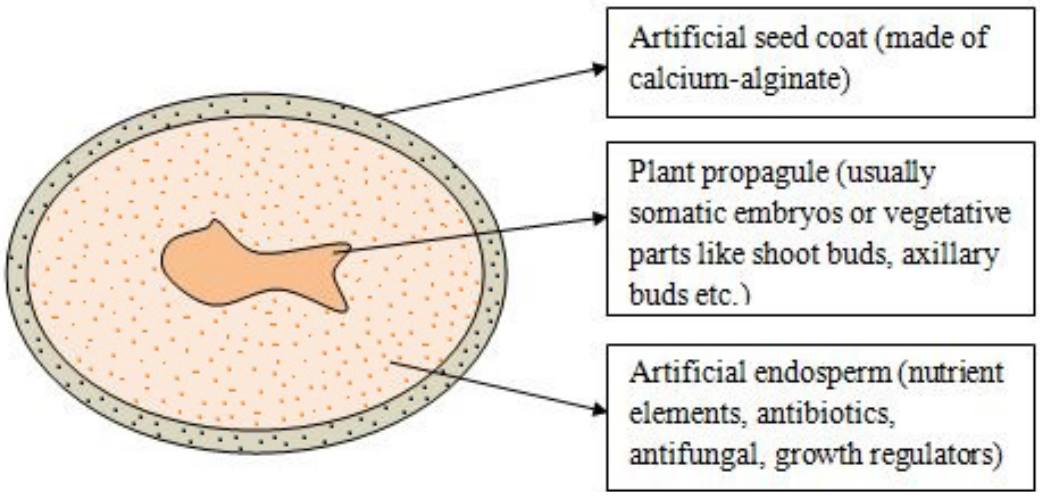

Fig. 3: Diagram of Artificial seed or Synthetic seed

The production of encapsulated seeds involves following steps:

(a) Induce artificial embryos from cell suspension culture.
(b) Mix embryos well with 2% Na-alginate.
(c) Transfer embryos in a solution of calcium salt, $Ca(NO_3)_2$ for 30 minutes. Here, individual embryo gets enclosed into clear and hardened beads of about 4 mm.
(d) Sieve the bead through a nylon mesh.
(e) Test the growth vigour of encapsulated embryos by plating in sand or soil amended with pesticides.

Further, for increasing viability of embryo for longer time superfluro chemical oils are mixed with gel which increases oxygen supply. There are four types of artificial seeds that have been proposed – **Coated desiccated somatic seeds** (somatic embryos, coated with polyoxyethylene and then desiccated, e.g. carrot, celery embryos etc.); **Coated hydrated somatic seeds** (coated somatic embryos are hydrated by hydrating gels- sodium alginate, e.g. alfalfa, barley, sandlewood etc.); **Uncoated desiccated somatic seeds** (uncoated somatic embryos are desiccated, 8-15% moisture content, e.g. carrot, *Glycine max* etc.); **Uncoated hydrated somatic seeds** (uncoated somatic embryos are hydrated through fluid drilling, e.g. carrot, tomato, lettuce

etc.). S. L. Kitto and J. Janick (1985), produced citrus embryos in vitro. They tested 8 compounds for their synthetic coating properties on embryos and a polyethylene oxide (polyox WSR-N75) revealed good encapsulating properties. Similarly, E. M. Muralidharan and A. F. Mascarenhas in 1987 had done production of artificial seeds in *Eucalyptus* sp.

Advantages of Artificial seeds:

1. They can be stored for long period without loss of viability.
2. Easy to handle and transportation for transplantation.
3. They can be directly sown in the soil like natural seeds.
4. They do not need hardening in greenhouse.

High cost of production is the only limitation of artificial seeds but with further research and advances surely this will go down and commercial production of synthetic seed will increase.

5.8. PRODUCTION OF HYBRIDS AND SOMACLONES

In 1960, E. C. Cocking at the University of Nottingham (U.K.) demonstrated that the protoplasts can be obtained through enzymatic degradation of cell walls. This became a new tool of genetic manipulation of plants and the fusion of protoplasts of genetically different species showing physical or chemical incompatibility has also been possible.

Protoplast fusion or somatic hybridization is the most important use of protoplast culture and is significant for hybridisation between species or genera which cannot be crossed through conventional method of sexual hybridization. Somatic hybridization is playing an important role in plant breeding and crop improvement. It has been possible to transfer useful genes (e.g. nif genes, disease resistance genes) from one species to another, thus widening the genetic base for plant breeding. Major aspects of protoplast fusion are - development of fertile amphidiploids from sexually incompatible species; production of heterozygous lines within one plant species e.g. potato; transfer of a part of genetic information from one species to another through chromosomes elimination and the transfer of cytoplasmic genetic information from one to a second species. During fusion of cytoplasm, the nuclei of two protoplasts may or may not fuse together. When two nuclei present within a cell, and then this binucleate cell are known as **heterokaryon or heterocyte.** When the two nuclei fuse during cytoplasmic fusion then the resultant cell is known as **hybrid or synkaryocyte** and when the genetic information from one of the two nuclei is lost during cytoplasmic fusion then the cells are known as **cybrid or cytoplasmic hybrid or heteroplast (Fig. 4)** (Doods and Roberts, 1985; Dubey, 2016). Production of cybrid is called cybridisation, while production of hybrid is called hybridisation. Cytoplasmic genetic information controlled by cytoplasmic genes like male sterility in some plants, susceptibility and resistance to some of the pathogens and drug etc. can be transferred from one plant to another through cybrid formation and this information can be applicable in plant breeding experiments. Cybrid technology has successfully been applied to carrot, *Brassica* sp., *Citrus*, tobacco and sugar beet.

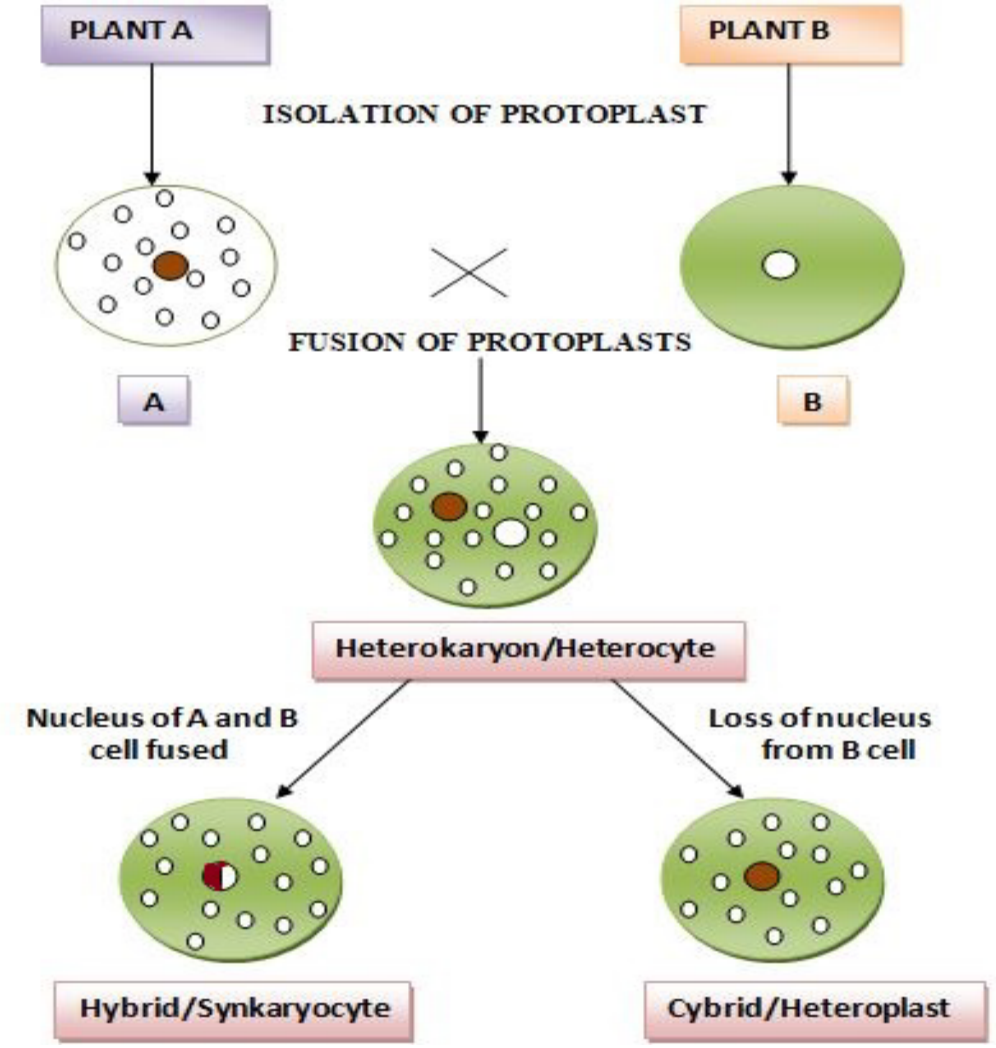

Fig. 4: Hybrid/Cybrid production through protoplast fusion

Steps involved in somatic hybridisation are:

1. Isolation of protoplasts from suitable plants.
2. Mixing of protoplasts in centrifuge tube containing fugigenic chemicals like polyethylene glycol (PEG), sodium nitrate ($NaNO_3$), maintenance of high pH 10.5 and temperature 37^oC, resulting into viable heterokaryons.
3. Wall regeneration by heterokaryotic cells.
4. Fusion of nuclei to produce hybrid cells.
5. Plating and production of colonies of hybrid cells.
6. Selection of hybrid and induction of organogenesis.
7. Transfer of mature plants.

As mentioned above, after fusion of protoplasts wall regeneration takes place and cells undergo mitosis. The resultant colonies are a mixed population of both homokaryotic fusion product and hybrids. Hybrid cell should be differentiated from other cells through various selection methods dependent on physical and biological properties of fused cells and their colonies. Somatic

hybrids can be identified with the help of biochemical markers. P.S. Carlson and co-workers (1972) produced first interspecific somatic hybrids between *Nicotiana glauca* and *N. langsdorfii*. In 1978, Melchers and workers developed first intergeneric somatic hybrids between potato (*Solanum tuberosum*) tomato (*Lycopersicon esculantum*) and which was known as **pomato** or **topato (Fig. 5)** (Dubey, 2016)**.**

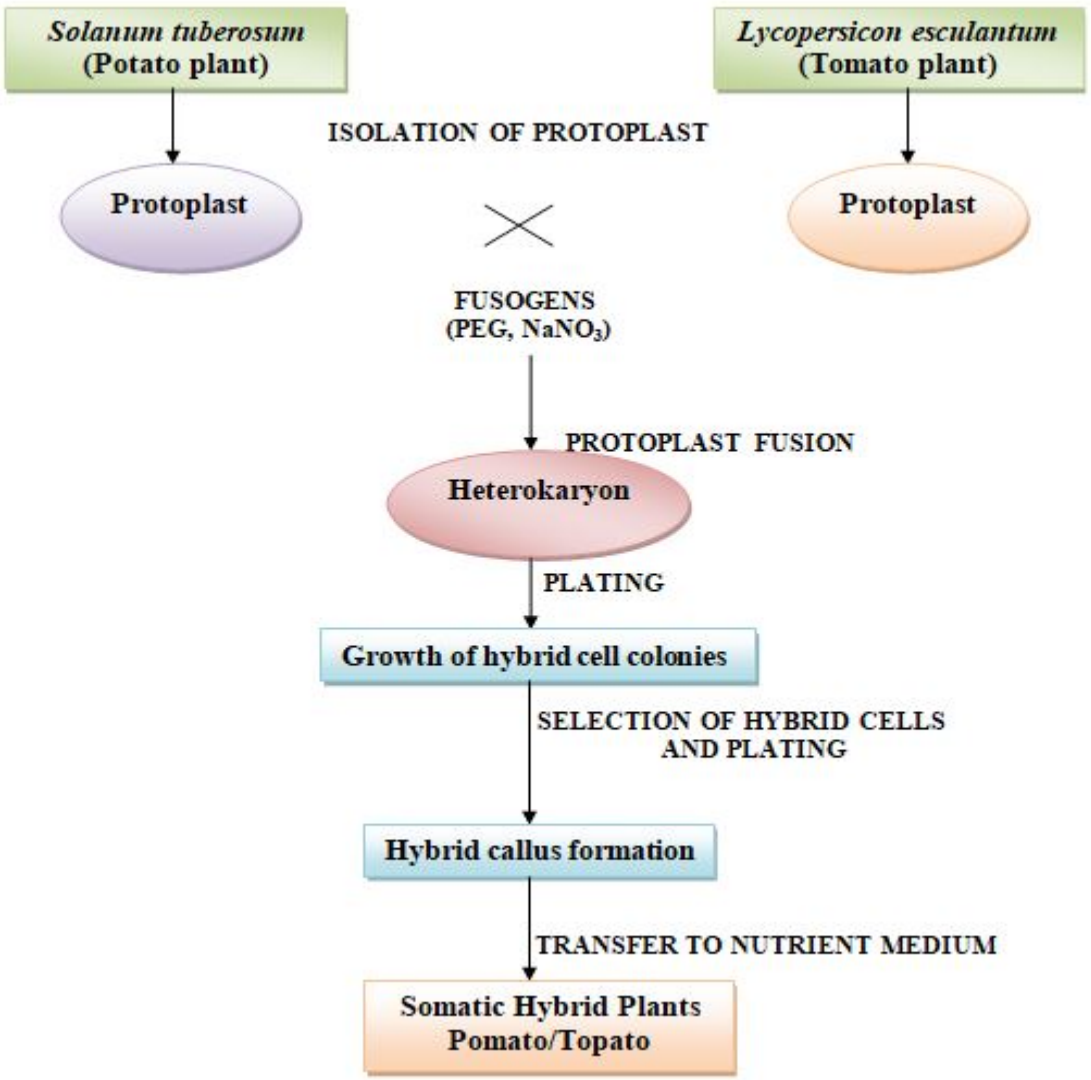

Fig. 5: Hybrid plant (Pomato/Topato) produced through protoplast fusion of potato and tomato plants

During plant tissue culture it is observed that genetic variability occurs spontaneously. P.J. Larkin and W.R. Scowcroft (1981) coined a term '**Somaclonal variation**' for indicating genetic variability that occurs during plant tissue culture, and plant variants obtained is called **somaclones**. According to them, this variation may be due to heterogeneity between cells and explant tissue, spontaneous mutation and transposition of genetic materials in culture environment. Evan *et al.* (1984) introduced the term gametoclonal variation for plant variant

generated from gametic cells. Genetic variation occurs during *in vitro* culture and it may occur in isolated protoplast, undifferentiated cells, calli, tissues etc. Variant selected from tissue cultures have been referred as 'calliclones' (from callus cultures; Skirvin 1978) or 'protoclones' (from protoplast culture; Shepard *et al.* 1980). The cause of variation is mostly due to chromosomal alterations (change in structure or number of chromosomes) and there are several factors that contribute to chromosomal alterations such as (i) pre-existing genetic variations in explants tissues; (ii) occurrence of sponataneous mutations; (iii) Structural and numerical alterations in chromosomes during in vitro culture; (iv) intracellular mutagenic agents produced during in vitro growth; (v) activation of transposable elements (Bhaskaran, 1987; Larkin, P. J. and Scowcroft, W. R., 1981; Razdan,2019) .

Somaclonal variation is one of the aspects of tissue culture and variation in characteristics of somaclones indicates that it can be an additional tool for crop improvement. Possible advantage of somaclonal variation over induced mutagenesis are – (i) frequency of variation is greater than induced mutation; (ii) changes does not involve drastic genetic alterations; (iii) It occurs for trait of both nuclear and cytoplasmic origin, so it has distinct advantage due to variations in cytoplasmic genes. J. E. Shepard and co-workers (1980) screened about 100 somaclones of Russet Burbank and found a significant and stable variation in its various traits - growth habit, maturity, date, tuber uniformity, tuber skin colour and photoperiodic requirements and here greater tuber uniformity and early onset of tuberization were agronomic improvements over the parent variety. Thus, somaclonal variation has proved an alternative tool to plant breeding for generating new varieties that can exhibit disease resistance and improvement in quality and yield in many plants (**Table-3**) (Dubey, 2016; Purohit, 2005).

Table-3: Somaclonal variation in some crop plants

Crop	Variant traits/Character
Brassica spp. (Brassicaceae)	Flowering time, precocious flowering from apex, stem or leaf, growth habit, gross morphology
Nicotiana tabacum (Solanaceae)	Yield, plant height, leaf size, type of inflorescence, alkaloids
Oryza sativa (Poaceae)	Plant height, number of tillers per plant, panicle size, seed fertility, flowering period.
Saccharum officinarum (Poaceae)	Cane diameter, stalk length and number, sugar yield, pathogenic disease
Solanum tuberosum (Solanaceae)	Plant habit, disease resistance, shape, yield and maturity period of tubers, photoperiod requirement, tuber uniformity
Zea mays (Poaceae)	Twin stalk formation, pollen fertility, endosperm and seedling mutant

In India, Sugarcane Breeding Institute, Coimbatore has released many varieties produced through the process of somaclonal variation, eg. sugarcane variety - **Co 2001-13** (Sulabh) which is resistant to red rot and drought. **Bio-13** (somaclonal variant of *Citronella java* with 37% more oil and 39% more citronellon) has been released for commercial cultivation by Central Institute

for Medicinal and Aromatic Plants (CIMAP), Lucknow. Heinz Co. and DNA Plant Technology Laboratories (USA) developed **supertomatoes** with high solid component which reduced shipping and processing costs.

5.9. PRODUCTION OF SECONDARY METABOLITES/ NATURAL PRODUCTS

The use of plant tissue culture for the production of useful compound has become a subject of interest for biotechnologists in various biotechnological programmes. Microorganisms have already been utilized in cultural practices for industrial production of drugs, breweries etc. Similarly higher plants which are important source of natural products like pigments, sweeteners, flavours, fragrances, antimicrobials etc. are also utilized in production of these compounds using cultural techniques. Majority of these compounds belongs to a metabolic group called as secondary metabolites and chemically these are of different types e.g. latex, alkaloids, tannins, resins, terpentines etc. **Secondary metabolites** are generally not involved in vital metabolic functions of plants but act as a chemical interface between plants and its surrounding environment like protecting them from predators and attracting pollinators. These substances are useful in combating many infectious disease or act as anticancer agents (Whitaker and Hashimoto, 1986; Wink *et al.*, 2005). As these compounds are naturally produced by plants in small amount, therefore their production in large amount is done by cell suspension culture technology. Plant cell culture has been considered as potential source of specific secondary metabolites and cell cultures have following advantages in natural products/metabolite production –

1. Rate of biochemical synthesis from small amount of plant material is quite high in short time period.
2. Cultures are maintained in controlled conditions (environmental and nutritional) ensuring continuous yields of metabolites.
3. A new means of product synthesis from plants difficult to grow or establish e.g. quinine, pyrethroids etc.
4. A new route of noval compounds synthesis from deviant and mutant cell lines.
5. Some cell cultures have the capacity for biotransformation of specific substrate to more valuable compounds. e.g. digoxin synthesis (Fowler, 1983).
6. Cell cultures are more economical for those plants which attain maturity very late e.g. *Papaver bracteatum* (source of thebaine) attain maturity in 2-3 seasons.

Production of secondary metabolites can be achieved by selection of specific cells and the development of a suitable medium. Through clonal selection such strains of cells are selected that produces greater amount of these substances than those found in the intact plants. For this purpose two methods are used – single cell cloning and cell aggregate cloning. The latter process is easier than the first one as isolation and culture of single cell is comparatively difficult. Nowadays in vitro production of useful compounds or secondary metabolites has increased and

due to economical and pharmaceutical importance of these compounds, plant tissue culture technology are utilized for large scale production of these substances (**Table-4**)(Dubey, 2016; Razdan, 2019).

Table – 4: Secondary metabolites/natural products produced by plants and their uses -

S. no.	Plant species	Secondary metabolites/ Natural Products	Uses
1	*Artemisia* spp.	Artemisin	Antimalarial
2	*Azadirachta indica*	Azadirachtin	Insecticidal, antiprotozoan, anti-worm
3	*Barbaris* sp.	Barberine	Antibacterial, antiprotozoan, anti-inflamatory
4	*Catharanthus roseus*	Vinblastine, vincristine	Anticarcinogenic
5	*Cinchona officinalis*	Quinine	Antimalarial
6	*Coffea arabica*	Caffeine	Stimulant
7	*Crocus sativa*	Saffron	Flavouring/colouring agent
8	*Digitalis lanata*	Digoxin	Cardiac tonic
9	*Dioscorea deltoidea*	Diosgenin	Antifertility
10	*Ephedra gerardiana*	Ephidrine	Spasmolytic
11	*Jasmium* sp.	Jasmine	Perfume
12	*Papaver sominiferum*	Codeine	Sedative, analgesic
13	*Rauwolfia serpentina*	Resperine	Hypotensive
14	*Taxus buccata*	Taxol	Anticarcinogenic

5.10 CRYOPRESERVATION & GERMPLASM PRESERVATION

The sum total of all genes present in a crop constitutes its germplasm or it refers to the heredity material transmitted to offspring. In recent years, with the advances of modern tools and technology, introduction of exotic species and due to the course of evolution many new introduced plants species with desired traits have started replacing the primitive plants with some valuable genetic traits. Hence, it is necessary to conserve these endangered species so that their valuable traits can be retained. For this purpose, a global body namely **International Board of Plant Genetic Resources (IBPGR)** has been established in **1974**. Main objective of this organization is to advance the conservation and use of plant genetic resources for the present and future generations.

The main objective of **germplasm conservation** is to preserve the genetic diversity of plant for its future use. There are two main approaches for germplasm conservation **– in-situ and ex-situ conservation**. **In-situ** means conservation of germplasm in their natural environment i.e. national parks, sanctuaries, biosphere reserves etc. Important aspect of this type of conservation

is that it enables the natural evolutionary process among species allowing the appearance of new recombinant forms. But this conservation process require controlled monitoring, thus have expensive maintenance cost for large number of species. Also in nature, there is probability of germplasm degradation due to various environmental hazards. **Ex-situ** means conservation of genetic materials in form of seeds or in form of in vitro cultures (plant cells, tissues or organs cultures) preserved in gene banks for long term storage under suitable conditions. In ex-situ conservation, genetic resource is not conserved in its natural environment so it does not allow the natural evolutionary process to continue, but it ensures the safety of genetic materials and ensures its availability in need.

Traditionally, germplasm is conserved as seeds stored in required temperature. But conventional methods fail to prevent losses due to disease, pathogens, pests, climatic disorders and other economic causes. It could not save the viability of short lived seeds of economic plants like oil palm, rubber, *Citrus* sp. and *Coffea* sp. (Dodds and Roberts, 1985). Biological activities of these materials can be conserved for long time by storing at low temperature at which growth rate of cells retards. It has been found very effective for tissue culture of many plant species such as potato, cassava, pea, rice, wheat, coconut, palm, strawberry and sugarcane etc. There are three main ways of in-vitro germplasm preservation: (i) **Cryopreservation**, (ii) **Cold storage** and (iii) **Low pressure and low oxygen storage**.

I - CRYOPRESERVATION

Cryopreservation (gr. Kayos meaning “frost”) refers to preservation in the “frozen state”. It is the process of storing cells, tissues or organs at very low temperature such as over solid carbon dioxide (-79^{o}C), in deep freezers (-80^{o}C), in vapour phase nitrogen (-150^{o}C) or in liquid nitrogen (-196^{o}C) in the presence of cryoprotectants. Various difficulties are also associated with cryopreservation like certain features of plant cells (large size, water abundance and strong vacuolization), cell damage during freezing and thawing process and large crystal formation that often ruptures cell membranes etc. But due to presence of cryoprotectants these difficulties can be overcome.

Steps of Cryopreservation:

(i) **Selection of plant material** – Preferential plant materials that are used in cryopreservation are apical meristem, plant organs, protoplasts, anthers, pollen grains, ovules and young embryos etc. Cultured cells are not ideal material for cryopreservation.

(ii) **Cryoprotectants** – Addition of mixture of few cryoprotectors like dimethyl sulfoxide (DMSO), sugars, sugar alcohols, alcohols, glycols, polyethylene glycol (PEG), dextrins, glycerine, sucrose and some amino acids etc. These cryoprotectants protect the preserved materials from cryodestruction.

(iii) **Freezing of material** – freezing of material should be done in regulated rate (desired rate is commonly not less than 1^{o}C per minute) in order to prevent intercellular freezing and crystal formation. Types of freezing can be rapid, slow or stepwise.

(iv) **Storage of material in liquid N_2** – For prolonged storage of frozen material very low temperature is required and this can be achieved by the use of liquid N_2 that maintains the temperature at -196^oC. At this temperature all the metabolic activities of cells get retarded and it also prevents biochemical injury.

(v) **Thawing and washing** – This process involves the elevation of temperature (between 35-45^oC) of frozen vials containing preserved material and as the last ice crystal disappear, quickly transferring the vials to water bath (20-25^oC). After thawing the obtained plant materials should be washed to remove the toxic cryoprotectants. This process involves – dilution, resuspension, centrifugation and removal of cells.

(vi) **Reculturing** – During above process there is possiblilty that some cells die due to storage stress and strong one survive. In order to find out viable one there culturing on growth medium is essential. Further, cell viability test can be done by using FDA (Fluorescein Diacetate) staining and growth measurement (cell number, dry and fresh weight, mitotic index).

Mitotic Index = $\frac{\text{Total number of dividing cells}}{\text{Total number of cells (dividing and undividing)}} \times 100$

(vii) **Regeneration** – The selected viable cells are then cultured on growth media to regenerate into plantlets.

II – COLD STORAGE

In this method, germplasm is conserve at a low and non-freezing temperature (1-9^oC). In this case growth activity of plant material is slowed down. This process prevents preserved materials from cryogenic injuries and yields germplasm with good survival rate. It is simple and cost effective process. Through this techniques grape plants and virus free strawberry have been stored for fifteen years (9^oC) and for six years (10^oC).

III – LOW PRESSURE AND LOW OXYGEN STORAGE

In low pressure storage method, atmospheric pressure around the plant material is reduced whereas in case of low oxygen storage method, the oxygen concentration is reduced which ultimately reduces the in vitro growth of plants. This method is useful in increasing the shelf life of many fruits, vegetables and flowers.

Significance of germplasm preservation

1. Preservation of genetic diversity.
2. Conserving plant materials of endangered species.
3. Long term conservation of cell cultures of several species through cryopreservation.
4. Preservation of disease free plant material that can be propagated when it is required.
5. Conservation of somaclonal variations.
6. Long term maintenance of recalcitrant seed.
7. Enhancing pollen longevity by conserving it.
8. Development of germplasm banks.

9. Important in breeders programmes as it provide raw materials to breeder for producing various crops.

The main limitation of germplasm storage is that this process requires expensive equipment and trained persons for executing it in proper way. Organisations associated with germplasm are International Plant Genetic Resources Institute (**IPGRI**), National Bureau of Plant Genetic Resources (**NBPGR**), Forest Research Institute (**FRI**) and Botanical Survey of India (**BSI**).

5.11. SUMMARY

- Plant tissue culture is a collection of techniques used in maintaining and growing plant cells, tissues or organs under sterile conditions on a nutrient medium.
- A German botanist, **Gottlieb Haberlandt** (1898,1902) has done intensive effort and developed the concept of *in vitro* cell culture and is thus regarded as father of tissue culture.
- In India, tissue culture work was pioneered by **Panchanan Maheshwari** who is regarded as father of embryology in India.
- **Guha** and **Maheshwari** (1964-67), first time developed the haploid through anther and pollen culture.
- **Murashige and Skoog** (1962) formulated MS medium suitable for many applications and most widely used in plant tissue culture. Other media used in plant tissue culture are proposed by Gamborg *et al* (1968), White (1963), and Smith (1967) etc.
- The general techniques used in plant tissue culture are cleaning of glassware, sterilization of glassware and media, sterilization of plant material and culture.
- **Totipotency** is the basis of plant cell and tissue culture. The term 'totipotency' is coined by T. H. Morgan (1901).
- The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular **totipotency**
- The reversion of mature differentiated cells of explant to the meristematic state - undifferentiated callus tissue is called '**dedifferentiation**'. The conversion of component cells of callus to whole plant or plant organ is described as '**redifferentiation**'. The phenomenon of dedifferentiation and redifferentiation are described as '**cellular totipotency**' which is found only in plant cells.
- The basic event in the development of higher organisms is the specialization of cells i.e. **cytodifferentiation**. For the development of whole plant from callus totipotent cell, there should be organogenesis and this can be accomplished either through '**shoot bud differentiation**' or through '**somatic embryogenesis**'.
- **Shoot bud differentiation** involves formation of shoot buds and this is initiated by both chemical and physical factors. Besides genotypic influence it is also dependent on the auxin/cytokinin ratio in the medium.

- **Somatic embryogenesis** is an in vitro method of plant regeneration in which an embryo or a complete plant development is initiated from a single somatic cell or a group of cells and this is widely used as important biotechnological tool.
- Somatic embryogenesis was reported in carrots by **Steward *et al***. (1958) and later, It has been studied on other plants like *Citrus, Coffea* sp. etc.
- Plant tissue cultures have wide application in several areas like agriculture, horticulture, forestry etc. and it is an important tool for crop improvement, micropropagation, production of disease resistant plant, commercial production of natural compounds, conservation of germplasm etc.
- **Micropropagation** is an alternative means of asexual propagation of important plants through tissue culture or cell culture techniques. It offers many advantages over traditional methods of plant propagation like millions of clonal plants can be regenerated from small explant in a short time and space, developing disease free plants, continuous production of plants round the year etc.
- **Artificial or synthetic seeds** are the somatic embryos encapsulated in a suitable matrix (e.g. sodium alginate), along with substances like mycorrhizae, insecticides, fungicides and herbicides. The concept of artificial seed was first time given by T. Murashige (1977).
- **Protoplast fusion or somatic hybridization** involves hybridisation between species or genera during protoplast culture. It is playing an important role in plant breeding and crop improvement.
- During protoplast fusion, when the two nuclei also fuse then the resultant cell is known as **hybrid or synkaryocyte** and when the genetic information from one of the two nuclei is lost then the cells are known as **cybrid or cytoplasmic hybrid or heteroplast**
- **Somaclonal variation** is the term used for indicating genetic variability that occurs during plant tissue culture, and plant variants obtained is called **somaclones**. It is an alternative tool to plant breeding for generating new plant varieties with useful traits like disease resistance, improvement quality and high yield.
- Plant tissue culture is also used for the production of useful compound. Microorganisms are utilized in industrial production of drugs, breweries etc. Similarly higher plants which are source of **natural products/ secondary metabolites** are also utilized in production of these compounds using cultural techniques.
- Germplasm refers to the sum total of all genes present in a crop. Through **germplasm conservation** the genetic diversity of plant can be preserved for its future use.
- G**ermplasm conservation –** in-situ and ex-situ conservation. **In-situ** - conservation of germplasm in their natural environment (national parks, sanctuaries, biosphere reserves etc). **Ex-situ** - conservation of genetic materials in form of seeds or in form of in vitro cultures (plant cells, tissues or organs cultures) preserved in gene banks.
- There are three main ways of in-vitro germplasm preservation - Cryopreservation, cold storage and low pressure and low oxygen storage.

- **Cryopreservation** is the process of storing cells, tissues or organs at very low temperature (in solid carbon dioxide.-79^{o}C; deep freezers,-80^{o}C; vapour phase nitrogen,-150^{o}C; liquid nitrogen,-196^{o}C) in the presence of cryoprotectants.

5.12. GLOSSARY

Agar – a polysaccharide powder obtained from algae (e.g. *Gracilaria, Gelidium*) used to gel a medium.

Anther culture – culture of single pollen grains or anther for producing monoploid plants.

Artificial seed – encapsulated somatic embryos that are treated like seed.

Aseptic – free of microorganisms; free of pathogens, contaminants, algae, bacteria, fungi, viruses, etc.

Autoclave – a machine used for sterilizing wet or dry items with steam under pressure.

Auxin – Phytohormone that promotes callus growth, cell enlargement, adventitious buds and lateral rooting.

Callus – mass of unorganized plant parenchyma cells.

Clone – asexually produced plant from a single source plant.

Clonal propagation – asexual production of genetically uniform plants from an explant.

Contamination – contaminated with unwanted microorganisms such as bacteria or fungi.

Cryopreservation – low temperature storage of cells, tissues, embryos, seeds etc.

Cryoprotectant – an agent that prevent freezing and thawing damage to cells during cryopreservation.

Culture – in vitro growing of a plant.

Cybrid – a cytoplasmic hybrid cell with the nucleus of one fusing cell and cytoplasmic organelles of another, or of both cells.

Cytokinin – a phytohormone that regulate growth, morphogenesis and cell division.

Differentiated – Cells with the specialized structure and function, typical of the cell type in vivo.

Explant – the excised plant portion taken from its original site and transferred to culture medium for growth or maintenance or for tissue culture.

Gibberellins – a phytohormone that influences cell enlargement.

Hormones – growth regulators like cytokinins, auxins and gibberellins etc.

In vitro – to be grown within the glass or performed outside of living organism.

In vivo – to be grown naturally or within a living organism.

Medium – a nutritive solution for culturing cells.

Micropropagation – In vitro clonal propagation of plants from shoot tips, axillary buds, adventitious roots etc.

Organ culture – culture of plant organs such as roots or shoots.

Somaclonal Variation – Phenotypic variation seen in plants (somaclones) produced by plant tissue culture.

Somaclones – Plants obtained through cell culture by using somatic plant cells.

Somatic embryos – embryo like structure obtained from somatic cells.

Sterile – without life or a culture free from microorganisms.

Tissue culture – in vitro maintenance or growth of tissue (explant) in a culture medium under sterile condition.

Totipotency – a potential of a cell to divide and give rise to entire plant.

Transgenic – plants having a piece of foreign DNA.

Undifferentiated – plant cells, existing in a state of cell development.

5.13. SELF ASSESSMENT QUESTION

I. Very Short Answer Type Questions:

Q1. Who is known as the father of plant tissue culture?

Q2. Who executed the experiments on wound healing in plants through callus formation?

Q3. Who prepared the first synthetic seeds?

Q4. Who produced first interspecific somatic hybrids between *Nicotiana glauca* and *N. langsdorfii*?

Q5. Conservation on Micropropagation Research and Technology Development (CMRTD) is involved in ..... related activities of plant species.

Q6. Mention one of the main objectives of germplasm preservation?

Q7. Name any two varieties produced through the process of somaclonal variation?

Q8. What is the full form of NBPGR?

II. Multiple Choice Questions (MCQs):

Q1. What is the term used for unorganized mass of cells?
(a) Explants (c) Totipotency
(b) Callus (d) Regeneration

Q2. Who developed first haploids through anther and pollen culture?
(a) Guha and Maheshwari (1964) (c) T. H. Morgan (1901)
(b) Larkin and W.R. Scowcroft (1981) (d) None of Above

Q3. Which term is used for the ability of a cell to divide and regenerate into a whole plant?
(a) Callus (c) Regeneration
(b) Explants (d) Totipotency

.Q4. Haploid plants can be obtained from……culture.
(a) Bud culture (c) Root culture
(b) Leaf culture (d) Anther culture

Q5. What occurs at high auxin:cytokinin ratio?
(a) Somatic embryo (c) Root Initiation
(b) Callus (d) Shooting

Q6. What is the term used for the plant propagation through tissue culture?
(a) Hybrid (c) Micropropagation
(b) Cybrid (d) Regeneration

Q7. Name the chemicals used as fusogen during protoplast fusion.
(a) Polyethylene glycol (PEG) (c) MS Medium
(b) $CaCl_2$ (d) None of Above

Q8. Who coined the term somaclonal variation?
(a) Larkin and W.R. Scowcroft (1981) (c) Guha and Maheshwari (1964)
(b) S. L. Kitto and J. Janick (1985) (d) Steward *et al.* (1958)

Q9. Which of the following substance is used as cryoprotectant?
(a) Nitrous oxide (c) Dimethyl sulfoxide (DMSO)
(b) MS Medium (d) None of Above

Q10. Most commonly used medium for culturing plant tissue.
(a) White medium (b) Gamborg medium

(c) MS medium (d) Gautheret medium

Answers –

I. Very Short Answer Type Questions:

1. Gottlieb Haberlandt; **2.** Henri-Louis Duhamel du Monceau; **3.** Kitto and coworkers (1982); **4.** P.S. Carlson and co-workers (1972); **5.** Micropropagation; **6.** Save plants from extinction; **7.** Sugarcane variety - Co 2001-13 (Sulabh) and somaclonal variant of *Citronella java* - Bio-13; **8.** National Bureau of Plant Genetic Resources

II. Multiple Choice Questions (MCQs):

1. (b) Callus; **2. (a)** Guha and Maheshwari (1964); **3. (d)** Totipotency; **4. (d)** Anther culture; **5. (c)** Root Initiation; **6. (c)** Micropropagation; **7. (a)** Polyethylene glycol (PEG); **8. (a)** Larkin and W.R. Scowcroft (1981); **9. (c)** Dimethyl sulfoxide (DMSO); **10. (c)** MS medium.

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5.15. SUGGESTED READINGS

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- Elements of Biotechnology – P. K Gupta
- Introduction to plant tissue culture – M. K. Razdan

- Biotechnology Fundamentals and Applications – S. S.Purohit
- Plant Tissue Culture: Theory and Practice - S. S. Bhojwani, and M. K. Razdan

5.16. TERMINAL QUESTIONS

Q1. Give a brief history of development of plant tissue culture.

Q2. What is micropropagation? What are its advantages over traditional methods of plant propagation?

Q3. Give a brief account of 'shoot bud differentiation' and 'somatic embryogenesis'

Q4. What is somatic hybridisation? Describe the various steps involved in somatic hybridisation.

Q5. What are cybrids and how these are produced? Mention some of the uses of cybrids in crop improvement.

Q6. What is somaconal variation and how it can be induced? What are the advantages of somaclonal variation over induced mutagenesis?

Q7. Give an account of production of secondary metabolites through plant cell culture.

Q8. What is cryopreservation? What are the steps involved in cryopreservation?

Q9. Discuss the application of plant tissue culture in agriculture, forestry, medicines and industries.

Q10. Write a short note on the following?

(i) Callus
(ii) Totipotency
(iii) Cytodifferentiation
(iv) Steps of clonal propagation
(v) Artificial seeds
(vi) Protoplast fusion
(vii) Hybrid and Cybrid
(viii) Somaclonal varients
(ix) Germplasm conservation
(x) Cryoprotectants

UNIT-6 ORGANOGENESIS AND ADVENTIVES EMBRYOGENESIS

Contents:

6.1 OBJECTIVE

After reading this unit students will be able to-

- Understand the fundamentals of organogenesis and adventive embryogenesis.
- Understand the concept of morphogenesis.
- Understand the concept of androgenesis.
- Understand the technique of haploid production via anther culture and microspore cultures.
- Understand the techniques and utility of these processes.

6.2 INTRODUCTION

The intact plant represents a highly organized and coordinate system in which number of factors correlatively operates in integrated manner to form a plant with its several organs. Regenerating complete plantlet from a single cell or tissue *in vitro* on an artificial culture media is the one of the biggest achievement of the twentieth century. The technique which deal with regeneration is called as "Plant tissue culture", which is generally define as the aseptic culture of tissue or cell *in vitro*. Plant tissue culture exploits the totipotent nature of the plants and manipulates other conditions to regenerate the plantlets *in vitro*. Totipotency is the ability of plant cell to perform all functions of development, which are characteristic of zygote i.e its ability to develop into a complete plant. Besides this, this technique based on two more fundamental process i.e., dedifferentiation and rededifferentiation, with the use of some phytoregulators to regenerate plantlet. Primarily using plant tissue culture we regenerate callus by using almost any part of the plant i.e., leaf, pith, root, floral part, petiole, tubers, apical meristem etc and from every calli under appropriate conditions and manipulations we may have organs of interest, embryo or complete plantlet.

This biotechnological approach or in other word bio- technological approach promise the supply of plant throughout the year irrespect of season, further besides complete plantlet this technique promise for producing organ (shoots or roots) of interest via organogenesis. Organogenesis deals with an organ's initiation and development and we get organ of our desire by just manipulating the culture condition. Besides the organ this technique also provides a pure homozygous line in one generation or provides unlimited colons throughout the time. Further, using this technique we may regenerate haploid, triploid, adventive embryos which otherwise very tedious under natural conditions.

In this unit we are going to disuse some fundamental aspects, techniques and mechanism in plant tissue culture.

6.3 ORGANOGENESIS AND ADVENTIVE EMBRYOGENESIS

6.3.1 Organogenesis

Plants, the eukaryotic multi-cellular organism consists of many specialized cell organizations. Tissues and organs are among the many specialised cell groups seen in an adult plant. Meristem, cortex, phloem, and epidermis are tissues made up of cells with a uniform shape

and particular function. Leaves, roots, flowers, and the vascular system are examples of tissues that are grouped together to form an organ.

Organogenesis is the process of an organ's initiation and development. Under normal in vivo condition this process is under the control of number of genes (*LEARY GENE, APETALA*1, *APETALA*2, *APETALA*3,*PISTILLATA, AGAMOUS, STM, CLAVATA* etc) and the expression of each and every gene is under the control of provided conditions in the unit time (temperature, humidity, season, water, stress etc).

During *in vitro* the induction of different adventitious organs of the plant from cultured tissues under *in vitro* condition is known as organogenesis (Fig 6.1). The organogenesis includes two steps, Caulogenesis (development of shoots) and rhizogenesis (development of roots). Organogenesis begins with the production of primodium, which commences with the development of a clump of meristematic cells termed meristamoids. Organogenesis begins in the callus in response to the substances in the media stimulating it. Inducing organogenesis in plant tissue culture is an important approach to regenerate plants from the culture. White (1939) reported the first *in vitro* stimulation of shoot organogenesis using a tobacco hybrid, while Nobecourt (1939) reported the first detection of root development using carrot callus. The primary regulatory mechanism driving organogenesis was unknown until the late 1950s. By growing explants, calli, and cell suspension in a specified medium, it is now able to attain organogenesis in a significant variety of plant species. Organogenesis, which is basically based on the concept of dedifferentiation and redifferentiation is a monopolar structure for whole plant regeneration. It grows procambial threads that connect to the pre-existing vascular tissue scattered throughout the callus or cultured explant. Organogenesis is significantly more prevalent than somatic embryogenesis, and therefore has much more-greater potential for clonal plant growth. Torrey (1966) proposed that organogenesis in callus begins with the formation of a group of meristematic cells, that is, meristemoids, that can respond to factors inside the system to generate a primordium, which can trigger the formation of either shoot or embryoid depending on the types of inputs.

Organogenesis can be accomplished in two ways:

1. Direct organogenesis i.e., emergence of adventitious organs directly from explants.
2. In direct organogenesis i.e., development of organs passed via callus formation.

6.3.1.1- Organogenesis via callus formation

Callus is generally defined as the group of parenchymatous cells that are produced from the explant via dedifferentiation and have potential for the redifferentiation. Organogenesis via callus culture require, first establishment of successful aseptic culture from the selected explants (cotyledons, hypocotyl, stem, leaf, shoot apex, root, immature inflorescence, flower petals, petioles, embryos, etc) and then exposing the callus to different PGRs for organogenesis. The explant may be *in vitro* grown or wild depending upon the conditions and needs. Further, some time a specific explant may be needed for successful plant propagation in any given species or variation. Callus formation can be induced in explants from both mature and immature organs further; the explants with mitotically active cells are usually preferred for callus induction. The explant's size and shape are also important aspects during incubation. It has been discovered that just a tiny percentage of cells in a specific

explant contribute to callus development. In explants peripheral surfaces or the excised surface are the most common sites for callus formation. *In vitro,* callus is formed on explants as a result of injury and in reaction to hormones provided in the medium, either endogenous or exogenously. Because of their clonal qualities, culture survival, growth rates, and totipotency *in vitro,* meristematic are always preferred over other tissues. Explants from cereals such as meristems, shoot tips, axillary buds, immature leaves, and immature embryos are particularly well suited. Explants from herbaceous species such as mature leaves, roots, stems, petioles, and flower parts can often be effectively grown to generate plantlets through organogenesis. The form of every callus is determined by the explant tissue or tissues from which it emerged, as well as the medium employed to initiate and nourish it. Callus can be sequentially subcultured and harvest for long periods of time, however the structure and composition of the callus can alter over time as the medium favours certain cells and they begin to dominate in the culture.

During organogenesis from the callus, the redifferentiation process came to take part and this process is under the control of plant growth regulators and after any attempts and failures it was established by Skoog and Miller (1957) and many other workers of the time that successful redifferentiation can be achieved by just altering the concentrations of auxin and cytokinin and according to their findings, a high ratio of auxin to cytokinin facilitated root production whereas the opposite favoured shoot proliferation.

6.3.1.2- Direct Adventitious Organ formation

In some plants (generally woody) they have ability to give rise their clones directly during *in vitro* cultures from their nodal explant and rarely from their apical shoot meristem. Besides this, other ways of propagation is to induce adventitious shoots directly from intact bulb scales, and basal disc (in case of globular herbs) particularly well suited to herbaceous species in culture. Numerous examples of shoots developing accidentally on a variety of organs have been documented in the literature across the plant world. Direct organogenesis has been reported from stem segments in *Acacia nilotica,* hypocotyl explants has been reported in *Tamarindus indica.* Direct organogeneis from cotyledon explants has been reported in *Tamarindus indica, Albizzia falcataria, and Sesbania grandiflora*. Buds in Begonias, for example, usually start along the injured leaf during *in vitro* culture.

The choice and nature of explant is further affecting the potential of direct organogenesis, in some cases shoot production from explant (nodes or any other meristem) take place devoid of growth regulator in the culture medium, but for other cytokinin are must be there in the culture medium. Moreover, the amount of exogenous auxin and cytokinin required in the process varies per tissue system, and appears to be dependent on endogenous hormone levels in the tissue. These findings gave rise to the idea of totipotency, or the ability of all cells to regenerate a whole new plant even after differentiation inside the plant's somatic tissues. As a result, the reactivation of genes associated with the embryonic development stage will be required for the production of adventitious structures. The rate of shoot formation from in direct method *in vitro* regeneration may be significantly higher than that from the in direct organogenesis, further these are the clone copy of the parent and rhizogenesis responses are also high when placed in rooting cultures.

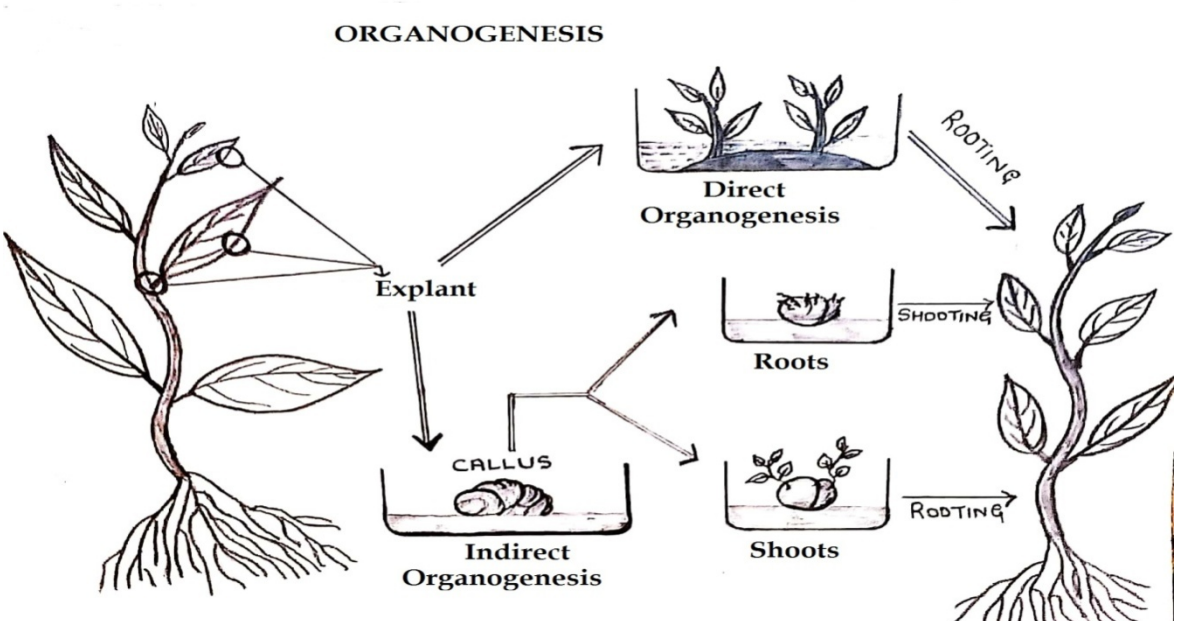

Figure* 6.1*: Plant regeneration via Organogenesis

6.3.1.3- Mechanisms during organogenesis

Organogenesis is thought to occur by a process of differentiation in an undifferentiated mass of parenchyma. The majority of parenchymatous cells are severely vacuolated, with small nuclei and cytoplasm, and some have lignification. Random cell division would occur in this set of cells, resulting in radial files of differentiated tissues. Meristematic centres, also known as meristemoids, would arise as a result of these scattered cell division zones forming regions of intense mitotic activity. These meristemoids might be found on the calli's surface or embedded in the tissue. Continuing cell development in these meristemoids would result in tiny protuberances on the calli's surface, giving the tissues a nodular look. According to Torrey (1966), the primordia of organs develop either a shoot or a root from the meristemoids by recurrent mitotic activity. Meristemoids are spherical masses of tiny isodiametric meristematic cells with packed cytoplasm and a high nucleo-cytoplasmic ratio. Before organogenesis, callus tissues store starch and other crystals, but these substances vanish during meristemoid development. The cytoplasmic protrusions enter the vacuoles during the early stages of meristemoid development, dispersing the vacuoles around the edge of each cell or distributed all through the cytosol.

6.3.1.4- Factors affecting Organogenesis

The following factors directly or indirectly affects the process of organogenesis:

1. **Explant Size**- The size of the explant has a good impact on organogenesis. The larger explant, which contains parenchymatous cell, vascular tissue and cambium, has a higher regeneration capacity than the smaller explant.
2. **Explant source**- The explant source is one of the rate limiting and important aspects of plant tissue culture, with the vast diversity of plants the source of organogenesis

also vary from plant to plant. Depending on the species, the best part of the plant to use for starting culture will vary. Like many plant species' leaves and leaf fragments are good for callus induction but in some other petiole is best. Such as *Begonia, Solanum, Nicotiana, Crepis*, and others have the ability to regenerate the shoot buds from leaves explants. In *Thymus Spp* nodal part is of preference for shoot regeneration. Likewise, bud production capacity is high in many monocot species with specialised storage organs.

3. **Explant Age-** The age of the explant is another important factor that affects organogenesis. In the case of *Nicotiana sp.*, regeneration of an adventitious shoot bud is only observed when leaf explants are gathered during the vegetative phase, prior to flowering. Young *Echeveria sp.* leaf explants produce just roots, whereas older leaf explants produce only shoot buds, and medium-aged leaves produce both shoots and roots.
4. **Light quality and intensity**-Shoot formation is promoted by blue light, while rooting is induced by red light. The organogenesis phenomena, is also stimulated by the application of blue light followed by red light. Artificial fluorescence light promotes rooting in some cultures while inhibiting it in others. In the case of Pisum sativum, shoot bud initiation occurs in the dark, followed by a brief exposure to light.
5. **Temperature**-The majority of tissue cultures can be successfully cultivated at twp. around 25 ^{o}C. The ideal temperature for a variety of bulbous species could be as low as 15-18°C. Increases in temperature up to 33^{o}C have been linked to increased tobacco callus formation, however for shoot bud initiation, a lower temperature of around 18 ^{o}C may be best.
6. **Culture Medium**-Although some reports show the production of leaf shoot buds on culture cultivated in a liquid medium, medium solidified with agar favours bud formation.
7. **Medium pH-** Organogenesis may be influenced by pH. Before sterilisation, the PH of the culture medium is usually adjusted to 5.6 to 5.8.
8. **Ploidy level-** Chromosomal stability is the most crucial component in sustaining callus tissue's organogenic potential. Ploidy level (Anuploidy, polyploidy, and other chromosome number variations) in cultured plant cells have been thoroughly described and it is observed that there is a general decrease in the morphogenetic potentiality of callus tissue as chromosomal instability increases. The morphogenetic capacity of a totipotent callus may be reduced or even lost during repeated culturing. Other cultivated tissues with a genetic imbalance have been documented, including pollen from Ginkgo, and stalk callus in Tobacco.
9. **Age of Culture-** Organs are typically produced by a young culture. However, in old culture, the organogenic potential may decline and eventually vanish. Plant regeneration potential can be maintained endlessly for many years in specific cultures of some plants.
10. **Chemical factors (Phytohormones)-**

a. **Ratio of Auxin to Cytokinin:** A high ratio of auxin to cytokinin promotes root development, whereas an elevated concentration of cytokinin and low auxin promotes shoot development.
b. **Auxin in low concentration:** The removal of auxin after pre-culture results in root development. Auxin 2.4-D is a powerful growth agent that is metabolised slower by tissues and hence stays in the medium longer than IAA or NAA. It is also more active in activating meristematic activity, which leads to cell proliferation. Whatever the type of the culture system utilised, tissue growth on a 2,4-D medium for a period of time followed by transfer to a 2,4-D free media causes differentiation/organogenesis. In most cases, the loss of morphogenetic potential is linked to 'habituation,' a condition associated with cytokinin-autotrophy in long-term cultures.

6.3.2- Adventive embryogenesis

Embryogenesis, a general term use to explain the one of the basic fundamental process of reproductive biology, which include's the syngamy followed by development of the embryo from the zygote. But some time embryos may developed from other ovular tissue i.e., nucellus and integuments or rarely from cells of embryo sac, since these cells are haploid in nature the embryo produced is diploid and called as somatic embryo, adventive embryo, embyoid, adventitious embryo and supernumerary embryo. These type of embryos naturally reported in *Citrus, Mangifera, Optunia, Limnathes* etc, and the path and factor contributing in the production is studied experimentally in *Citrus* by many workers.

As an alternative, the plant tissue culture technique exploiting the totipotent nature of plant cells to regenerate the plants and in this way it was reported that not only ovular tissue but all other somatic cells may have the potential to produce the embryos and the *in vitro* produced embryos are called as somatic embryos. The initiation and development of embryos from somatic tissues in plant tissue culture was first recognised by Steward *et.al.,* 1958 and Reinert, 1958-1959 in *Daucus carota*.

Majorly the somatic embryos are in demand due to the following reason

1. Large Scale Propagation Compared to Zygotic Embryos
2. Feasibility of single cell origin and the possibility of automating the large-scale production of embryos in bioreactors.
3. Production of synthetic seeds from these somatic embryos.
4. This technique is more useful than Organogenesis and use in mutant production.

6.4 FUNDAMENTAL ASPECTS OF MORPHOGENESIS AND ANDROGENESIS

6.4.1- Morphogenesis

Plant morphogenesis is a biological mechanism in which plant assumes its specific forms in relation to time (pre to post) during development under the expression of both external and internal signalling. In other word this process in under the controls of the spatial patterns of

cells during embryo development to cause a tissue or organ to establish its shape. Morphogenesis command tissue organisation, which affects a lot of an organisms anatomy, physiology, and behaviour. This process involves in integrating growth and differentiation through cell division and specialisation as a consequence of spatial and temporal hormonal control mediated by the regulation and expression of multiple gene systems, the relating action of meristem and their derivatives, and environmental variations. Plant hormones and mineral nutrient requirements for morphogenetic responses were considered to be the only important aspect of the morphogenesis. But later it was realized that not only hormonal and nutritional interaction, but physiological interaction at cellular levels are also essential to understand morphogenesis.

Plants pass through three phases of development (morphogenic competence, development determination, and morphological differentiation) at both at the cellular level and in tissues. The morphogenic competence is defined as the cell's ability to recognize a specific signal that leads to a particular development under control conditions. Further, competent cells are decided by induction forcing them to reroute their development as per the received signals, which results, some of the cells differentiate, resulting in a new tissue organisation. Extrinsic variables, whether biotic or abiotic, regulate the process of morphogenesis in addition to a number of cell intrinsic factors. These factors will function by either influencing cell activity to direct a specific development in a defined way, or by converting cells to restore their totipotent traits and then again using their totipotenty nature to remodelling if required. Apart from the plant growth regulator, the morphogenic process includes cell competence, polarity, habituation, and gene control performance. So, Morphogenesis can be induced by the combination of chemical as well as mechanical factors.

6.4.1.2- Cell Competence

The morphogenesis process, such as the growth of new cell structures, is closely linked to the cell's ability to respond to extrinsic and intrinsic signals, which begins with the rupturing of cell determination and the first cell divisions that give rise to meristemoids. Meristems' ability to generate a new organism from an explant is determined by several steps, including competence acquisition, induction or morphogenic commitment to a certain route, differentiation, and eventually development. There is a direct link between a cell's ability to generate different cell types and its degree of dedifferentiation and morphogenic competence. So, the cells can be classified as multipotent, totipotent, or pluripotent depending on the degree of dedifferentiation.

6.4.1.3- Cellular determination

Cellular determination is the process by which a cell's development competence is limited to a specific route that is dependent prior to acquisition or a target cell's ability to respond toward the specific developmental signals, such as metabolic, molecular, or hormonal signalling and cellular positioning. The formation of cell polarity, asymmetric division, and cell placement in the plant body are all required for differentiation. In tissue culture, however, the extent of differentiation can be changed, causing the cells to become less differentiated. This happens when cells are released from the body's regulation and exposed

to a new situation in the growth media, causing them to dedifferentiate and display their genome in a different way, resulting in novel patterns of differentiation and the formation of new ordered structures. The age of the cell and its degree of differentiation, determination, and/or residual memory retention of the original somatic cell have a direct influence on the dedifferentiation and subsequent differentiation and development of new cellular lineages Cellular differentiation is defined by Moore (1979) as the change of genetically similar cells generated from a zygote or any other cell into biochemically, physiologically, and architecturally differentiated cells. Finally, cellular differentiation is a result of at least three factors: genetics, which is established at fertilisation, and incorporation of "stock" of potential, which may be expressed throughout progression; ontogeny, which began as a response to environmental stimuli but, once formed, tends to remain in a consistent condition; and attributes, which originated in ontogeny, initially as a response to environmental stimuli but, once established, tend to remain in a stable state.

6.4.1.4- Cellular habituation

Habituation is a persistent and hereditary decrease of growth factor needed by grown plant cells. According to Meins (1989), auxin and cytokinin cellular habituation is caused by transitory changes in cellular heredity known as epigenetic alterations. Epigenetic modifications, in comparison to mutations, are reversible and directed, that is, they occur in response to a specific inductor and suggest alterations in DNA that influence gene expression. The stage of growth of the cells has a substantial influence on the inclination to habituation and the competence for cytokinin habituation varies by tissue.

The main obstacle for commercial production may be cellular habituation associated with extended periods of *in vitro* subcultures, as a result of the progressive loss of plant vigour for example, tobacco callus habituated to cytokinin, where it has been shown that the higher the amount of these endogenous substances, the lesser the capacity to develop adventitious buds, implying an inversely proportional relationship for the both procedures. Because it endures when cells are cloned and is relatively durable, the habituated state entails a change in cellular heredity. When habituated cells may be forced to form full they may revert to the cytokinin-requiring condition, indicating expression of a pre-existing potentiality. These tissues are frequently identical to fully changed crown gall tissues and hereditary tumour tissues, and they are capable of manufacturing large amounts of the growth factor to which they have become accustomed. This disorder is thought to be caused by somatic mutations in the gene complex that controls hormone regulatory systems, as well as chromosomal anomalies.

6.4.1.5- Growth regulators in Morphogenesis

Plant growth regulators (endogenous or either applied or exogenous) have a direct influence in morphogenesis of plants, which is different for different species and in different ways. Auxin one of the dynamic plant hormone a variety of developmental processes and adjust the growth and morphology of plants according to external environmental conditions, few examples for auxins are apical dominance, the induction of vascular differentiation and the retardation of leaf abscission and fruit development are also controlled by the levels of auxin and many other process transportation of organic solute which indirectly contribute in

morphogenesis. Number of similar effects has been reported for cytokinins also. In conclusion, these growth regulators have their role in morphogenesis as they are involve in altering the gene expression.

Further, exposure the culture to various growth regulator or their combinations triggers a pre-programmed series of events within the cell, culminating in a heavily regulated sequence of cellular division that leads to the creation of root or shoot primordia. Partially structured meristematic aggregates can occur, which then get obstructed at a later stage of development and growth. To bypass this stumbling block, the meristemoids may need to be withdrawn from the original medium and transplanted to a new medium that contains either a different combination of growth regulators or none at all. Even if a growth substance's morphogenetic effects in cultured cells are well known, it is currently hard to provide adequate explanations for the mechanism of its action in formation.

Many, though not all, of the following features must be included in any understanding of the role of growth regulators in morphogenesis.

a. In most cases, growth regulators have a generic impact. Even distinct growth regulators from the same family can cause morphogenetic changes in the same tissue.
b. The amalgamation or more growth regulators at sufficient levels or in certain ratios causes some morphogenetic responses, whereas other doses of the same growth regulators have no impact or have a markedly distinct effect.
c. In most plant cell cultures, there is a lag period of several days to several weeks between the application of growth regulators and the morphogenetic action.
d. In some circumstances, sequential dosing with multiple growth factors is more successful than simultaneous treatment with the same growth regulators in inducing morphogenesis.
e. The response of cells from the same plant or cells growing *in vitro* to the same growth regulators varies significantly over time.

Growth regulators are thought to regulate morphogenesis through the following mechanisms:

a. Inside the cell, by using relative or absolute concentrators of two or more growth regulators.
b. Through the growth regulator's metabolism.
c. Through interaction with particular receptors at the membrane level.
d. Through indirect effects on nutrient uptake
e. But majorly at gene expression level.

6.4.1.6- Specific receptor interactions

The production of new shoot and root primordia from callus cells does not always necessitate the formation of many different kinds with radically different sets of proteins, nor does it necessitate a significant shift in cell division rate. The disorganised callus mass was separated from the root or shoot primodium by the orderly pattern of cell wall development. Membranes have been proposed as a primary target for growth regulator activity in a number of ways, the majority of which are dependent on the presence of certain receptors. Even

though the binding of numerous growth regulators to presumed proteinaceous receptors on a surface has been proven in several plant tissues, it is currently unknown how such associations induce the change in cell division pattern that eventually leads to the creation of an ordered meristem. The metabolic machinery of the cell must be ready to understand the signals resulting from the interaction of the two in order to activate these reactions. The growth regulator is usually thought to be a limiting factor when an exogenously provided growth regulator generates a morphogenetic response, however this isn't always the case. Although the mechanism governing the number and distribution of receptors on the membrane is unknown, a variety of unrelated factors such as minerals, temperature, osmotic stress, light, sugars, and genetic and intrinsic factors that affect specific growth and morphogenetic processes may all play a role. Treatment with growth regulators may potentially affect the number of receptor sites on the cell, similar to what is seen in animal cell systems.

Modifications of the affinity of the receptors for the growth regulator is another mechanism by which cell sensitivity can be modified in response to varied external variables, such as varying concentrations of the same growth regulator. It's possible that the receptor is a complex protein that can go through one or more structural changes based on the growth regulator's concentration in the environment. Moreover, the varying affinity of the receptor for these compounds could explain differences in sensitivity of cells to different growth regulators of the same class.

6.4.2 Androgenesis

Sporophyte is the resultant of fertilization of male and female gamete and contains a set of chromosomes from each parent with genomic constitution of 2n (Figure 6.2). Haploids plants are defined as sporophytes having only a single set of chromosomes. The ability of plant regeneration directly from anther or microspore culture under *in vitro* conditions is referred to as androgenesis which is of tremendous importance in plant breeding, plant physiology and embryology studies because either spontaneous doubling or an application of the chemical colchicines to double the chromosome number gives rise to homozygous plant in a single generation. Further, androgenesis is based on the notion of stopping pollen cells from developing into sexual cells and forcing them to develop directly into a mature plant via embryogenesis or developed into calli and then into a plant. Till date it’s reported in many families majorly dominating families Solanaceae and Poaceae. Besides, the androgenic methods, gynogenic method are also in practice to produce homozygous plant but gynogenic method gave result inferior than the androgenesis. Immature pollens are driven to pursue the sporophytic mode of development in Androgenesis by a variety of physical and chemical stimuli. The essential premise is to prevent pollen cells from developing into gametes, or sexual cells, and instead drive them to develop directly into plants. Extracted anthers and separated pollen can both be cultured to produce haploids.

6.4.2.1- Mechanism of Anther Culture-

It is the process of using anthers in the culture to produce haploid plantlets. The technique was discovered by Guha and Maheshwari in 1964. Anther culture success is determined by

the variety chosen, the plant's growing condition, and the quality of the donor material. Natural flowering settings are usually the ideal for donor plants to yield anthers that can be used in regeneration trials.

1. **Pre-treatment of anthers**- The technique of anther culture is rather simple, quick and efficient. The selected microspore requires specific pre-treatment conditions for anther callus induction. Young flower buds with immature anthers are surface sterilised and rinsed with sterile water to ensure that the microspores are confined within the anther sac at the right stage of pollen formation. Flamed forceps are used to extract the calyx from the flower bud. The stamens are removed and placed in a sterile petri dish after the corolla is sliced open. To determine the stage of pollen development, one of the anthers is crushed in acetocarmine. Each anther is gently detached from the filament and the intact undamaged anthers are injected horizontally on nutrient media if it is confirmed to be in the correct stage. When working with plants with small blooms, such as Brassica and Trifolium, a stereo microscope may be required to dissect the anthers.
2. **Media and Growth regulators**- The medium requirement may vary with the type of specie used. The most commonly used medium for anther culture is MS (Murashige and Skoog, 1962) medium. The constituents and basal media, as well as the combinations of growth regulators, all play a role in effective androgenesis. Agar is used to solidify anther culture media. In some species, agar may include chemicals that hinder the androgenic process. Some studies have proposed for the use of liquid medium as a strategy to circumvent possibly inhibiting components in gelly agents. The wall tissue of responding anthers turns dark over time, and within 3-8 weeks, they break open due to pressure applied by the expanding pollen callus or pollen plants. Individual plantlets or shoots coming from the callus are detached and put to a medium that will allow further development after they reach a height of about 3-5 cm. In the pots, the rooted plants are transferred to sterile soil mix. Most species required a comprehensive nutritional medium (mineral salt, vitamins, sucrose) as well as growth regulators during androgenesis. Auxin, on the other hand, is only required in low concentrations in the medium in most species. Auxin, which is required for pollen development and callus formation, is occasionally employed in conjunction with Cytokinin. Other ingredients such as glutamine, cesine, proline, biotin, ionositol, coconut water, silver nitrate, and polyvinylpyrrolidon can be added. Adding glutamine and glutathione to the culture medium improves the embryogenic process as well.
3. **Induction and stages of Pollen development:** After inoculation, haploid plants develop from anther culture either directly or indirectly (through callus phase).
   - **3.1 Direct Androgenesis**- It is also known as pollen derived androgenesis. In this case, pollen grains act as zygotes and go through numerous embryogenic stages that are analogous to embryogenesis.
   - **3.2 Indirect Androgenesis**- Instead of typical androgenesis, the pollen grain divides to form callus and then the callus further differentiate into shoots and roots

in appropriate medium. This type of androgenesis is reported in Rice, tomato, and wheat where callus tissue redifferentiated to generate haploid plantlets.

6.4.2.2- Mechanism of Microspore Culture:

During the microspore culture, anthers are gathered from sterilised flower buds in a tiny beaker with basal medium in a standard process for microspore culture (e.g 50 anthers of Nicotiana in 10 ml media, fig 6.3). The microspores are then pushed out of the anthers using a glass rod against the side of the beaker. Filter the suspension through a nylon sieve, with a pore diameter slightly larger than the diameter of the pollen and then removes residue from the suspension. It has been observed that smaller microspores do not regenerate, thus larger, good and viable microspores can be concentrated by filtering the microspore suspension through nylon sieves. This pollen solution is then agitated for 5 minutes at a low speed of 150× g. The fine debris-containing supernatant is removed, and the pollen pellet is resuspended in fresh medium and washed at least twice. After that, the microspores are mixed with a suitable culture medium at a density of 103-104 microspores/ml. Pipette the completed suspension into miniature petridishes. The liquid layer in the disc must be as thin to promote proper aeration. To avoid dehydration, each dish is then wrapped with parafilm and incubated. The responding microspores form embryos, or calli, which can then be transferred to suitable environments for further growth into plants.

Microspore culture have some advantage over the anther culture, because during anther culture this has been reported that some time if proper screening is not conducted or some other reason the callus from the other sections of the anthers get involved in the cultures then the resulting population of plants show various ploidy levels. This problem can be solved by cultivating isolated microspores, which has the following benefits:

1. The anther wall and other accompanying tissues have no uncontrollable consequences, and many parameters influencing androgenesis can be better regulated. However, where the anther wall has a stimulatory impact, this is a disadvantage.
2. Starting with a single cell, the sequence of androgenesis can be observed.
3. Because microspores can be equally exposed to chemicals or physical mutagens, they are appropriate for absorption, transformation, and mutagenic research.
4. Higher plant yields per anther could be achieved.

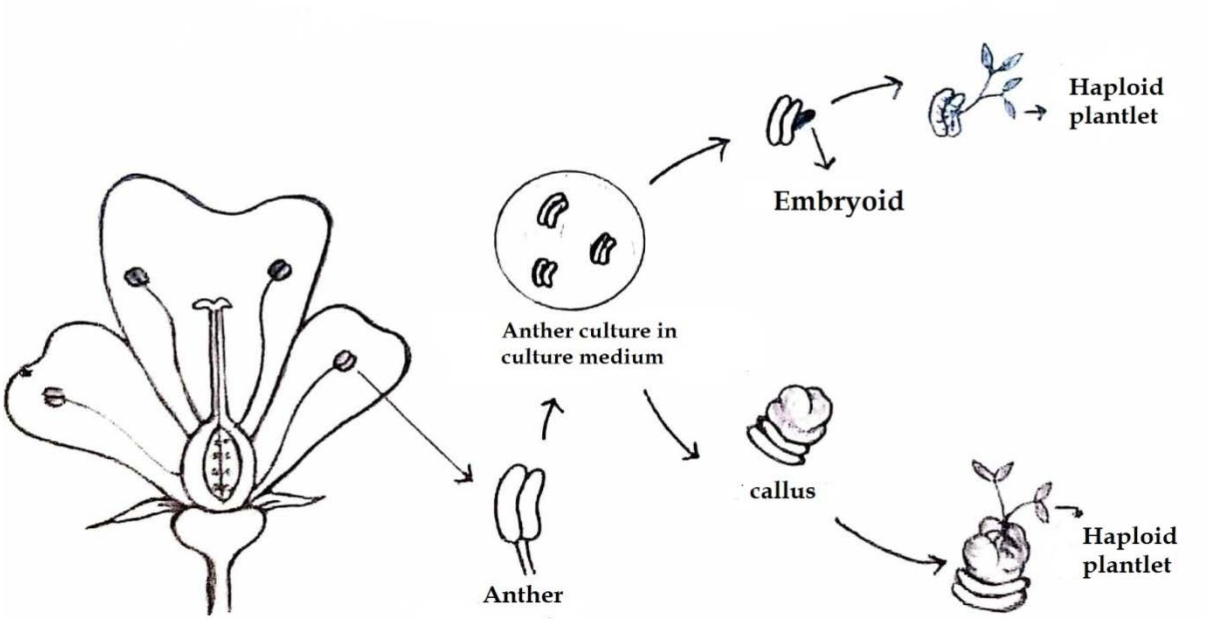

Figure 6.2: Androgenesis via anther culture

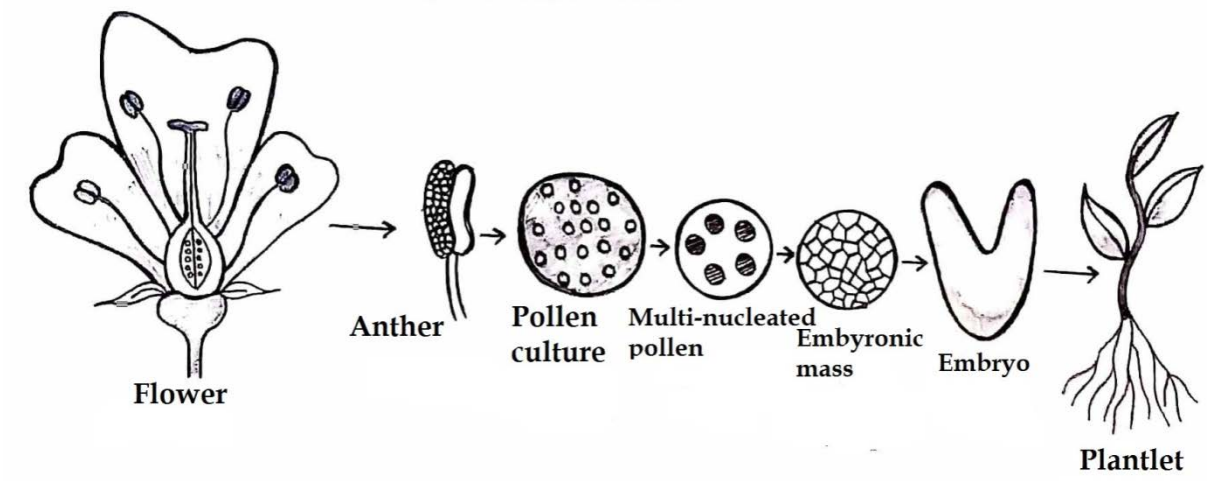

Figure 6.3: Plant regeneration via androgenesis through microspore culture

6.4.2.3- Factors affecting Androgenesis:

1. **Physiological Status of the donor plant**- The age and conditions in which the plant was grown have an impact on its physiology, and this feature of the donor plant has an impact on *in vitro* androgenesis. Flowers that bloom early, for example, have a stronger response to culture than flowers that bloom later. In anthers excised near the end of the season, sporophyte development has also been seen in a low frequency. In some plants (such as Brassica rapa), anthers removed at the old and sticky phases produce more embryos than anthers removed at the juvenile stage. A stress situation or treatment of plants with substances such as feminising agents and gametocidal compounds (which interfere with normal growth) in some other plants.The differences in anther responses from plants growing under various environmental circumstances could be attributed to differences in endogenous growth regulator levels. Light intensity, temperature,

photoperiod, nutrition, and carbon dioxide content are all important environmental elements.

2. **Stage of Pollen**-It has been determined that the stage of anther selection is the most crucial. Microspores ranging from tetrad to binucleate are sensitive in anthers. However, once starch deposition begins in the microspore, there is no sporophytic development and, as a result, no macroscopic structure construction.
3. **Anther walls**-The epidermis, endothecium, middle layers, and tapetum make up the anther wall of pollens. The efficiency of the anther wall in pollen cultures has been demonstrated in several investigations. Even when pollen from one cultivar of a plant is introduced to the anther cultures of another cultivar, it develops into an embryo. Other investigations have found that anther extracts in pollen cultures improve productivity and embryo development. The nursing nature of anther walls in pollen cultures has been proven to be an important element in androgenic response in these investigations.
4. **Genotype**-Plant genotype is one of the most important elements influencing proliferation and differentiation in microscopic cultures. Some plant species respond more positively to pollen-derived embryogenesis than others. Japanese rice, for example, has a higher androgenic reaction than indica rice. Some plant hybrids exhibit a wide range of responses. Some hybrids have a stronger androgenic response than others, whereas others are unresponsive. The interaction between the genotype of the plant and the environment in which this is raised could explain the heterogeneity of microscopic culture responses. Some plants' poor-responsive lines (such as *Solanum tuberosum*) may be intercrossed to develop lines with better androgenic responses. In tetraploid Melandrium, however, the presence of X-chromosomes enhances the androgenic response, whereas the presence of even one chromosome decreases it.
5. **Pre-treatment of Anthers**-The androgenic response is enhanced when anther/pollen culture is pre-treated.

The embryo yield is increased when *Nicotiana tabacum* anthers/pollen are cold treated (at 5°C for 72 hours) before being heated to 25 °C. White burley buds treated at 7-9 °C are more effective than those treated at 5 °C. After a high-temperature shock of 30-35°C for 1-4 days, some Brassica species and wheat genotypes show a higher androgenic response.Cold treatment followed by centrifugation improves androgenic anthers as well as the quick and synchronised development of embryos in some species.

Low-dose irradiation of anthers before culture has been reported to stimulate callusing and embryogenesis in several plants, such as *Datura* and *Nicotiana* species.

6. **Culture Media-**In tobacco, adding ethereal to the nutrient culture mix or putting excised anthers on agar-sucrose plates has been shown to increase the androgenic response. The inclusion of sucrose in various quantities has been shown to aid pollen callus formation. Potato and wheat, for example, require 6% sucrose, while Brassica plants require 12-13 percent. For optimal embryo growth, iron is essential in the culture media during the post-inductive stages of anthers culture. Low nitrogen levels in the media have been proven to enhance androgenesis in some studies.

7. **Light**-Light is not required for the growth of anther cultures. Incubation of cultures in the dark for the first 24 hours followed by diffuse lighting was found to induce embryogenesis in anther cultures.
8. **Culture Density**-Many studies have indicated that a minimum culture density of 3000 pollen/ml of growth media is needed for embryogenesis, but that a culture densities of 10000-40000 pollen/ml of culture medium produces the maximum embryo yield.

6.5 Techniques

6.5.1- General technique in in-vitro cultures.

Stage-0 (Pre-propagation stage)

The pre-propagation stage requires proper maintenance of the mother plants in the greenhouse under disease and insect free conditions with minimal dust. Clean enclosed areas, glasshouses, plastic tunnels and net covered tunnels, provide high quality explant source plants with minimal infection. Collection of explants for clonal propagation should be done after appropriate pre-treatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro* cultures. This improves the growth and multiplication rates of *in vitro* cultures. The control of contamination begins with the pre-treatment of the donor plants. The choice of explant depends on the methods.

Stage-I (Initiation of aseptic culture)

In this stage sterilization of explants and establishment of explants were done. The plant organ used to initiate a culture is called explant. For sterilization number of different methods and chemicals are available and they are used depending upon the need and utility. Further, the choice of explant depends on the methods to be followed.

1. For organogenesis it may be leaf, node, root, or any part.
2. For androgenesis anther or pollen is choice.
3. For somatic embryogenesis callus from leaves, petiole or any other tissue or some time it might be from single cell.

Stage-II (Multiplication of culture)

The success of any *in vitro* technique is largely dependent on the efficiency of this stage. In this stage multiplication of cultures take place and shoot germinates either from the callus or direct. But as in case of somatic embryo the embryo has fate for both root and shoot so this step is to be followed by acclimatization.

Stage-III (Rooting)

In-vitro grown shoots lack root system. For induction of roots they were transferred to rooting medium. For rooting half strength MS medium supplemented with auxin is used.

Stage-IV (Acclimatization)

The ultimate success of plant tissue culture depends on the ability to transfer the *in vitro* raised plantlet to field or soil conditions with their high survival rate which otherwise will

lead to high rate of mortality. Because under *in vitro* condition the high humidity, low light and poor gaseous exchange in the culture vessels and this allows the plant to grow well but the natural protective covering of cuticle is not fully developed, they have green leaves but their chloroplast and other cells are primarily immature in comparison to the field grown plants and same is true for root system. Thus the direct exposure, from *in vitro* condition to the outer atmosphere results in photoinhibition and chlorophyll photobleaching to these plants results in the high rate of mortality.

Thus the transplantation from completely controlled conditions to the outer environment should be gradual or step wise and this process of gradually preparing the plants to survive in the field conditions is called acclimatization. Gradual transfer must include, transferring them in the paper cups with soil but keeping these plant in controlled condition within the growth chambers for few days and then only the exposure to low humidity, high light for few hours to increase a full day before planting in the field.

6.5.2- Organogenesis

Organogenesis is the formation of organs in culture via direct or indirect methods and follows all above steps (Stage I to Stage IV). Organogenesis is majorly is under the control of growth regulators and choice of development of particular organs.

6.5.3- Technique in androgenesis

Androgenesis refers to the development of plants (sporophytes) from microspores or immature pollen (male gametophyte). The basic techniques are same as in case of general *in vitro* studies i.e., from stage 0 to stage IV, **but this involve the concept of stress in stage 0**. Two techniques are used to produce androgenic haploids, viz. anther culture and isolated pollen culture.

Anther culture:

Anther culture is one of the simple and efficient methods for haploid production, further this technique requires minimum facilities. Flower buds, with pollen grains at the most responsive stage (to be sure that the anthers are at the right stage for culture some of them are selected anthers are break on glass slide and stain with acetocarmine and examine the anther under microscope) are surface sterilized (sterilization is generally done with 70 % ethanol and some time with sodium hypochloride or any other chemicals). Than the anthers excised under aseptic conditions and culture on semi-solid or in liquid medium. In some cases, where the flower buds are small, whole buds or inflorescences enclosing the anthers at the appropriate stage of pollen development are cultured.

Generally it is thought to be put our plant under suitable stress treatment before, the explant to be collected for culture. Once the calli become visible in the cultures they are further placed for organogenic or embryogenic differentiation depending upon the objectives on different medium.

Microspore culture

Parallel to the anther culture, it is now possible to achieve androgenesis in the cultures of mechanically isolated pollens. But the special pretreatment is required in some cases further the plating density is a critical factor for the induction of androgenesis in pollen cultures. 1×10^4 pollen per ml of the medium is satisfactory.

Further, the application of a stress is necessary to induce microspore embryogenesis. Because the stress is required to switch the default developmental pathway of microspores toward embryogenesis and this vary with the species and even within a species. Cold treatment is preferred stress for further inductions while some time heat treatment or heat shock also used.

6.5.4- adventive embryogenesis

In vitro adventive embryogenesis also know as somatic embryo is one of the popular technique in plant tissue culture, in this studies the first two stages and fourth stage i.e., stage-0, stage-I and stage IV remain same as in case of general *in vitro* studies, but stage- II and stage III, get merged because in somatic embryo as somatic embryo have fate for both root and shoots and further they germinate as zygotic embryos to form plants with a primary root system, so rooting step in not required in this technique.

6.6 Utility

1. A clone is a group of individuals or cells derived from a single parent individual or cell through asexual reproduction. All the cells in callus or suspension culture are derived from a single explant by mitotic division. Therefore, all plantlets regenerated from a callus/suspension culture generally have the same genotype and constitute a clone. These plantlets are used for rapid Clonal propagation. Besides the calli, nodal explant is also use for the faster clonal propagation.
2. Genetic variation present among plant cells of a culture is called Somaclonal variation. Sometime these variations prove to be useful develop such verities which are resistant to pathogens or stress resistant. Further, some time new mutation may be isolated example jointless pedicel mutant in tomato.
3. Callus produced via tissue culture is use in secondary metabolite production.
4. The main advantage of haploids in plant breeding programmes is to achieve complete homozygosity in a single step by doubling the chromosome number.
5. The haploid produced via tissue culture may use for the identification of mutants, since even recessive mutant alleles if present will expressed in the next generation or in the regenerated plantlets.
6. Encapsulation of somatic embryo will produce artificial seeds, thus can be store and use for long time.
7. Production of virus free plants can be raised via plant tissue culture.
8. Seed dormancy, the term means the failure of seeds to germinate although environmental conditions including water, temperature, light and gases are favorable for germination or the incapacity of a viable seed to germinate under favorable conditions. So using this technique seed germination can be foster.
9. Plant tissue culture is used to regenerate plants produced by distant crosses, because otherwise these may fail due to several reasons.

10. This technique is useful in many early ripening varieties such as cherry, apricot, plum, produced seeds are not able to germinate and dies under the soil due to incomplete embryo development.
11. This technique is used in the production of transgenic plants; a gene of desirable character is inserted in the cell or transgenes and than the plantlets can be regenerated from these cells.

6.7 SUMMARY

In present unit, we discuss the different fundamental approaches of plant tissue culture. The science of plant tissue culture comes with the work of Haberlandt 1902. He himself was unsuccessful, but subsequent efforts by his successors were also dogged by failures, with the efforts of many workers and many trials present day this technique became a popular tool in the field of conservation, gene manipulation, mutant regeneration and many other. Development of organ from the callus is called as organogenesis. We have two approaches for the organogenesis in first we can raise our plantlet from explant (node, meristem, bulbs or embryo) without forming callus and in second we first form callus from any part of the plant and from the callus we raise plantlets. Besides, organogenesis we may study plant morphogenesis using this technique, plant morphogenesis corresponds to a biological process in which the plant assumes its specific form during their development in relation to its external form and to its internal organization, thus encompassing all levels from the cellular components until the complete plant is produced. Somatic embryogenesis occurs only under the controlled environment of plant tissue cultures. Since 1958, when it was reported for the first time in carrot today somatic embryogenesis is choice of technique for the large scale plant regeneration, somatic embryo produce plantlet as true as zygotic embryos. Somatic embryogenesis also serve in the synthesis of artificial seeds. Further, a fascinating outcome of tissue culture studies initiated at the University of Delhi has been the spectacular demonstration for the first time of the development of pollen embryoids and plantlets from anther culture of Datura by Guha and Maheshwari. Their work stumbled into the discovery which revolutionizes plant breeding programmes of the future world. Anther culture is a technique of culturing anther/pollen under aseptic *in vitro* condition to raise haploid plantlets. Plants breeders are interested in haploid plants because either spontaneous doubling or an application of the chemical colchicines to double the chromosome number gives rise to homozygous plant. The main factors that hinder the application of anther and pollen culture to cereals are low rates of androgenesis and the high frequency of albinism.

6.8 GLOSSARY

Anther culture: Production of haploid plant by culturing anther on suitable culture medium.
Artificial Seeds: A gel bed containing somatic embryo shoot bud, necessary nutrient and growth regulators etc, needed for the development of complete plantlet.
Auxins: A class of growth regulators, that are primarily associated with enhancing cell division, cell elongation and root initiations in cultured cells. IAA, IBA, NAA and 2,4-D are some commonly used auxins in Plant tissue cultures.
Caulogenesis: Shoot regeneration from the cultured cells or stem organogenesis.

Colchicine: An alkaloid from the bulb of *Colchicum autumnale* that interferes with the formation of spindle apparatus.
Contaminations: Presence of unwanted cells or micro-organism in an pure or aseptic culture.
Dedifferentiation: Conversion of differentiated plant cells or from specialized organ into meristematic ones.
Differentiations: Development of different organs from the meristematic cells.
Haploid plants: Plants having half or gametic chromosome number of the species.
Hybrid rescue: Culture of embryo to save hybrid that otherwise die due to degeneration of endosperm.
Meristem : A group of actively dividing cells from which permanent tissue systems such as root, shoot, leaf, flower etc are derived
Meristemoid : A group of meristematic cells with in a callus with a potential to form primordial Embryoid / Somatic embryos : Non zygotic embryo's formed in culture.
Organogenesis : Type of morphogenesis which results in the formation of organs and / or origin of shoots roots. The floral organs from tissue culture (or) suspension culture
Rhizogenesis: Root regeneration from the cultured cells in plant tissue culture.
Sub culture : Aseptic transfer of a part of a culture to a fresh medium.
Totipotency : The ability inherent property of a cell (or) tissue to give rise to whole plant irrespective of their ploidy level and the form of specialization

6.9 SELF ASSESSMENT QUESTIONS

6.9.1-Short notes

Q1. Define organogenesis.
Q2. What is morphogenesis?
Q3. Write a short note on adventive embryogenesis.
Q4. Differentiate between direct organogenesis and in-direct organogenesis.
Q5. Note on factor affecting androgenesis.
Q6. Define explant and its importance.

6.9.2- Multiple choice questions

Q1.The experimental plant piece subjected to tissue culture is referred to as

(a) *In vitro* culture
(b) Nurse tissue
(c) Explant
(d) Callus

Q2. Artificial seeds are

(a) seeds produced in laboratory condition
(b) seeds encapsulated in a a gel
(c) somatic embryos encapsulated in a gel
(d) zygotic embryos encapsulated in a gel

Q3. The pair of hormones required for a callus to differentiate

(a) Ethylene and Auxin
(b) Auxin and cytokinin
(c) Auxin and Abscisic acid
(d) Cytokinin and gibberellin

Q4. Totipotency refers to

(a) capacity to generate genetically identical plants
(b) capacity to generate a whole plant from any plant cell / explant.
(c) capacity to generate hybrid protoplasts.
(d) recovery of healthy plants from diseased plants.

Q5. Androgenic haploid were first produced by
(a) Guha and Maheshwari (b) Maheshwari and Johri
(c) Maheshwari and White (d) Rangaswamy

Q6. What is an explant?
(a) A part of plant grown under soil
(b) Any part of a plant taken out and grown in a test tube
(c) A specific part of a plant grown in a test tube
(d) Leaves grew under test tube

Q7. What is Callus?
(a) Tissues that grow to form an embryoid
(b) An unorganised actively dividing the mass of cells maintained in a culture
(c) An insoluble carbohydrate
(d) A tissue that grows from an embryo

Q8. Haploid plants are produced in large numbers by
(a) anther culture (b) Ovary culture
(c) both a and b (d) embryo culture

Q9. Cybrids are
(a) nuclear hybrids
(b) hybrid plants derived from cross pollination
(c) cytoplasmic hybrids
(d) cytological hybrids

Q10. In plant tissue culture, what is term ORGANOGENESIS mean ?
(a) formation of callus culture
(b) formation of root and shoot from callus culture
(c) genesis of plants
(d) none of the above

Answers: 1= c ; 2 = c; 3 = b; 4 =b; 5 =a; 6 = b ; 7 =b ; 8 = c; 9 = c ; 10 = b.

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6.12 TERMINAL QUESTIONS

Q1. Discuss the factors that may influence anther response and how can anther responses be increased during culture.

Q2. What function does ABA have in embryo development?

Q3. Explain somatic embryogenesis. Draw the stages of embryogenesis and note when the different stages were first visible.

Q4. Discuss the anther culture in detail.

Q5. Discuss the morphogenesis and factor affecting morphogenesis.

Q6. Define Haploids. Describe the haploid production via anther culture and their applications.

Q7. Discuss the utility of haploid culture in horticulture sector.

UNIT-7-GENETIC ENGINEERING

Contents:

7.1-OBJECTIVES

After going through this unit learners will be able to answer the following questions.

- What is recombinant DNA technology?
- What are the tools of genetic engineering?
- What is the role of enzymes in recombinant DNA technology?
- What is vector? How can we use the vector for cloning?
- Why different types of vectors required in cloning experiments?
- What do you mean by expression vector? Is it like cloning vector?
- What are different methods of transfer of genes?
- What are protoplasts?
- What is somatic hybridization? What are the advantages of somatic hybridization?
- What is transgenic? What is the need of transgenics? How transgenics produced?
- What is molecular marker? What is the difference between co-dominant and dominant marker?
- What is polymorphism?
- Why there is a need of molecular marker?

7.2-INTRODUCTION

The concept of the gene as a unit of hereditary information was introduced by the Austrian monk Gregor Mendel in an 1866 paper entitled 'Experiments in plant hybridization'. Although Mendel introduced the concept, the word gene was not used until 25 years after his death. It was coined by Wilhelm Johansen in 1909 to describe a heritable factor responsible for the transmission and expression of a given biological trait.

The first evidence as to the physical and functional nature of genes emerged in 1902. Chromosome theory of inheritance was put forward by William Sutton in 1902. Archibald Garrod showed that the metabolic disorder alkaptonurea resulted from the failure of a specific enzyme and could be transmitted in an autosomal recessive fashion in 1902. Hunt Morgan and colleagues performed the first genetic linkage experiments in 1911 in the fruit fly *Drosophila melanogaster*, and hence showed that genes were located on chromosomes and were physically linked together. In 1942, George Beadle and Edward Tatum found that X-ray induced mutations in fungi often caused specific biochemical defects, reflecting the absence or malfunction of a single enzyme. This led to the one gene one enzyme model of gene function. In 1944, Oswald Avery and colleagues showed that DNA was the genetic material. Thus evolved a simple picture of the gene– a length of DNA in a chromosome which encoded the information required to produce a single enzyme.

The definition of recombinant DNA is any artificially created DNA molecule which brings together DNA sequences that are not usually found together in nature. It is not surprising that the first cloning experiments were undertaken in *E. coli* and that this organism became the primary

cloning host because gene manipulation in this bacterium is technically easier than in any other organism. Until the mid-1980s, all cloning was cell-based (i.e. the DNA molecule of interest had to be introduced into *E. coli* or another host for amplification). In 1983, there was a further mini-revolution in molecular biology with the invention of the polymerase chain reaction (PCR). This technique allowed DNA sequences to be amplified *in vitro* using pure enzymes. The ability to insert new combinations of genetic material into microbes, animals, and plants offers novel ways to produce valuable small molecules and proteins; provides the means to produce plants and animals that are disease resistant, tolerant of harsh environments, and have higher yields of useful products; and provides new methods to treat and prevent human disease. The alien gene or foreign gene can be transferred into target host by means of the use of different methods like direct gene transfer methods and/or vector mediated gene transfer.

As we know totipotency is a characteristic feature of a plant cell and when protoplast is isolated by removing the cell wall of plasmolysed cell by using different methods, it also shows potential of regeneration of cell wall, growth and division (Vasil, 1976) under suitable conditions. So it too can be cultured and regenerated into a whole plant. The absence of cell wall facilitates the uptake of various organelles including DNA so could be useful in genetic transformation experiments. Protoplasts can also be useful in the production of hybrid plants (somatic hybrid) by the fusion of protoplasts which cannot be produced by conventional plant breeding due to incompatibility. The process of producing somatic hybrids is known as somatic hybridization. Somatic hybridization is very important for plant breeding as it overcoming common crossing barriers among plant species and in organelle genetics and breeding so can be used as a tool for crop improvement.

Different techniques like cloning and PCR amplification are extensively used for number of purposes in the field of biotechnology including the development and use of DNA based markers (molecular markers). The molecular markers are based on the polymorphism detected at the level of small DNA fragments. Till date a broad range of molecular markers are available which are being used in a variety of ways in plant breeding.

7.3 GENETIC ENGINEERING

Genetic engineering or recombinant DNA technology is a process in which the alteration of the genetic makeup of cells is done by deliberate and artificial means. Alteration of the genetic makeup of cells by deliberate and artificial means is possible which could be done by either adding or replacing or transferring gene(s) to create recombinant DNA in *in vitro* conditions.

Recombinant DNA can be defined as “DNA molecules constructed outside living cells (*in vitro*) by joining two or more natural or synthetic DNA segments of different sources (different species) to form new DNA molecules that can replicate in a living cell and which would never occur naturally.”

Basic steps required in genetic engineering or recombinant DNA technology

i) identification of DNA with desirable genes (identifying genes)

ii) isolation of identified DNA with desirable genes
iii) introduction of identified DNA with desirable genes into the host
iii) maintenance and re-expression of introduced DNA in the host and transfer of the DNA to its progeny

Uses of genetic engineering or recombinant DNA technology

It can be used to:

- Increase the yield and quality of existing products
- Improve the characteristics of existing products
- Produce existing products by new routes
- Develop novel products not previously found in nature

Tools of genetic engineering

The basis of recombinant DNA technology is the ability to manipulate DNA molecules in the test tube which, in turn, depends upon the availability of purified enzymes whose activities are known and can be controlled. So they can therefore be used to make specified changes to the DNA molecules that are being manipulated. Therefore to manipulate DNA in the test tube, 1) the availability of purified enzymes is must, 2) activities of purified enzymes should be known and 3) activities of purified enzymes can be controlled. So the purified enzymes can be used to make specified changes to the DNA molecules that are being manipulated.

Enzymes for DNA manipulation

DNA Polymerases

DNA polymerases are enzymes that synthesize new polynucleotides complementary to an existing DNA or RNA template. In other words an enzyme that synthesize DNA is known as DNA polymerase and when it copies an existing DNA or RNA molecules, is known as template-dependent DNA polymerase.

Mode of action of a template-dependent DNA polymerase

A template-dependent DNA polymerase forms a new polynucleotide whose sequence is dictated, via the base-pairing rule, by the sequence of nucleotide in the template (DNA or RNA molecule) that is being copied. The new polynucleotide is always synthesized in 5’ to 3’ direction. New nucleotide attached at 3’ end with the help of phosphodiester bond.

Important features of template-dependent DNA polymerase

In template-dependent DNA synthesis, DNA polymerase is unable to use an entirely single-stranded molecule as the template. Why it is so? Answer is because for initiation of DNA synthesis there must be a short, double- stranded region to provide a 3’ end onto which the enzyme will add new nucleotide. This short strand is called as **primer.** So we can say that a DNA polymerase requires a primer to initiate the synthesis of a new polynucleotide.

In the test tube, a DNA copying reaction is initiated by attaching to the template a short, synthetic oligonucleotide**,** usually about 20 nucleotides in length, which act as a primer for DNA synthesis. Annealing of the primer to the template depends on complementary base-pairing. The position within the template molecule at which DNA copying is initiated can be specified by synthesizing a primer with the appropriate nucleotide sequence. In other words, the primer determines which part of a DNA molecule is copied.

A second general feature of template-dependent DNA polymerases is that many of these enzymes are multifunctional, being able to degrade DNA molecules as well as synthesize them. In addition to 5'to 3' DNA synthesis capability, DNA polymerases can also have one or both of the following exonucleases activities.

a) 5'to 3' exonuclease activity

b) 3' to 5' exonuclease activity

a) A 5'to 3' exonuclease activity is less common, but it possessed by some DNA polymerases.

b) A 3' to 5' exonuclease activity enables the enzyme to remove nucleotides from the 3' end of the strand that it has just synthesized, if it is incorrect. This is called the **proofreading activity** because it allows the polymerase to correct errors by removing a nucleotide that has been inserted incorrectly.

Types of DNA polymerases

E.coli* DNA polymerase I:** It is also known as **Kornberg polymerase** after its discoverer Arthur Kornberg. This enzyme plays a main role in replication of *E.coli* genome and possess both 3' to 5' and 5' to 3' exonuclease activities. It is unmodified ***E.coli enzyme and DNA dependent DNA polymerase**.**

There is a modified version of Kornberg enzyme, the **Klenow polymerase.** It was initially prepared by cutting the natural *E.coli* DNA polymerase I enzyme into two segments using a protease. One of these segments retained the polymerase and 3' to 5'exonuclease activities but lacked the 5' to 3' exonuclease function of the untreated enzyme.

Dear students do you know that an optimum reaction temperature is required for proper functioning of *E.coli* DNA polymerase. *E.coli* DNA polymerase I enzyme has an optimum reaction temperature of 37°C, a usual temperature of the natural environment of the bacterium, inside the intestine of mammals such as humans. Therefore , for its proper functioning , the test tube reactions are incubated at 37 °C and terminated by raising the temperature to 75 °C or above, destroying its enzymatic activity.

T4 DNA polymerase: T4 is a bacteriophage of *E.coli*. It's activities are very much similar to Klenow fragment of DNA polymerase I. It functions as a 5' to 3' DNA polymerase and 3' to 5'exonuclease but does not have 5' to 3' exonuclease activity.

Reverse transcriptase: It is an additional type of DNA polymerase, which is an RNA-dependent DNA polymerase so makes DNA copies of RNA rather than DNA template. It is obtained from various retroviruses. This enzyme plays a main role in replication cycle of

retroviruses, including the human immunodeficiency viruses that causes acquired immunodeficiency syndrome (AIDS).
In the test tube, a reverse transcriptase (RNA-dependent DNA polymerase) can be used to make DNA copies of RNA molecules. These copies are called complementary -DNAs (cDNAs).

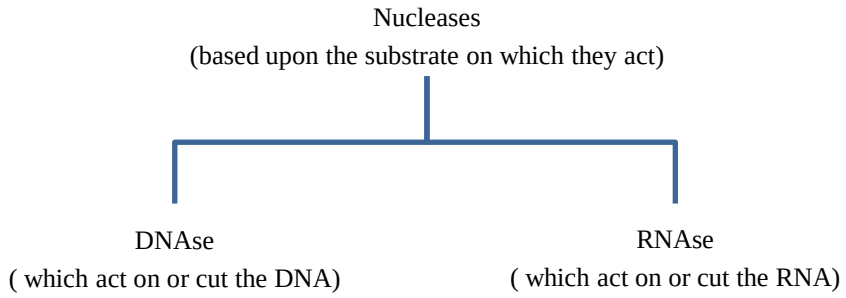
Taq Polymerase: Taq Polymerase is also known as *Thermus aquaticus* DNA polymerase I, obtained from *T. aquaticus. T. aquaticus* is a thermophilic bacterium which lives in hot springs at temperature up to 95 °C. Therefore, DNA polymerase I enzyme obtained from it has an optimum working temperature of 72 °C, hence known as thermostable DNA polymerase. Taq polymerase is used in PCR.

Nucleases

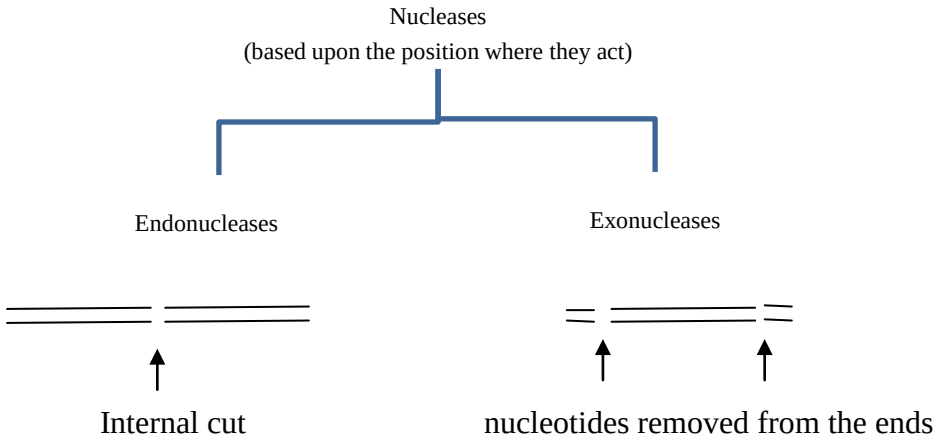
Nucleases are those enzymes which cleave or cut genetic material (DNA or RNA) by breaking the phosphodiester bonds that link one nucleotide to the next. Some nucleases are specific for DNA and some are specific for RNA. Some nucleases works only on double-stranded DNA and others only on single-stranded DNA. Some are not fussy what they work on.

Types of Nucleases

Nucleases based upon the substrate on which they act are of two types- DNAse or RNAse



On the basis of position where they act, nucleases are either **endonucleases**, making cuts at internal phosphodiester bonds or **exonucleases**, removing nucleotides from the ends of DNA/or RNA molecules.



Endonucleases	Exonucleases
DNAse which act at a non-specific region in the centre of DNA	DNAse which act at the ends or terminal regions of DNA
Do not require any free DNA ends (i.e. 5' and 3' ends)	Require a DNA strand with at least two 5' and 3' ends
Can act on circular DNA	Can not act on circular DNA
They release short segments of DNA	They release nucleotide

DNAse which act on specific position or sequences on the DNA are called as **restriction endonucleases** and the sequences which are recognized by the restriction endonucleases are known as restriction sequences or restriction sites or recognition sequences. These sequences are palindromic. Palindromic sequences are the nucleic acid sequence in a double strand wherein reading in a certain direction on one strand matches the sequence reading in the same direction on the complementary strand. Example- 5'-GAATTC-3'

3'-CTTAAG-5'

Thus restriction endonuclease binds to a DNA molecule at a specific sequence and makes a double stranded cut at or near that sequence. In this way the position of the cuts within a DNA molecule can be predicted (an important feature of restriction endonuclease beneficial for all those aspects of recombinant DNA technology in which DNA fragments of known sequence are required**).**

The 1978 Nobel Prize in Medicine was awarded to **Daniel Nathans, Werner Arber and Hamilton Smith** for the discovery of restriction endonucleases, leading to the development of recombinant DNA technology. The first practical use of their work was the manipulation of *E. coli* bacteria to produce human insulin for diabetics.

Types of restriction endonucleases:

Type I and Type III restriction endonucleases - Restriction sequences and position of cut may not be same, therefore less useful because the sequence of the resulting fragments are not precisely known.

Type II restriction endonucleases: Cut is always at the same place either within the recognition sequence or very close to it. For example- the type II enzyme, *Eco*RI (isolated from *E.coli*) cuts DNA only at the hexanucleotide 5'-GAATTC-3'.

So digestion of DNA with a type II enzyme gives a reproducible set of fragments whose sequences are predictable if the sequence of the target DNA molecule is known. Therefore they are very important because of their specificity. These enzymes require Mg^{++} as cofactor for cleavage activity and can generate 5'- PO_4 and 3'-OH.

The first Type II to be discovered and utilized was *Eco*RI, which is staggered and its recognition sequence is 5′-GAATTC-3′ as discussed above.

When restriction enzymes cut they produce either blunt ends or sticky ends (Figure 7.1). Based upon the mode of cutting, they are two types. Blunt end cutters and cohesive (sticky) end cutters.

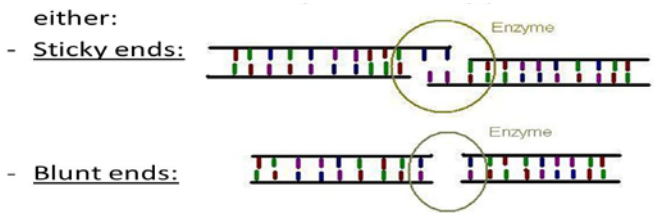


Figure 7.1 *–Sticky ends and blunt ends produced by the action of restriction enzyme*

1. Blunt end cutters: Blunt end cutters are those Type II restriction enzymes that recognize a particular recognition site and cut the DNA strand at the same point on both the strand of DNA within this recognition site. As a result of which the generated DNA strands are completely base paired. Such fragments are called as blunt ended or flush ended fragments and restriction enzyme is called as blunt end cutter (Figure7.2).

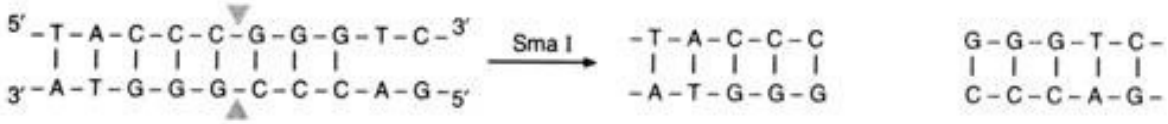
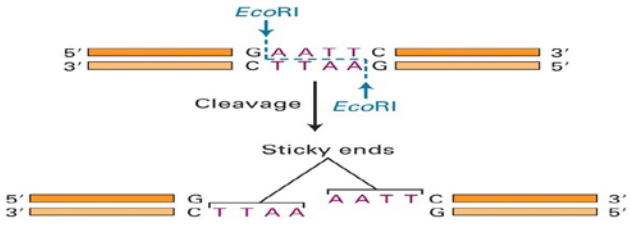


Figure 7.2*- Restriction endonuclease SmaI, a blunt end cutter produced blunt ended fragments*

2. Cohesive (sticky) end cutters: Type II restriction enzyme of this class cut the DNA strand at different points on both the strands of DNA within the recognition sequence. They generated a short single-stranded unpaired sequence at the end (Figure 7.3). The short single-stranded sequence is called as sticky or cohesive end because base pairing between the sticky ends stick the DNA molecule back together again. This cohesive end may contain 5'- PO_4 or 3'-OH, based upon the terminal molecule (also called as 5' overhangs or 3' overhangs). Therefore again classified as 5'end- cutters or 3'end- cutters as the case may be.



Or

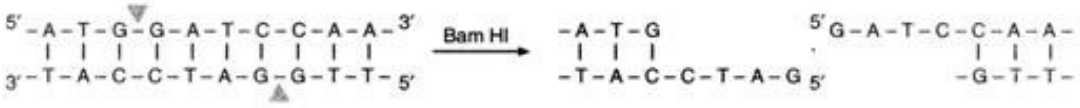


***Figure 7.3**: Action of sticky end cutters (EcoRI and BamHI) produces sticky ends*

Table1: Characteristics of some restriction endonucleases

Restriction enzyme	Source	Recognition sequence and cleavage site	Nature of cut ends
Eco RI	*Escherichia coli* RY13	5′-G/AATTC-3′ 3′-CTTAA/G-5′	Sticky
*Hin*dIII	*Haemophilus influenza* Rd	5′-A/AGCTT-3′ 3′-TTCGA/A-5′	Sticky
*Bam*HI	*Bacillus amyloliquefaciens* H	5′-G/GATCC-3′ 3′-CCTAGG/G-5′	Sticky
*Hae*III	*Haemophilus aegiptius*	5′-GG/CC-3′ 3′-CC/GG-5′	Blunt
Alu I	*Arthrobacter luteus*	5’-AG/CT-3’ 3’-TC/GA-5’	Blunt

The naming of restriction endonucleases: Restriction endonucleases are named in such a manner that the name provides information about their sources. For this a system was given by Smith and Nathans (1973) and a simplified version of this is in use today. The key features of this system are:

- The first letter written in capital letter tells about the genus of the host organism and the next two letters written in small letters identified species of that particular genus. This abbreviation is always written in italics.
- Where a particular strain has been the source then this is identified.
- When a particular host strain has several different R-M systems, these are identified by roman numerals.

Example: *Eco*RI, In this restriction enzyme *E*= Genus, *co*= species, R= Strain, and I= Order of identification in a bacterium. Table1is showing few characteristics of some restriction enzymes.

Ligases

Ligases are those enzymes which join DNA molecules together by synthesizing phosphodiester bonds between nucleotides either at the ends of a single molecule or at the ends of two different molecules as shown below-

DNA molecule ligated to itself two DNA molecules ligated

DNA fragments that have been generated by treatment with restriction endonuclease can be joined back together again, or attached to a new partner, by a DNA ligase. Energy is required for this reation which is provided by adding either adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD) to the reaction mixture, depending upon the type of ligase that is being used.

The most widely used DNA ligase is obtained from *E.coli* cells infected with T4 bacteriophage. This enzyme is involved in the replication of Phage DNA and is encoded by the T4 genome. Its **natural role** is to synthesize a missing phosphodiester bond between unlinked nucleotides present in one polynucleotide (one strand) of a double stranded molecule. In order to join together two restriction fragments ***in vitro***, the ligase has to synthesize two phosphodiester bonds, one in each strand. So we can say that ligases are the enzymes which seal the nick by synthesize a phosphodiester bond at this nick

End modification enzymes: End modification enzymes are those enzymes which make changes to the ends of DNA molecules.

1. Terminal deoxynucleotidyl transferase is one example of end modification enzymes. It is obtained from calf thymus tissue. It helps in addition of nucleotides one after the other to the 3' terminus at a blunt end strand, thus modifying a blunt end into sticky end (**Homopolymer tailing**). So it is, in fact, known as template independent DNA polymerase because it is able to synthesize a new DNA polynucleotide without base pairing of the incoming nucleotides to an existing strand of DNA or RNA.

> **Homopolymer tailing-** Homopolymer tailing is a method of adding similar nucleotides to the 3 prime end of the DNA strand (blunt end strand) with the help of **terminal deoxynucleotidyl transferase.**

2. Alkaline phosphatase is another example of end modification enzymes. It is obtained from various sources, including *E.coli* and calf intestine tissue. It helps in removing phosphate groups from the 5' ends of DNA molecules, thus prevents these molecules from being ligated to one another. Phosphatase which acts in basic buffers with pH 8 or 9 is called as alkaline phosphatase. Application of alkaline phosphatase treatment is to prevent recircularization of vector plasmid without insertion of foreign DNA. In this case circularization of the vector can occur only by insertion of non-phosphatase-treated foreign DNA which provides one 5'terminal phosphate at each join. One nick at each join remains unligated but after transformation of host bacteria, cellular repair mechanism reconstitute the intact duplex (Figure 7.4).

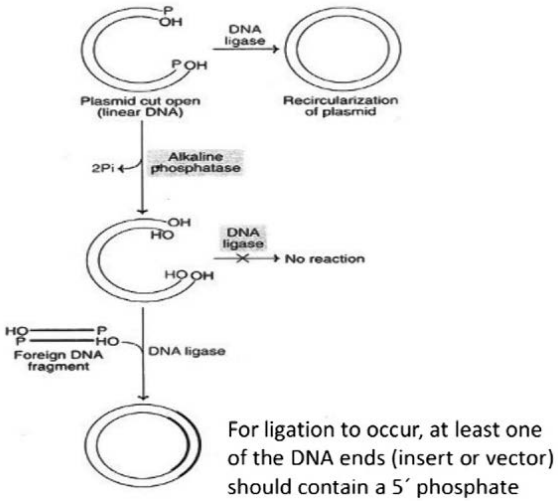


***Figure 7.4** –Action of Alkaline phosphatase*

3. T4 polynucleotide kinase is also an end modification enzyme. It is obtained from *E.coli* cells infected with T4bacteriophage. Function of T4 polynucleotide kinase is just opposite to that of alkaline phosphatase means it performs the reverse reaction to alkaline phosphatase. It helps in adding phosphate to 5' end by transferring the phosphate from ATP to the 5'end of DNA or RNA.

You can understand the role of different enzymes used in genetic engineering by going through Table 2.

Vectors

Vectors used in genetic engineering are plasmids, cosmids, lamda phage vectors, shuttle vectors, BACs and YACs etc.

"**Vector**" is an agent that can carry a DNA fragment into a host cell. If used for reproducing the DNA fragment, it is called a **cloning vector**. If used for expressing certain gene in the DNA fragment, it is called an **expression vector**.

For efficient cloning experiment, the same restriction enzyme must be used to cut both the vector and the DNA sample. Therefore, a vector usually contains a sequence (**polylinker**) which can recognize several restriction enzymes so that the vector can be used for cloning a variety of DNA samples.

Cloning Vectors

Cloning vectors are carrier vehicles used for gene transfer and reproducing the DNA fragment.

Features required to facilitate cloning into a vector are i) Origin of replication, ii) Selectable marker and iii) Cloning sites. Commonly used vectors for cloning include plasmid, Lambda phage, cosmid and yeast artificial chromosome (YAC).

Table 2- Enzymes used in genetic engineering

Enzymes	Functions
Alkaline phosphatase	Removes phosphate groups from 5' ends of DNA (prevents unwanted re-ligation of cut DNA)
DNA ligase	Joins compatible ends of DNA fragments (blunt/blunt or complementary cohesive ends). Uses ATP
DNA polymerase I	Synthesizes DNA complementary to a DNA template in the 5'-to-3'direction. Starts from an oligonucleotide primer with a 3' OH end
Exonuclease	Digests nucleotides progressiviely from a DNA strand in the 3' -to-5' direction
Polynucleotide kinase	Adds a phosphate group to the 5' end of double- or single-stranded DNA or RNA. Uses ATP
RNase A	Nuclease which digests RNA, not DNA
Taq DNA polymerase	Heat-stable DNA polymerase isolated from a thermostable microbe (*Thermus aquaticus*)

Vectors based on *E.coli* plasmids

Plasmids

Extrachromosomal element of DNA in bacteria is termed as plasmid. Term 'plasmid' was coined in 1952 by Lederberg for all extrachromosomal, covalently closed circular (CCC) DNA molecules. These are small double-stranded, CCC DNA molecules carrying genes for antibiotic resistance. They do not occur free in nature but are found in a bacterial cell. Plasmids are naturally occurring, self replicating (replicate independently of the host cell), present in a bacteria and in the nuclei of some eukaryotic cells. The size of plasmids ranges from a few kb to near 100 kb. Plasmids in bacteria exist in supercoiled form.

A plasmid can be considered as suitable vector if it posses the following features:

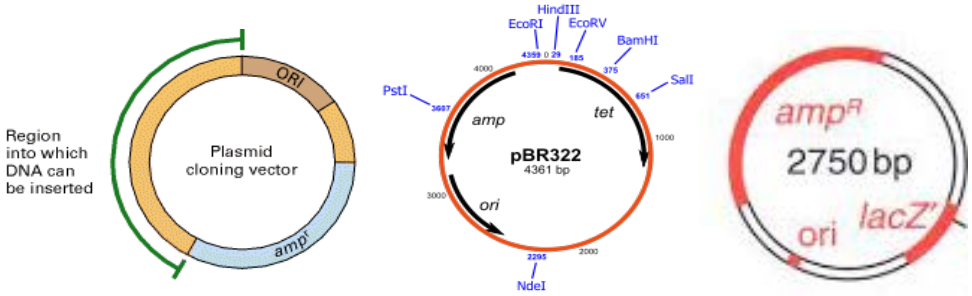
- It can really be isolated from the cell.
- It possesses a replication origin (ORI) sequence **(**Origin of replication**).**
- It possesses a single restriction site for one or more restriction enzymes, called cloning sites. Insertion of a linear molecule at one of these sites does not alter its replication properties.
- Plasmids contain genes encoding proteins that make the bacteria resistant to antibiotics such as tetracycline, ampicilline. These are known as selectable markers.
- It can be reintroduced into a bacterial cell.

- It must have self transfer capacity.
  Plasmid's small size (usually about 3kb) makes them easy to purify from bacterial cultures, allow the cloned DNA to be recovered easily. Plasmid vectors are ≈1.2–3kb in size.

Drawbacks:

- A small size and efficiency to transfer maximum of 15 kb of foreign DNA
- Single restriction endonuclease site
- One or more selectable genetic markers

Natural Plasmids: Natural plasmids are those plasmids which occur naturally in bacteria and are not constructed *in vitro* for the sole purpose of cloning. Examples- pSC, Col E1 and RSF 2124. In early cloning experiments, the cloning vectors used were natural plasmids such as Col E1, RSF2124 and pSC101. These plasmids are small and have single site for the common restriction endonucleases and they have limited genetic markers for selecting transformants. Figure 7.5A is showing plasmid as a cloning vector.



A B C

***Figure 7.5A**- Plasmid as cloning vector, **B**- pBR322, C-* pUC 8

For this region, *in vitro* superior cloning vectors were constructed. In early 1970s many natural plasmids were artificially modified and constructed as cloning vectors.

pBR322: Plasmid pBR322 was named after its developers Bolivar and Rodriguez in 1977. Here p stands for plasmid, B for Bolivar, R for Rodriguez and 322 is the number used by them to designate the plasmid (strain number). Plasmid pBR322 was constructed by ligating restriction fragments from three naturally occurring *E.*coli plasmids: RSF2124, pSC101 and Col E1-like plasmids (Figure 7.5B). It contains the Ap^R and Tc^R genes. It has an origin of replication (ori) that functions only in *E. coli.* It is maintained at a high copy number in *E.coli.* Plasmid pBR322 contains 4361 bp.

pBR325: It encodes additional chloramphenicol resistance in addition to Ap^R and Tc^R and has a unique *Eco*RI site in the Cm^R gene.

pBR327: It was derived from pBR322, by deletion of nucleotides between 1427 to 2516. These nucleotides were deleted to reduce the size of the vector and to eliminate sequences that were known to interfere with the expression of the cloned DNA in eukaryotic cells. pBR327 still contains genes for resistance against ampicillin (Ap^R) and tetracycline (Tc^R).

pUC: Another cloning vector is pUC where p means plasmid, U means University and C means California. pUC series was constructed by enhancing the valuable features of pBR322. It was named so because it was produced at the University of California. So we can say that pUC series is derived from cloning vector pBR322.

When the pUC plasmid has been used to transform the host cell *E. coli* the gene in it (lac Z) may be switched on by adding the inducer IPTG (isopropyl-β-D-thiogalactopyranoside). Its presence causes the enzyme β-galactosidase to be produced. The functional enzyme is able to hydrolyse a colourless substance called X-gal (5-bromo-4-chloro-3-indolylb-galactopyranoside) into a blue insoluble material (5,50-dibromo-4,40–dichloro indigo). However if the gene is disrupted by the insertion of a foreign fragment of DNA, a non-functional enzyme results which is unable to carry out hydrolysis of X-gal. This makes the recombinant pUC plasmid to be easily detected as it is white or colourless in the presence of X-gal (Figure 7.6). An intact non-recombinant pUC plasmid will be blue since its gene is fully functional and not disrupted. This elegant system, termed **blue/white selection**, allows the initial identification of recombinants to be undertaken very quickly and has been included in a number of subsequent vector systems.

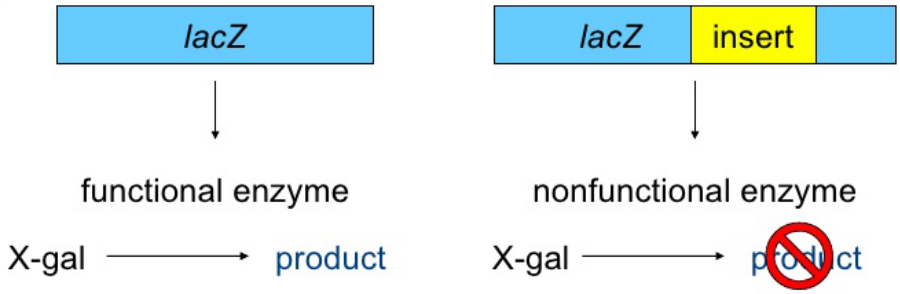


***Figure 7.6**– Lac selection*

So we can say that lac selection or Blue white screening is a method for selection of recombinants**.** New recombinant can be identifies by using insertional inactivation. Identifying recombinant is important because manipulation (process of formation of recombinants) results a variety of ligation products, including plasmids that have recircularized without insertion of new DNA.

pUC8: A most popular pUC plasmid is pUC8, a small plasmid having a size of just 2.7 kb. Along with its origin of replication, it carries two genes -Ap^R and lac Z' gene. lac Z' gene has a cluster of restriction sites (Figure 7.5C).

A gene for ampicillin resistance (*Ap*^{R})- Presence of this gene in pUC 8 means that a bacterium having a pUC 8 plasmid, is able to synthesize an enzyme, called β-lactamase. It enables the cell to withstand the growth inhibitory effect of the antibiotic. Normal *E.coli* cells (without pUC8 plasmid) are sensitive to ampicillin and can not grow when the antibiotic is present. Ampicillin resistance is therefore a selectable marker for pUC8.

The lac Z' gene- It codes for a part of the enzyme β-galactosidase (α-peptide portion of β-galactosidase). β-galactosidase is one of the enzymes involved in the breakdown of lactose to glucose and galactose. This lac Z' gene in pUC 8, contains a cluster of unique restriction sites. Ligation of new DNA into any one of these sites results in insertional inactivation of the gene and hence loss of β-galactosidase activity. This is the key to distinguish a recombinant plasmid (one that contain an inserted piece of DNA) from a non-recombinant plasmid that has no new DNA.

[Insertional inactivation- Inactivation of any gene by inserting new DNA somewhere in-between that gene.]

Screening for β-galactosidase presence or absence is quite easy. The presence of functional β-galactosidase molecules in the cells is checked by a histochemical test with a compound called X-gal (5-bromo-4-chloro-3-indolyl- β–D-galactopyranoside). β-galactosidase convert X-gal into a blue product.

After manipulation, bacteria having pUC8 plasmid allowed to grow in nutrient medium having agar, ampicillin and X-gal plus an inducer of the enzyme such as isopropyl-β-D-thiogalactopyranoside (IPTG). Non- recombinant colonies, the cells of which synthesize β-galactosidase appeared blue in colour. Recombinant colonies with the disrupted lac Z' gene, unable to make β-galactosidase appeared white (Figure 7.7).

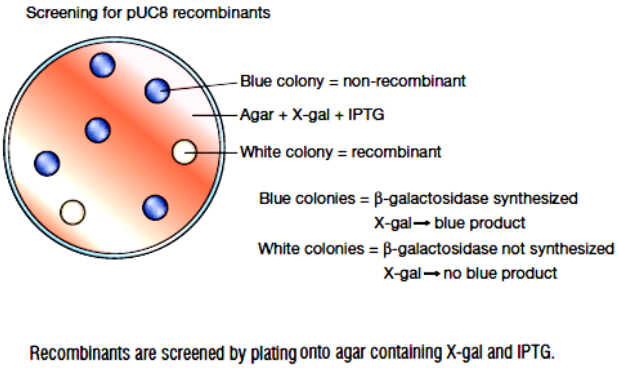


Figure 7.7*- Screening for pUC8 recombinants*

Image courtesy: Fig 5.10, Gene Cloning & DNA Analysis- An Introduction, T.A.Brown. fifth edition, blackwell publishing, 2010

This will be clearer by going through Figure 7.8. Plasmids can insert pieces upto 10kb.

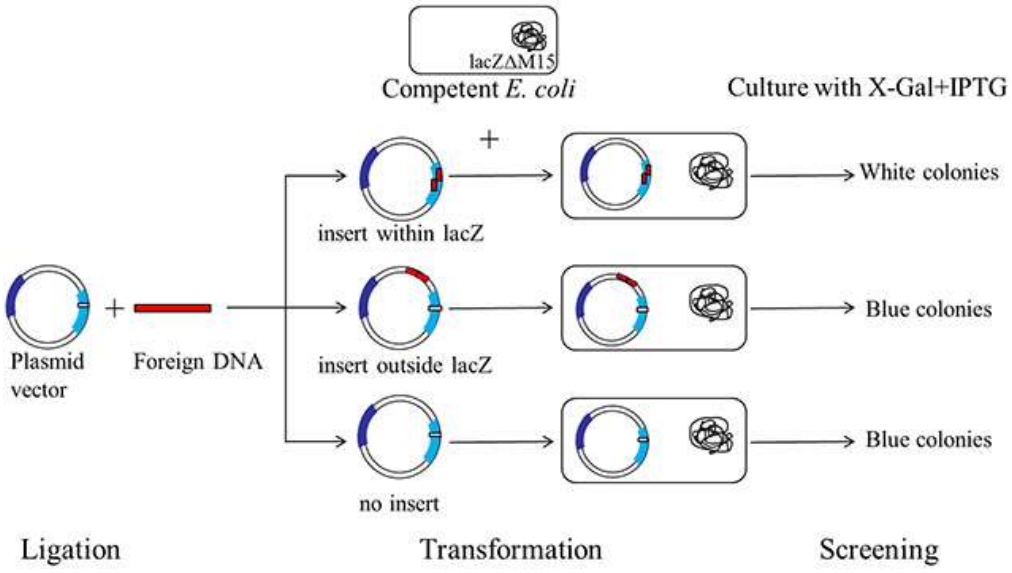


Figure 7.8*- Screening for pUC8 recombinants*

Cloning vectors based on *E.coli* bacteriophage genome (Virus based vectors)

Different types of cloning vectors other than plasmids are required. The reason for developing cloning vectors based on *E.coli* bacteriophage genome and others was the inability of plasmid vectors to handle DNA fragments **greater than about 10 kb in size**. When insert size was large, either it interfere with the plasmid replication system or rearranged itself in such a way that the recombinant DNA molecule become lost from the host cell. In addition to this, for preparing a genomic library for a eukaryote, the cloned fragment should be large enough to contain a whole gene

Lambda Phage

The first attempt to develop vectors able to handle DNA fragments greater than about 10 kb in size focused on bacteriophage λ. Lambda (λ) phages are viruses that can infect bacteria. The major advantage of the λ phage vector is its high transformation efficiency, about 1000 times more efficient than the plasmid vector. Phage λ genome is 48.5 kb linear double stranded DNA having 'sticky end' (cohesive ends) of about 12 bases at both ends which are complementary in sequences. These cohesive ends are called **'cos sites'**. Approximately 15 kb of λ genome is 'optional' (non-essential region) because it contains genes that are only needed for integration with the bacterial chromosome (Figure 7.9). So this segment can be deleted without affecting the ability of phage to infect bacteria for lytic life cycle. Due to the presence of complementary sequences in cos- sites DNA could adopts a circular structure when it is injected in the host cell and ligase may seal the gap of the COS sites.

Phage λ genome can be manipulated in the test tube like plasmid. Reintroduction of manipulated Phage λ genome into *E.coli* is known as **transfection**, the term used for uptake of naked phage DNA. The more efficient uptake system is called '***in vitro* packaging**'. DNA cloning using λ phages as vectors is given in Figure 7.10.

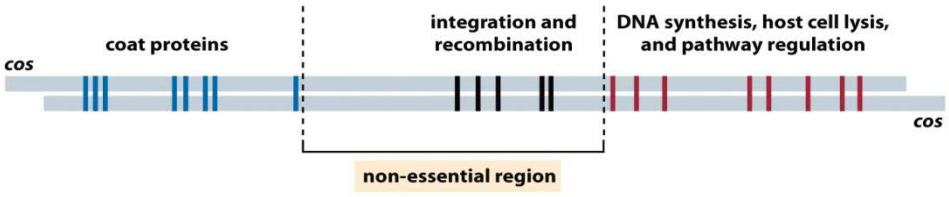


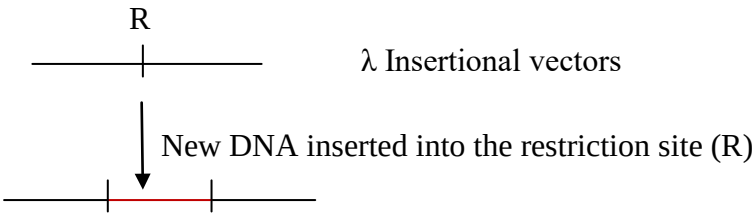
Figure 7.9*- Phage λ genome*
Source- human Molecular Genetics, 4ed.Garland Science 2004

The λ phage particle can accommodate upto 52 kb of DNA, so if the genome has 15 kb removed, then upto **18 kb of new DNA** can be cloned. This limit is higher than that for plasmid vector (10 kb).

Wild type λ DNA is not itself suitable as a vector because it's DNA contains several target sites for most of the restriction endonucleases, so not itself suitable as a vector. Therefore derivatives of wild type λ DNA have been developed that either have a single target site at which foreign DNA can be inserted (*insertional* vectors) or have a pair of sites defining a fragment that can be removed (*stuffer*) and replaced by foreign DNA (*replacement* vectors).So two basic types of phage λ vectors have been developed:

1.Insertional vectors
2.Replacement/ substitution vectors.

1. Insertional vectors: In which part or all of the optional DNA has been removed and a unique restriction site introduced at some position within the trimmed-down genome. They have a single target site at which foreign DNA can be inserted. Example- λgt 10, λgt 11 and λ ZAP vectors.



λgt 10 and λgt 11 are modified lambda phages designed to clone cDNA fragments.

λgt 10 vector: is a 43 kb double stranded DNA for cloning fragments that are only 7kb in length.

λgt 11 vector: is a 43.7 kb double stranded DNA for cloning fragments that are less than 6kb in length

λ ZAP vectors: λ ZAP is a commercially produced cloning vector that includes unique cloning sites clustered into a multiple cloning site (MCS).

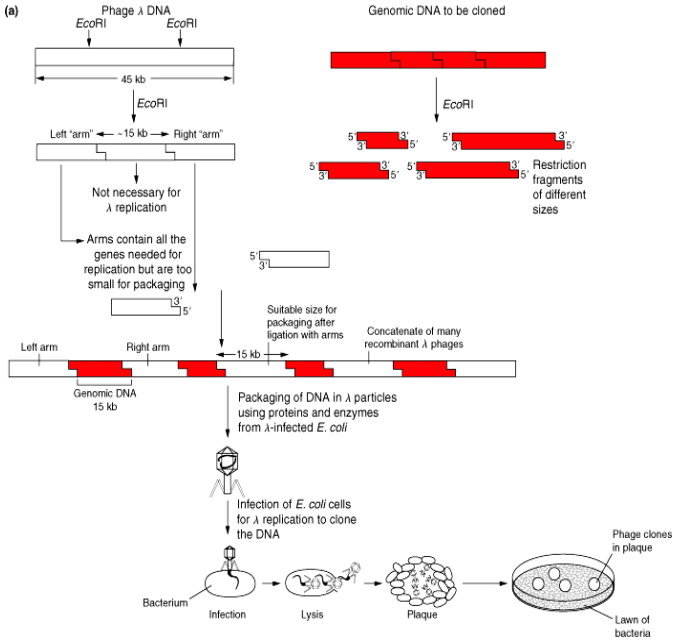
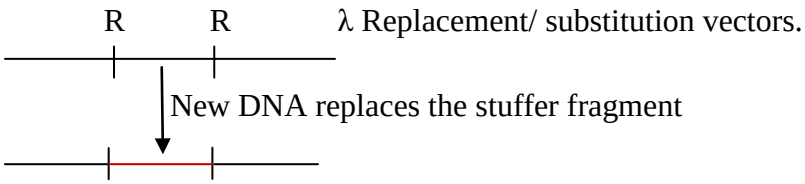


Figure 7.10*- DNA cloning using λ phages as vectors. The DNA to be cloned is first inserted into the λ DNA, replacing a nonessential region. Then, by an in vitro assembly system the λ virion carrying the recombinant DNA can be formed.*

2. Replacement/substitution vectors: In which the optional DNA is contained within a stuffer fragment, flanked by a pair of restriction sites that is replaced when the DNA to be cloned is ligated into the vector.

It means replacement vectors have a pair of sites defining a fragment that can be removed (a stuffer fragment) and replaced by foreign DNA. Example- λ EMBL3 and λ EMBL4, charon etc.



EMBL 3 and EMBL4 are used to clone a large fragment (9-25kb) of genomic DNA. These are used for the construction of genomic libraries. In these, a central non-essential part (about 14kb), called stuffer can be replaced by a foreign DNA. These two vectors have poly-linkers with reverse orders of restriction sites with respect to each other (Figure 7.11). They can be used for preparing genomic libraries in eukaryotes.

Phasmids:

Phasmids are those vectors which have combined properties of phage and plasmid. If phasmid has M13 ORI region, then it is known as **phagemid**. So we can say that a phagemid is an

engineered vector that contains plasmid and M13 components. Phage M13 is a filamentous phage of *E.coli* with a single stranded circular DNA genome. The genomes are enclosed in a protein coat forming a long filamentous form.

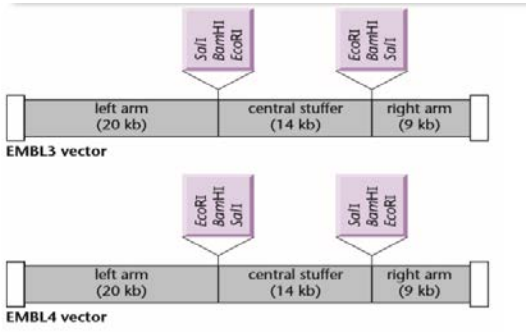


Figure 7.11*- EMBL3 and EMBL4*

Source-Principles of Gene Manipulation and Genomics, S.B.Primrose and R.M.Twyman, 2006

Advantages of Phagemids

Like plasmid vectors, they give high yield of double stranded foreign DNA

Cosmid-based vectors

A cosmid is a plasmid that contains phage sequences that allow the vector to be packaged and transmitted to bacteria like a phage vector. So cosmid is a plasmid having cos sites. Cosmids can efficiently clone large pieces of foreign DNA (32-47 kb) so they are attractive vectors for constructing libraries of eukaryotic genome fragments.

Advantages-It has the following advantages:

- High transformation efficiency, enough to produce a complete genomic library
- The cosmid vector of 5 kb, can carry up to 32-47 kb of foreign DNA whereas plasmid and λ phage vectors are limited to 10 kb and 25 kb.

Drawback:

Possibility of two or more genome fragments joining together in the ligation reaction, hence creating a clone containing fragments that were not initially adjacent in the genome. This would give an incorrect picture of their chromosomal organization.

This problem can be solved by dephosphorylating the foreign DNA fragment so that they cannot ligate together. This method is very sensitive to the exact ratio of target-to-vector DNAs because vector-to-vector ligation can occur. Recombinants with duplicated vectors are unstable and break down in the host.

Cosmids are plasmids that can be packaged into bacteriophage λ particles Cosmid=cos site+plasmid

Fosmids:-

Fosmid contain the F plasmid origin of replication and a ʎ cos site. They are similar to cosmids but have a lower copy number in *E. coli*, which means that they are less prone to instability problems.

Cloning Vectors used in eukaryotes

Plasmid vectors are for bacteria, but they are also available for eukaryotic cells (Yeast and Fungi). Yeast has a natural plasmid, called the 2µm circle.

Yeast artificial chromosome vectors (YACs)

Szastak and Blackbwn (1982) developed the first vector which could be maintained as a linear molecule, there by mimicking a chromosome known as YACs. It is a high-capacity cloning system permitting DNA molecules greater than 40-50 kb to be cloned and can amplify large DNA molecules in a simple genetic background. Artificial chromosomes can be used to clone large pieces of DNA in yeast

These vectors are propagated in *S. cerevisiae*, rather than *E. coli* and are based on chromosomes, rather than on plasmids or viruses.

The development of YACs was based on the logic that an eukaryotic linear chromosome (Figure 7.12) needs for its replication and stability, not only **replication origin**, but also the **centromere** and the **telomere** means each chromosome has these three important components.

- The centromere, play a key role during cell division because the centromere sequence helps in efficient segregation of the chromosomes into the daughter cells by attaching to the mitotic spindle during cell division
- The telomere, special sequences that mark the ends of chromosomal DNA molecules preserve the integrity of the ends of the linear chromosome.
- One or more origin of replication, which initiate synthesis of new DNA when the chromosome divides.

Ones these elements were provided, the vector could replicate stably like a chromosome and could accommodate chromosomes sized inserts.

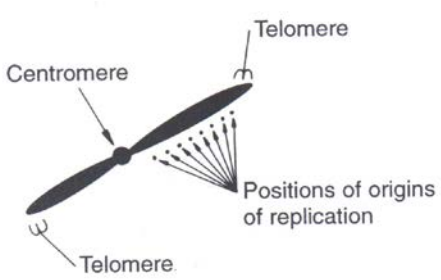


Figure 7.12*- Chromosome structure*

In YACs, the DNA sequences that underlies these chromosomal components are linked together with one or more selectable markers and at least one restriction site into which new DNA can be inserted.

So we can say that "YACs are artificially constructed linear molecules composed of a centromere, telomere and replication origin termed an ARS element (autonomous replicating sequence) which are required for replication and preservation of YACs in yeast cells." (Figure 7.13)

***Figure 7.13**- YAC vector* *pYAC3: a pBR322*

YACs are replicated in yeast cell, however the external cell wall of the yeast needs to be removed to leave a spheroplast.

Standard YACs can accommodate around 600 Kb DNA inserts; special types of YACs can accommodate upto 1400 Kb DNA inserts. Yeast artificial chromosomes can be used to clone very large fragments of DNA (50-1800Kb).

Key regions of pYAC vector are:-TEL= yeast telomeres, ARS1= autonomously replicating sequences, CEN4= centromere from chromosome 4 of yeast, URA3 & TRP 1= yeast maker genes, Ori= origin of replication of pBR322, YAC vector contain ori compitable in *E. coli* in addition to yeast replication origin or an yeast ARS element.

YAC are linear molecules composed of a centromere, telomere & a replication origin termed as ARS (Autonomous Replicating Sequence). They have restriction enzyme sites and genetic markers so that they can be traced& selected. 100-2000 Kb of DNA fragments can be inserted in YACs between centromere & telomere. They can be placed in *S. cerevisiae* & will be replicated along with the host chromosome.

Essential components of YAC vectors

- Centromers (CEN), telomeres (TEL) and autonomous replicating sequence (ARS) for proliferation in the host cell.
- *amp*^{r} for selective amplification and markers such as TRP1 and URA3 for identifying cells containing the YAC vector.
- Recognition sites of restriction enzymes (e.g., EcoRI and BamHI)

Large insert capacity vectors

BACs and PACs

Other new vector systems for cloning very large DNA inserts in *E.coli* are the bacterial artificial chromosomes (BACs) and P1- based artificial chromosomes (PACs). These BACs and PACs are based on replication control elements from factor F and bacteriophage P1, respectively (Shizuya et al. 1992; Ioannou et al. 1994).

Bacterial Artificial Chromosomes (BACs):

In order to overcome the difficulties associated with YAC vector, a bacterial cloning system based on *E.coli* F factor was designed which was capable of cloning fragments of upto 300-350kb. These are known as Bacterial Artificial Chromosomes (BACs). BACs are capable of maintaining human and plant genomic fragments of greater than 300Kb for over 100 generations with a high degree of stability and have been used to construct genome libraries with an average insert size of 125 Kb.

- BACs are constructed by using fertility or F factors (present on F plasmid) of *E.coli*.
- BAC vectors contain ori gene, rep E gene for maintenance of F factor, par A, B, C genes for plasmid copy number, an antibiotic resistance gene for selection of plasmid, MCS for insertion of foreign DNA.
- BACs are maintained as single copy plasmids in *E.coli*.
- Used in genome sequencing projects.
- BAC clones are stable for many generations.

PI derived artificial chromosomes or PACs:-

PACs have combine features of PI vectors and BACs. PACs enable genomic inserts upto 300 Kb in size to be stably maintained. PACs are vector that can carry much larger fragments of DNA than cosmids because they do not have packaging constraints.

An expression vector is a special cloning vector that can be used to clone the desired gene and which also contains the necessary regulatory sequences for obtaining high level of gene expression.

Table 7.3: Maximum DNA insert possible with different cloning vectors.

Vector	Host	Insert size
Plasmids	*E. coli*	~10kb
ʎ phage	*E. coli*	5–25 kb
ʎ cosmids	*E. coli*	35–45 kb
P1 phage	*E. coli*	70–100 kb
PACs	*E. coli*	100–300 kb

BACs	*E. coli*	≤300 kb
YACs	*S. cerevisiae*	200–2000 kb

Expression Vectors

In addition to incorporation of desired gene into host cell, the objective of recombinant DNA technology is to produce high amount of proteins encoded by the DNA insert. To achieve this goal, the introduced novel gene must express.

Hence, expression of cloned genes is carried by inserting a promoter sequence (signal for initiation of transcription) and a 'terminator sequence' (that provide signal for termination of transcription) into the vector. Near cloning site a translation initiation sequence (a ribosome binding site and short codon) is also incorporated into the vector.

The cloning vectors which contain these signals for protein synthesis are called expression vector.

In other terms those cloning vectors which in addition to act as vehicles for DNA insert, allow the DNA insert to be expressed efficiently, are called as expression vectors. Expression vectors are also known as expression constructs.

Main steps of recombinant technology

- Isolation of the genetic material
- Cutting of DNA at specific location
- Amplification of gene of interest using PCR
- Insertion of recombinant DNA into the host cell/organism
- Obtaining the foreign gene product

Artificial Chromosomes (Bacterial or Yeast) have all the elements necessary to propagate as a chromosome that can be used to clone DNA fragments from 100 Kb to 2000 Kb in length.

Applications of recombinant DNA technology

Now recombinant DNA technology has been used in various fields of life and has many applications like in agriculture, in medicine, in medical diagnosis of diseases, in gene therapy, in microbial cloning, in cell cloning, in plant cloning as well as in animal cloning.

7.4 METHODS OF TRANSFER OF GENES

Introduction of recombinant DNA molecule carrying desired gene into a suitable host is called gene transfer. The gene to be transferred in the host is called trans-gene and the organisms that developed after a successful gene transfer are known as transgenics .There are various methods of gene transfer or DNA uptake which can be classified into two broad classes (Table 4). They are (a) Vectorless or direct gene transfer and (b) Vector- mediated gene transfer.

(a) Vectorless or Direct gene transfer or Direct Genetic Transformation

Direct gene transfer has proved to be a simple and effective technique for the introduction of foreign DNA into the plant genome which takes place without the help of any vector or carrier vehicle. It has been further sub divided into two subcategories-

1. Physical gene transfer method
2. Chemical gene transfer method

Physical gene transfer methods

Physical gene transfer includes the following methods for uptake of naked DNA

1. Electroporation
2. Micro injection
3. Biolistics/ particle bombardment/microprojectile
4. Silicon carbide whiskers

Table 7.4-Gene transfer (DNA delivery) methods in plants

Method	**Salient features**
Direct or vectorless DNA transfer	
A.Physical methods	
Electroporation	Mostly confined to protoplasts
Micro injection	Limited use since only one cell can be microinjected at a time. Technical person should be highly skilled
Biolistics/particle bombardment/ Microprojectile	Very successful method used for a wide range of plants.
Liposome-mediated transformation	Confined to protoplasts
Silicon carbide whiskers	Requires regenerable cell suspensions
B.Chemical methods	
Polyethylene glycol mediated method	Confined to protoplasts
Calcium phosphate co-precipitation method	For the transfection of plant and mostly animal cells
DEAE dextran procedure	Does not yield stable trans-formants.
Vector-mediated gene transfer	
Agrobacterium mediated gene transfer	Very efficient
Plant viral vectors	Ineffective hence not widely used

Electroporation

Electroporation is a physical transfection technique for introducing DNA/RNA into plant cells (protoplast). In this method a precisely controlled brief electrical pulse of high field strength is used to generate transient, nanometer- sized pores in the cell membrane. For this cells are exposed to these pulse and reversible/temporary pores are generated. DNA enters the cell through these pores, and is transported to the nucleus. Process of electroporation can be seen in figure 7.14. Electroporation has also been successfully applied to rice and sugarcane. Pollen grains may also be transformed using this process.

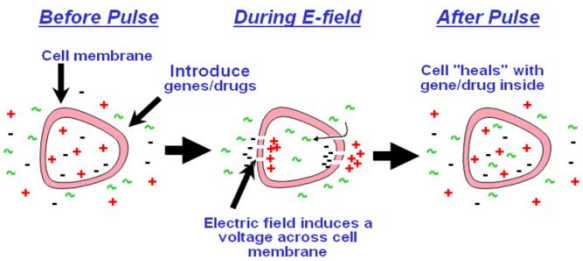


Figure 7.14- *Process of Electroporation*

Source: BTX, The electroporation experts. btxonline.com

Advantages

- It is a convenient, simple, fast and efficient method applicable to a wide range of plant protoplasts.
- No toxic side effects on the cells.

Disadvantage

- The most critical parameters are the intensity and duration of the electric pulse, and these must be determined empirically for different cell types. However, once optimal electroporation parameters have been established, the method is simple to carry out and highly reproducible.
- This process has high input cost.
- More number of cells may be required.
- The system requires protoplasts so regeneration protocol is prior requirement so that these protoplasts regenerate whole plants.

Principle- relatively weak nature of membrane.
A molecule is entered into the host cell through membrane.
Electric pulse is applied which temporarily disturb the phospholipid bilayer, and a compound enters 10,000 to 1,00,000 v/cm in millisecond or microsecond.

Microinjection

Microinjection is another method of gene transfer. The micro injection technique uses a fine capillary needle (0.5 to 5.0 micrometer) to deliver DNA precisely into cells in such a way that the cells are not killed but survive the treatment and can develop further into clones (Figure 7.15).

This procedure is performed under micromanipulator or phase contrast microscope. DNA transfer via microinjection is suitable in transgenesis, gene knockout study and genetic engineering.

Advantages

The main advantages of this technique, in addition to being host range independent, are:

(a) The delivery is precise, and can be even into the nuclei of target cells and the amount of DNA delivered per cells is not limited by the techniques and can be standardised, thus increasing the chance for integrative transformation.

(b) Small clumps of tissue containing a few cells such a proembryos with high regenerative potential can be injected.

(c) Desirable genes that are not accessible to *Agrobacterium* mediated transformation can be used by this method.

(d) Damage to the cell is less.

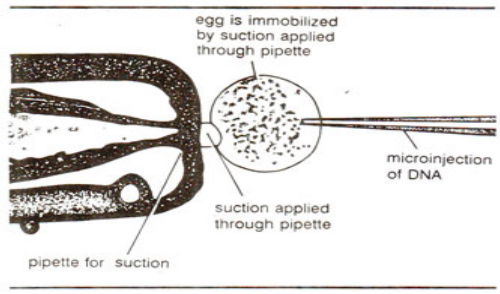


Figure 7.15*- Microinjection technique for gene transfer*
Source: Biocyclopedia

Disadvantage

Handling requires specialised skill and instrumentations.
Time consuming and laborious.

Particle bombardment/ Microprojectile(Biolistics) gun method

Particle bombardment or biolistics (short term for biological ballistics), is a commonly used method for genetic transformation of plants and other organisms. In this method particle bombardment device also known as the gene gun involves coating small metal particles (like gold, silver, tungsten) with biological molecules such as DNA/RNA and then accelerating them into target tissues using a powerful force, such as a blast of high-pressure gas, gun powder or an electric discharge through a water droplet. So millions of DNA-coated metal particles are shot at target cells or tissues. The DNA elutes off the particles that lodge inside the cells, and a portion may be stably incorporated in the host chromosomes.The target plant cells or tissues, which are regeneration competent and accessible to particle penetration, are used for bombardment (Figure 7.16). Microprojectile bombardment has been successfully employed for the transformation of many plants including a wide range of species such as onion, corn, rice, and wheat, soybean, cotton, maize, tobacco etc.

Advantages

Easy to use, rapid and versatile.
Small amounts of nucleic acids and few cells are required for efficient transformation
Many cells types can be transfected, including non-dividing cells and plants.

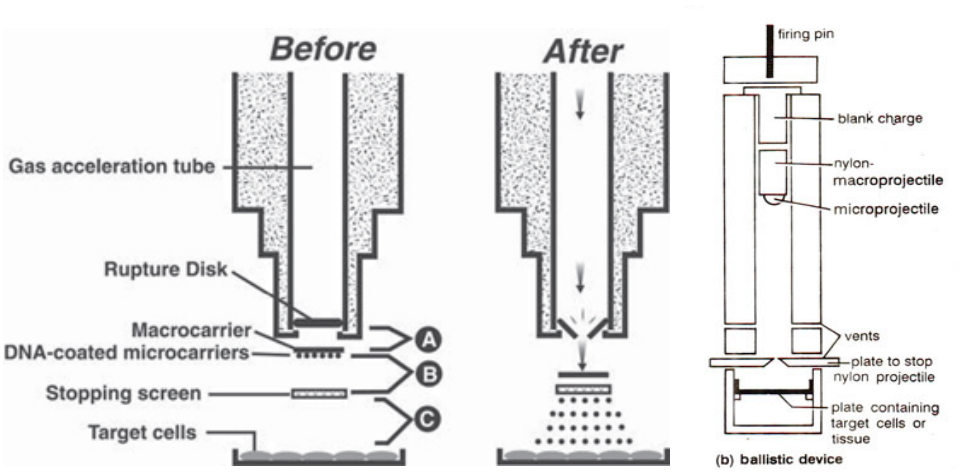


Figure 7.16*- Microprojectile gun method*
Source: https://link.springer.com/protocol/10.1385/1-59259-827-7:061

Disadvantages

May cause cell damage.
Requirement of equipment and skill personal is must.
Costly method.

Silicon Carbide

A new method of transforming plant cells is by the use of silicon carbide whiskers/fibres. It was used first in maize but is now being applied to other crops as well. Silicon carbide whiskers/fibres are very cheap and are capable of penetrating cells making pores which allow DNA uptake. The DNA coated silicon carbide fibres are vortexed with plant material (suspension culture, calluses). During the mixing, DNA adhering to the fibres enters the cells and gets stably integrated with the host genome. The silicon carbide fibres with the trade name Whiskers are available in the market.

Advantages

The process is simple and reproducible.
Direct delivery of DNA into intact walled cells. This avoids the protoplast isolation.
Procedure does not involve costly equipment.

Disadvantages

Silicon carbide fibres are carcinogenic and therefore have to be carefully handled.

Chemical gene transfer method

PEG (Polyethylene glycol) mediated gene transfer/ DNA uptake

It involves the use of 15-25% PEG, which stimulates uptake of DNA by endocytosis without any gross damage to protoplasts. The transformed protoplasts can be selected on a selection medium.

PEG, in the presence of divalent cations (using Ca^{2+}), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. In this way, the DNA enters nucleus of the protoplasts and gets integrated with the genome. The procedure involves the isolation of protoplasts and their suspension, addition of plasmid DNA, followed by a slow addition of 40% PEG-4000 (w/v) dissolved in mannitol and calcium nitrate solution. As this mixture is incubated, protoplasts get transformed.

Advantages:

A large number of protoplasts can be simultaneously transformed.
This technique can be successfully used for a wide range of plant species.

Disadvantages:

The DNA is susceptible for degradation and rearrangement.
Random integration of foreign DNA into genome may result in undesirable traits.
Regeneration of plants from transformed protoplasts is a difficult task.

Calcium phosphate co-precipitation method

The DNA is allowed to mix with calcium chloride solution and isotonic phosphate buffer to form DNA-calcium phosphate precipitate. When the actively dividing cells in culture are exposed to this precipitate for several hours, the precipitate is taken up by the cell by the process of phagocytosis. The recombinant DNA enters the nucleus and integrates into the host's genome. the cells get transformed. The success of this method is dependent on the high concentration of DNA and the protection of the complex precipitate. The transfection efficiency can be increased by exposing the host cell to 10-20% glycerol or Dimethyl sulfoxide (DMSO). This technique is used for the transfection of plants and mostly animal cells.

DEAE dextran procedure

The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The major limitation of this approach is that it does not yield stable trans-formants.

Liposome mediated transformation or lipofection or liposome transfection

Liposomes interact with DNA spontaneously, fuse with tissue culture cells, and facilitate the delivery of functional DNA into the cell. This technique is found very successful in the transfection of plant protoplasts and animal host cells. It is an alternative chemical transfection procedure to package DNA inside a *fusogenic* phosopholipid vesicle, which interacts with the target cell membrane and facilitates DNA uptake (Figure 7.17). This is also known as lipid mediated transfer. DNA or RNA encapsulated in liposomes can be transferred into plant protoplast by either direct fusion with the naked plasma membrane or through endocytosis of the liposome. Although it is efficient in terms of DNA encapsulation and transfer, the method once

again requires protoplasts and its application is limited to species for which protocols are available for protoplast regeneration.

Cationic lipid-mediated delivery is a fast, simple, and reproducible means for easily introducing DNA, RNA, siRNA, or oligonucleotides into eukaryotic cells. It allows the highly efficient **transfection** of a broad range of cell types, including adherent, suspension, and insect cells, as well as primary cultures.

Advantages:

It facilitates transient and stable transformation
The efficiency is also much higher than that of other chemical transfection methods

Disadvantages:

Requires protoplasts.
The major problem with liposome-mediated transformation is the difficulty associated with the regeneration of plants from transformed protoplasts.

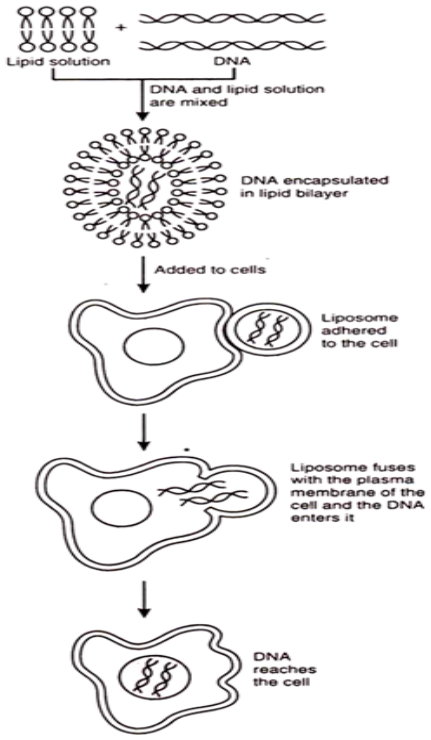
Vector mediated gene transfer

In this method of gene transfer vectors are used. The vector may be a circular double stranded DNA molecule or RNA molecule. Example- Plasmid of *E.coli.,* Ti plasmid of *Agrobactrium,* transposable elements and genome of viruses.

Vectors based on naturally occurring plasmids of *Agrobacterium*.

Agrobacterium tumefaciens: A plant pathogen, also known as nature's smallest genetic engineer. It is gram negative soil bacterium. Gene transfer from bacterium to plant occurs naturally and causes crown gall disease (the formation of tumors) in plants.

A. rhizogenes: Another species of *Agrobacterium* is *A. rhizogenes* which is responsible for hairy root disease in plants.



Liposomes can be unilamellar (20-25 nm), multilamellar (0.1 - 10 µM) vesicles prepared by sonication of phospholipids and buffer in organic solvent. Cationic/neutral lipid mixtures can spontaneously form stable complexes with DNA (*lipoplexes*) that interact productively with the cell membrane, resulting in DNA uptake by endocytosis (Felgner *et al.* 1987, 1994).

Figure 7.17*- Lipofection*

Both species of *Agrobacterium* are capable to transfer a particular DNA segment (T-DNA) of the tumour-inducing (Ti) plasmid/ hairy root inducing (Ri) plasmid into the nucleus of infected cells where it is stably integrated and transcribed, causing the crown gall disease/hairy root disease, respectively (Nester et al., 1984; Binns and Thomashaw, 1988).

Ti plasmid

As shown in figure 7.18, Ti plasmid consists of following segments-

T-DNA: It carries two types of genes (1) the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor/hairy root formation; (2) the genes encoding for the synthesis of opines which are produced and excreted by the crown gall/ hairy root cells and act as carbon and nitrogen sources for *Agrobacterium*.

Left border and right border: The T-DNA fragment is flanked by 25-bp direct repeats known as border sequences, which act as a *cis* element signal for the transfer apparatus.

Virulence region: The 30 kb virulence (*vir*) region is a regulon organised in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*)

The induction of vir gene expression results in the synthesis of proteins that form a conjugative pilus through which the T-DNA is transferred to the plant cell. Along with these, Ti-plasmid also contains Opine catabolism and origin of replication regions.

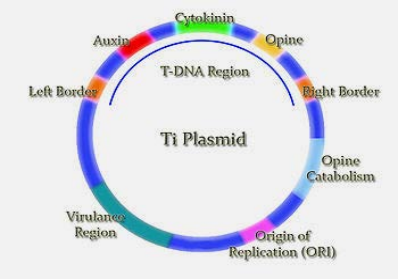


Figure 7.18*- Ti plasmid*

So we can say that the Ti-plasmid/Ri plasmid is a natural vector for genetically engineering plant cells because it can transfer its T-DNA from the bacterium to the plant genome. But wild-type Ti-plasmids are not suitable as general gene vectors because the T-DNA contains oncogenes that cause disorganized growth of the recipient plant cells. To be able to regenerate plants efficiently we must use vectors in which the T-DNA has been disarmed by making it non-oncogenic.

Ti plasmid of *A. tumefaciens* (causing crown gall disease) and Ri plasmid of *A. rhizogenes* (causing hairy root disease) have been used as vectors for gene transfer to plant cells. Ti or Ri plasmid have two regions called vir region and a T-DNA region. Vir region having six operons. These are *vir A, B, C, D, E and G.* Vir region is responsible for virulence towards host. T-DNA region is transferred to the host, along with the gene intended to be transferred.

Important points regarding T-DNA

1. The tumour formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes.
2. The T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process.
3. The genes on T-DNA are non-essential for transfer and can be replaced with foreign DNA.
4. Any foreign DNA placed between the T-DNA borders can be transferred to plant cell, no matter where it comes from.

Ti or Ri plasmid based vectors have been designed with the following properties:

- They do not cause the disease, since they are disarmed by removing disease causing genes.
- They carry sites for insertion of foreign gene intended to be transferred
- They carry selectable markers (genes which help in selecting the transformed cells), a powerful promoter (for high level of expression of markers) and a polyadenylation signal (for adding poly A to mRNA).

A vector having above properties, after insertion of the foreign gene, is transferred to *A. tumefaciens* or *A. rhizogenes*, as the case may be, and then the bacterium is used for infecting the host cells to which the gene is to be transferred. So we can conclude that during transformation,

several components of the Ti plasmid enable effective transfer of the genes of interest into the plant cells. These include:

- T-DNA border sequences, which demarcate the DNA segment (T-DNA) to be transferred into the plant genome
- Vir genes (virulence genes), which are required for transferring the T-DNA region to the plant but are not themselves transferred, and
- Modified T-DNA region where the genes that cause crown gall formation are removed and replaced with the genes of interest.

The first plant transformed by *A. tumefaciens* was tobacco (Herrera-Estrella, 1983). Figure 7.19 illustrates *Agrobacterium*-mediated plant transformation and Figure 7.20 represent flow diagram of it.

The *Agrobacterium*-mediated transformation process involves a number of steps:

(a) isolation of the genes of interest (target gene) from the source organism;

(b) development of a functional transgenic construct including the gene of interest; promoters to drive expression; and marker genes to facilitate tracking of the introduced genes in the host plant;

(c) insertion of the transgene into the Ti-plasmid;

(d) introduction of the T-DNA-containing-plasmid into *Agrobacterium*;

(e) Host cells (explants) are co-cultivated with *Agrobacterium* carrying the vector with the desired foreign gene to allow transfer of T-DNA into plant chromosome.

[Host cell may be any explants like protoplasts, suspension cultured cells, callus cells, leaf discs microshoots, root segments etc.]

(f) regeneration of the transformed cells into genetically modified (GM) plants; and

(g) testing for transgene expression at lab, greenhouse and field level.

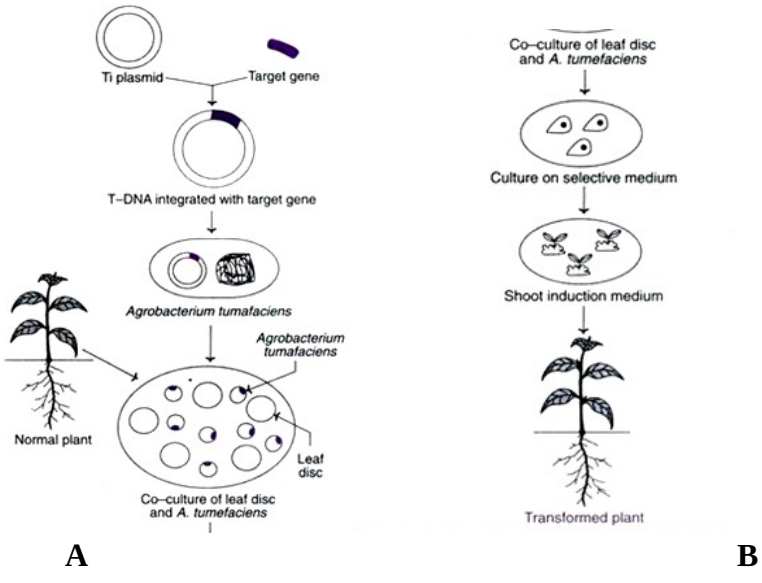


Figure 7.19*- A & B, Agrobacterium-mediated gene transfer (Plant Transformation Process)*

Advantages:

Stability of gene transfer is excellent.

Disadvantages:

Has the limitations of host range.

Virus mediated gene transfer (transformation)

Plant viruses can be employed as gene transfer and expression vectors. The vast majority of plant viruses have RNA genomes. However, the two groups of DNA viruses that are known to infect plants – the cauliflower mosaic virus (CaMV) and the geminiviruses – were the first to be developed as vectors because of the ease with which their small, DNA genomes could be manipulated in plasmid vectors.

Cauliflower Mosaic Virus (Caulimovirus):

The cauliflower mosaic virus (CaMV) is considered as most potential vector for plant transformation. Its genome consists of a small double -stranded circular DNA of 8 kb length. It has been used successfully as a vector to allow replication and expression of a foreign gene in dicotyledonous plants, especially the members of family Brassicaceae. The entire genome has been cloned in *E.coli* vector and cloned genome caused infection on turnips. Following infection, the virus spreads systematically and so does the foreign inserted gene, if any throughout the plant.

Disadvantage:

No heritable transformation via this vector as neither the gene gets integrated into plant genome nor the virus transmitted through seeds.

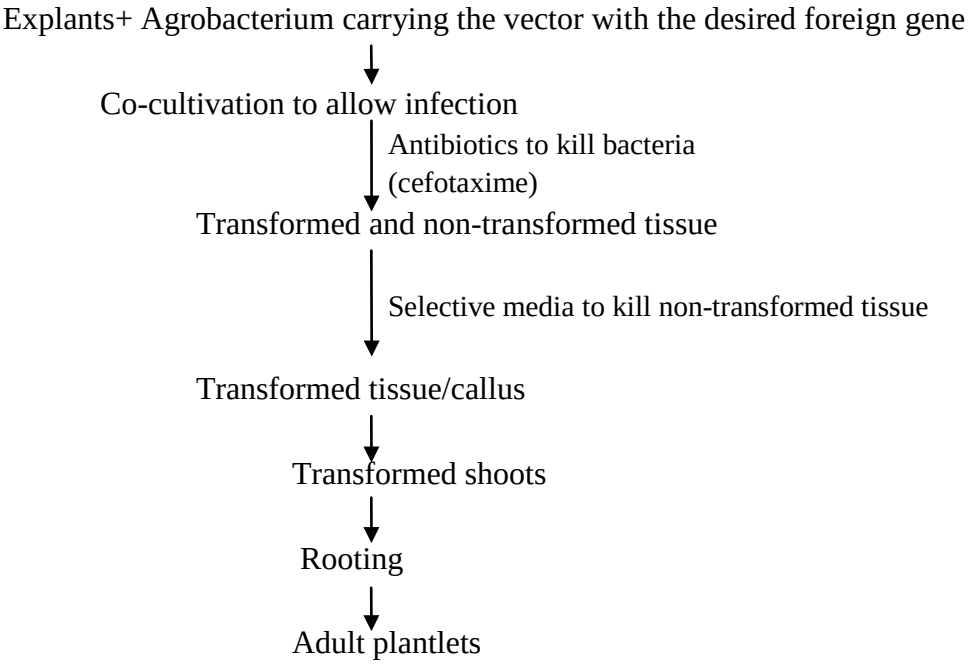


Figure 7.20*-Flow diagram of Agrobacterium mediated transformation of explants*

Gemini Virus:

A wheat dwarf virus, a member of gemini viruses, which infects several graminaceous members has also been cloned and reintroduced into wheat protoplasts.

Viral system usually does not result in stable transformation but they permit high level of transient expression of desired genes.

In plants the most popular method of transformation (gene transfer) is *Agrobacterium mediated* gene transfer, especially in dicotyledonous plants. This is followed by the physical method, especially particle bombardment in monocotyledonous plants. Chemical methods of gene transfer require protoplast.

7.5 PROTOPLAST AND SOMATIC HYBRIDIZATION

Protoplast

Hanstein (1880) introduced the term 'Protoplast' for living matter enclosed by plant cell membrane (or plasma membrane). Protoplast can be defined as a plant cell without cell wall. The term protoplast can also be defined as the spherical plasmolysed content of plant cell enclosed by plasma membrane.

As we know totipotency is a characteristic feature of a plant cell and when protoplast is isolated by removing the cell wall of plasmolysed cell by using different methods (will be discussed later in this unit), it also shows potential of regeneration of cell wall, growth and division (Vasil 1976) under suitable conditions. So it too can be cultured and regenerated into a whole plant. The absence of cell wall facilitates the uptake of various organelles including DNA. To exploit the above discussed properties of protoplast, there is a need of isolated protoplasts.

Isolation of Protoplast:

Isolation of protoplast means separation of protoplasts from plant cell/tissue. Plant protoplasts were first isolated enzymatically by Cocking in 1960 from tomato root cells. Isolation requires removal of plant cell wall. It can be done by means of two methods.

1. Mechanical method:

In this method cells are plasmolysed and protoplasts are released by cutting the tissue mechanically with the help of knife. This whole procedure is carried out under the microscope observation of cells. By this method most of the cells produced broken dead protoplasts. Number of uncut complete protoplasts is very less. Example- vacuolated cells of storage tissues like onion bulb scales, tissue of radish root and beet root.

For the first time in 1892, Klercker isolated protoplasts mechanically by micro-surgery of plasmolysed cells of *Stratiotes aloides*. Low yield and low viability of protoplast are the main problems of this method. Besides these it is also very laborious and tedious process.

2. Enzymatic method:

As the name indicates, this method involves the use of enzymes to dissolve the cell wall for releasing protoplasts. Cocking (1960) obtained protoplasts by degradation of cell wall using enzymes obtained from a fungus *Myrothecium verrucaria* (cells were tomato root tips and

enzyme was cellulase). Later this method become popular with suitable modifications. Enzymatic method is very easy process in comparison to mechanical method. High protoplast viability and high yield of protoplasts are the advantages of this method.

Best source for protoplast isolation-Mesophyll tissue from fully expanded leaves of young plants or new shoots. **Why it is so? Answer is-** A large number of relatively uniform cells can be isolated from mesophyll tissue and there is no need to kill the plants.

Enzymes required for digestion of cell wall

Enzymes required for digestion of cell wall should be very effective because we know that cell wall is made up of cellulose, hemicellulose and pectin. Cellulose, hemicellulose are the components of primary and secondary structure of cell wall while pectin is a component of middle lamella that joins the cells. Pectinase degrades the middle lamella while cellulase and hemicellulase are required to digest the cellulosic and hemicellulosic components of the cell wall.

Enzymatic method could be used as a one-step method or as a two-step method.

Two-step or sequential method

For isolation of protoplasts enzymes are used one by one. In the first step, the cells are isolated from callus or tissue by using macerozyme or pectinase that degrade middle lamella and then in the second step, cellulase is added to this cell suspension to digest the cell wall and release protoplasts.

One-step or simultaneous method

This method is known as one- step or direct or simultaneous method. The protoplasts are isolated directly from the tissue by using two enzymes cellulase and pectinase, simultaneously.

Importance of osmoticum

In addition to enzymes, osmoticum is also essential for isolation of protoplasts to prevent the plasma membrane from rupturing. During enzymatic isolation process an osmoticum is required which prevents the protoplasts from bursting as the mechanical barrier of cell wall for support is absent.

Osmoticum

Various osmoticums like ionic substances (potassium chloride or calcium chloride) and non-ionic substances (mannitol, sorbitol, glucose, fructose, galactose or sucrose) can be used during enzymatic isolation of protoplasts. For isolation of leaf mesophyll protoplast, mannitol is used as an effective osmoticum. In general, 0.3 to 0.7M sugar solution can generate suitable osmotic potential. Generally 50 mM $CaCl_2$ is added to increase the stability of released protoplasts (Rose 1980).

Purification of protoplast:

Pure population of intact and viable protoplasts is required for culture of protoplasts so purification of protoplasts is must. Purification is done by removing undigested material (debris), burst protoplasts and enzymes with the help of filtering, centrifugation and washing.

Protoplast viability

For further use of isolated protoplasts viability check is must which could be done by using various methods like fluorescein diacetate (FDA) staining method.

Applications:

- Reports about regeneration of complete plants from leaf protoplasts of tobacco by Takebe et al. (1971) increased the potential of prtoplast culture technique.
- Plant protoplasts can also take up foreign DNA, through their naked plasma membrane, under specific chemical and physical treatment, so could be useful in genetic transformation experiments.
- Protoplasts also provide an experimental system for a wide range of biochemical and molecular studies ranging from investigations into the growth properties of individual cells to membrane transport.
- Hybrid plants can be produced by the fusion of protoplasts which cannot be produced by conventional plant breeding due to incompatibility.
- Protoplast can be used to study wall synthesis and deposition.
- Protoplasts can also be studied as single cell systems

Somatic Hybridization

A hybrid produced by fusion of somatic cells of two varieties or species is called somatic hybrids and the process of producing somatic hybrids is known as somatic hybridization. Development of hybrid plants through the fusion of isolated somatic protoplasts of two different plant species/varieties under *in vitro* conditions is also known as somatic hybridization (Figure 7.21).

Somatic hybridization can also be defined as a technique which allows the manipulation of cellular genomes by protoplast fusion. Unlike sexual hybridization, the nucleus and cytoplasm of both parents are fused in the hybrid cells.

> When the nucleus of only one parent and cytoplasm of both the parents are fused in the hybrid cell, then instead of hybrid, it is known as **cybrid** and process is known as **cybridization.**

Plant cells have cell wall so it is very difficult to fuse them. Therefore for somatic hybridization process some conditions are required.

Requirements for Somatic Hybridization

Somatic cells (without cell wall) from two different parents along with fusogenic agent/ electrofusion method are the main requirements for successful somatic hybridization process. In addition to this a suitable regeneration system for that desired plant species is also an urgent requirement.

Basic steps required for somatic hybridization technique

The basic steps required for somatic hybridization technique are following:

1. Isolation of protoplast
2. Fusion of the protoplasts of desired species/varieties
3. Identification and selection of somatic hybrid cells
4. Culture of hybrid cells
5. Regeneration of hybrid plants

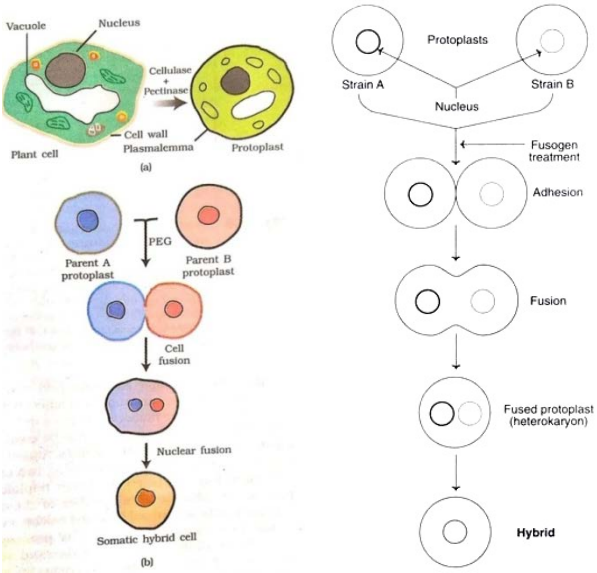


Figure 7.21-1*- Somatic hybridization. Protoplasts fusion induced by PEG ultimately yields somatic hybrid cells.****2****- A diagrammatic representation of protoplast fusion*

Fusion of the protoplasts of desired species/varieties

Freshly isolated/prepared protoplasts do not normally fuse together because they are negatively charged. Presence of negative charge on protoplasts causes repulsion. So the contact of membrane between the protoplasts is facilitated by the group of chemicals known as fusogens that compensate for their negative charge.

Chemical fusogens:

PEG (Polyethylene glycol), high concentration of calcium ions (Ca^{2+}) at high pH (8-10) and elevated temperature (37 °C), $NaNO_3$, Dextran, polyvinyl alcohol, synthetic phospholipids etc. may be used as fusogens.

Methods of Protoplast Fusion

Fusion of protoplasts can be achieved by applying either spontaneous fusion method or induced fusion method.

1. Spontaneous Fusion Method:

During enzymatic degradation of cell wall some of the adjacent protoplasts may fuse together to form homokaryons, sometimes more than two protoplasts fuse together and form multinucleate cells. Spontaneous fusion method is not in very much use because the spontaneous fusion products do not regenerate into whole plants except for undergoing a few divisions.

2. Induced Fusion Method:

Freshly isolated protoplasts can be induced to undergo fusion, irrespective of their origin. This is done by applying different kinds of fusogen (fusion inducing agents), as the case may be, during the process.

a. Sodium nitrate ($NaNO_{3)}$ Treatment:

It was for the first time reported by Power et al. (1970). In this method $NaNO_3$ is used as a fusogen. Isolated protoplasts are suspended in a mixture of 5.5% $NaNO_3$ and 10% sucrose solution and incubated in a water bath at 35°C for 5 min and centrifuged for 5 min at 200 x g. Following centrifugation the supernatant is decanted and the pellet is incubated in a water bath at 30°C for 30 min. During this period most of the protoplasts undergo cell fusion. After washing with osmoticum they are plated properly in culture medium for further growth.

b. High Calcium Ions at High pH:

Keller and Melcher (1973) found this method suitable for protoplasts fusion as positively charged ions (high concentration of Ca^{2+}) at high pH reduce the net negative charges of protoplasts. Here the isolated protoplasts are incubated in a solution of osmoticum containing 0.05 M $CaCl_2$ at pH 10.5 (using 50mM glycine-NaOH buffer). Temperature should be 37°C. Aggregation of protoplasts generally takes place at once and fusion occurs within 10 mins.

c. PEG Treatment:

Polyethylene glycol (PEG) is very effective fusogen. Rate of protoplast fusion is very high while using PEG as fusogen (Kao and Michayluk, 1974; Wallin et al. 1974). In this process, protoplasts are suspended in a solution having 28-56% PEG (1500-6000 MW). The tube is then allowed to settle for 10-40 min at room temperature. After PEG treatment the elution of fusogen is done by using high pH/Ca^{++} containing solution which is most effective in enhancing the fusion frequency. Survival rate of fused protoplasts is also very high in this method.

Electrofusion:

As developed by U. Zimmerman, protoplasts are placed in an electric field and are exposed to high intensity electric pulse for a short duration (nano to micro second). Due to potential difference protoplasts line up between the electrodes. An extremely short wave electric shock reversibly increases permeability of cell membrane and induces protoplast fusion.

In this fusion method, two step procedures is followed: a low voltage and rapidly oscillating AC field is applied, which causes the protoplasts to become aligned into chains by cell to cell contact. Second step is brief application of a high voltage DC pulse which induces reversible breakdown of the plasma membrane at the site of cell contact, leading to fusion and consequent membrane reorganisation. Heterokaryons produced by this electro-fusion divide normally in culture medium and have the capability of regenerating the plantlet. Yield of somatic hybrids by this method is very high. Fusion process is synchronous and extends over a short duration so the hybrids do not lose the viability.

There are different methods of protoplasts fusion but generally three methods are in frequent use for protoplasts fusion. These are (a) high Ca^{++} and high pH; (b) Polyethylene glycol (PEG) and (c) electric field.

Mechanism of Protoplast Fusion:

You can understand the mechanism of protoplast's fusion by going through the following three steps:

(i) Agglutination or adhesion:

When the protoplasts are treated with fusogen (any type), they come in close proximity and their membranes agglutinated.

(ii) Fusion of Plasma Membrane at Localised Sites:

As soon as the protoplasts come in close contact, specially at the point of adhesion. There is a formation of cytoplasmic bridges between the protoplasts. The high pH and high Ca^{++} ions have shown to neutralize the normal surface charge so that the agglutinated protoplasts can fuse due to intermingling of lipid molecules in membranes.

(iii) Formation of Heterokaryon:

Rounding off of the fused protoplasts occur due to the expansion of cytoplasmic bridges. This ultimately results in spherical heterokaryon or homokaryon formation. However some of the protoplasts remain unfused (Figure 7.22).

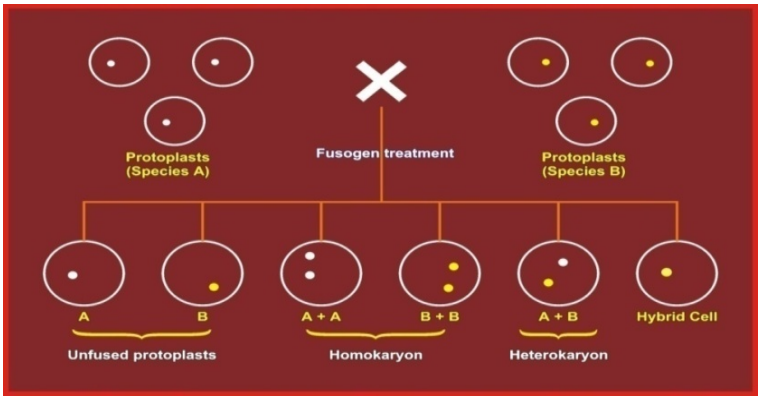


Figure 7.22– *Possibilities of fusion*

Identification and selection of hybrid cells:

Following fusion treatment the protoplast population consists of parental type protoplasts, homokaryotic fused products and also heterokaryotic fusion products (Figure 7.22). After this step selection of hybrid cells is must. Different methods have been developed for the selection of somatic hybrids. These are visual selection, selection by complementation, and/or use of metabolic inhibitors

Verification and Characterisation of Somatic Hybrid:

Verification of somatic hybrids after regeneration is must. It can be done by clear demonstration of genetic contribution of both the parents.

(i) Morphological Characters:

Morphological characters are leaf size, leaf surface, pigment, flower shape, pollen character, etc. In general, the somatic hybrids bear the characters from the parents. Sometimes they have the

somatic or sexual characters intermediate of both the parents. By going through the morphological characters somatic hybrids can be verified.

(ii) Isoenzyme Analysis:

Electrophoretic banding patterns of isoenzymes have been extensively used to verify hybridity. Somatic hybrids can be verified by going through the banding pattern of isoenzyme analysis. In this method electrophoretic banding pattern is observed. Somatic hybrids may show the banding pattern of used enzymes like the parents or it may be new type. The enzymes used in this analysis may be esterase, isoperoxidase, phosphatase, alcohol dehydrogenase, etc.

(iii) Chromosomal constitution:

Counting chromosomes is a reliable and easy method of detection. Cytologically the chromosome number should be the sum of chromosome number of two parents in somatic hybrid. Besides the number, the size and structure of the chromosomes of both the parents could be taken for verification of somatic hybrids.

(iv) Molecular Technique:

Various molecular markers such as RFLP, AFLP, RAPD, microsatellites, etc. are useful for verification of the somatic hybrids. PCR technology is being utilised for hybrid identification. Specific restriction patterns of chloroplast and mitochondrial DNA have been used with great advantage to characterise the somatic hybrids.

Culture and regeneration of the hybrid cells

Hybrid cells are cultured on suitable medium provided with the appropriate culture conditions so that a complete plant can regenerate from hybrid cells. An outline (Schematic representation) of protoplast fusion and regeneration of plant is given in Figure 7.23.

Advantages of somatic hybridization

- Its major contribution to plant breeding is in overcoming common crossing barriers among plant species and in organelle genetics and breeding.
- It can be used as a tool for crop improvement.
- Production of novel interspecific and intergenic hybrids like Pomato (hybrid of potato and tomato) is possible.
- Production of fertile diploids and polyploids from sexually sterile haploids, triploids and aneuploids.
- Gene for disease resistance, abiotic stress resistance, herbicide resistance and many other quality characters can be transferred by it.
- Production of heterozygous lines in the single species is possible by it.
- Production of unique hybrids of nucleus and cytoplasm.

Limitations of Somatic hybridization

- Poor regeneration of hybrid plants.
- Non-viability of fused products.
- Not successful in all plants.
- Production of unfavorable hybrids.
- Lack of an efficient method for selection of hybrids.

- No confirmation of expression of particular trait in somatic hybrids.

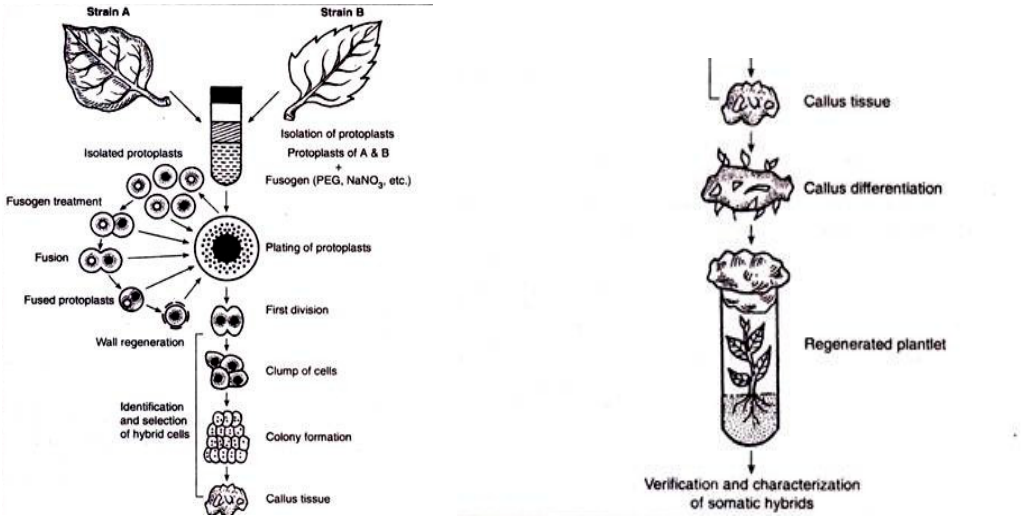


Figure 7.23*- Schematic representation of protoplast fusion and regeneration of plant*

7.6 TRANSGENICS

Transgenics (plants) are the ones, whose DNA is modified by means of recombinant DNA technology. Genetic transformation is a method of transferring the **gene** of interest to the host. The inserted gene sequence is known as the **transgene.** It is a key technique for plant molecular breeding to introduce desirable traits into the existing genomes while preserving the genetic identity of plants. The inserted genes can come from species within the same kingdom (plant to plant) or between kingdoms (bacteria to plant). In many cases, the inserted DNA has to be modified slightly in order to correctly and efficiently express in the host organism. All these require a laboratory step that is often called gene splicing, or genetic modification (GM).

There are number of tools or methods which could be applied to achieve the increase in productivity or change in various quality traits. Efforts have also been made to generate diversity and produce plants that would not exist in nature. These include use of wide crosses which may be inter- specific or inter –genetic, use of *in- vitro* techniques like- embryo rescue so that already existing variations can be used and protoplast fusion by over coming the species specific barrier or mutagenesis etc.

To broader the available genetic diversity in a given plant species, the most recent addition in the long list of methods is generation of transgenic plant by exploiting the techniques of genetic engineering to transfer a 'foreign' DNA into a plant cell.

> **Cisgenic plants** are made up of using genes, found within the same species or a closely related one, where conventional plant breeding can occur.

Genetically modified organisms (GMO)

Bacteria, fungi, plants and animals whose genes have been altered are called genetically modified organisms (GMO) or transgenics. **Transgenic plants** possess a gene or genes that have

been transferred from a different species. Although DNA of another species can be integrated in a plant genome by natural processes, the term "transgenic plants" refers to plants created in a laboratory using recombinant DNA technology. The aim is to design plants with specific characteristics by artificial insertion of genes from other species or sometimes entirely different kingdoms.

Food supply in the earth according to demand is still not enough to feed the growing human population, even after the Green Revolution which succeeded in tripling the food supply. Increased yields have been due to improved crop varieties, use of agrochemicals (fertilizers and pesticides). For most of the farmers agrochemicals are too expensive and conventional breeding is not helping in increasing the yield as per requirements. So there must be an alternative path for the farmers to obtain maximum yield from their field and to minimize the use of agrochemicals so their harmful effects on the environment are reduced.

Use of genetically modified crops (GM crops) may be a possible solution. Transgenic plants are used to express proteins, like the cry toxins from *Bacillus thuringiensis*, herbicide resistant genes and antigens for vaccinations.

This process provides advantages like improving shelf life, higher yield, improved quality, nutritional improvement, disease resistance, insect-pest resistance, herbicide resistance, virus resistance, tolerant to heat, cold and drought resistance, against a variety of biotic and abiotic (non-biological) stresses. Transgenic plants can also be produced in such a way that they express foreign proteins (diagnostic and therapeutic proteins) with industrial and pharmaceutical value. So we can say that transgenic plants have been deliberately developed for a variety of reasons as discussed above including edible vaccines. There are two common ways of introducing DNA into plant cells—indirect, and direct (discussed in gene transfer method section). Figure 7.24 is showing various steps essential for producing transgenic plants.

Benefits of GM crops

Genetic modification has made

- crops more tolerant to abiotic stresses (cold, drought, heat, salt)
- reduced dependency and reliance on chemical pesticides
- helped to reduce post-harvest losses
- enhanced nutritional value of food e.g., Vitamin A enriched rice.
- increased efficiency of minerals usage by plants (this prevents early exhaustion of fertility of soil).

First generation transgenic plants- are those transgenic plants which contain only marker genes, that are useful in the development of transformation systems.

Second generation transgenic plants- are those transgenic plants which in addition to selectable marker genes contain one or two transgenes, encoding simple agronomic traits such as pest and herbicide resistance.

Third generation transgenic plants-are those transgenic plants that contain multiple transgenes targeting multiple pests and diseases. These may also express additional value added or agronomic traits.

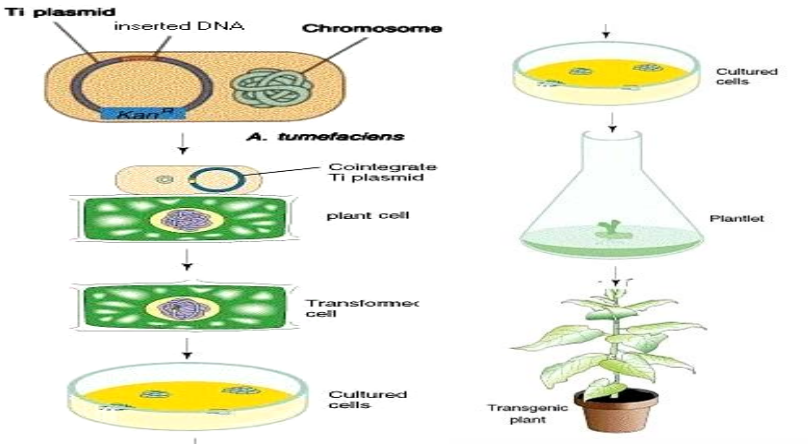


Figure 7.24- Steps for generation of transgenic plants
Source:Some Achievements http://www.biology-pages.info/T/TransgenicPlants.html

Some important points

- The first **genetically modified crop** plant was produced in 1982, an antibiotic-resistant **tobacco plant**. The first field trials occurred in France and the USA in 1986, when **tobacco plants** were engineered for herbicide resistance.
- The first modern recombinant crop approved for sale in the US, in 1994, was the FlavrSavr tomato, which was intended to have a longer shelf life.
- The first conventional transgenic cereal created by scientific breeders was actually a hybrid between wheat and rye in 1876 (Wilson, 1876).
- The first commercially genetically engineered crop marketed in Europe, in 1994, was tobacco engineered to be resistant to the herbicide bromoxynil, as approved by the European Union
- The country's first pesticide producing crop approved by the US Environmental Protection Agency, in 1995, was Bt Potato
- The first food with increased nutrient value, in 2000, was Vitamin A-enriched golden rice.
- A regulatory clean line with a β-carotene content of 1.6 µg/g was obtained in 2003.
- In 2004 – *Golden Rice* meets the sun in the open.
- The first *Golden Rice* field trial in the world was harvested in September 2004 in Crowley ("where life is rice and easy"), Louisiana, USA
- Herbicide tolerant soybean, *canola*, Bt maize, Bt cotton constitute the four major genetically modified (GM) crops.
- The two commercial transgenic vegetable crops are tomato with delayed fruit ripening and potato with insect and virus resistance.
- High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation.

Some examples of transgenics

Nutritional improvement

Transgenic plants have been developed to improve the nutritional value. For example –

Golden rice: Milled rice is the staple food for a large fraction of the world's human population. Milling rice removes the husk and any β-carotene it contained. **β-carotene** is a precursor to vitamin A. Vitamin A deficiency is widespread, especially in the countries of southeast Asia and causes half a million children to become partially or totally blind each year.

The transgenic rice (Golden rice) exhibits an increased production of β-carotene as a precursor to vitamin A and the seed is yellow in colour. Such yellow or golden rice may be a useful tool to treat the problem of vitamin A deficiency in young children.

A japonica variety of rice was engineered with three transgenes necessary for the rice grain to produce and store β-carotene. These included two genes from the daffodil plant and a third from a bacterium. These t**hree** transgenes incorporated into rice that enabled the plants to manufacture β-carotene in their endosperm. *Golden Rice* is a new type of rice that contains β-carotene (provitamin A), which is converted into vitamin A as needed by the body and gives the grain its golden colour. The original golden rice was called SGR1, and under greenhouse conditions it produced 1.6 µg/g of carotenoids. Figure 7.25 shows different steps for formation of golden rice.

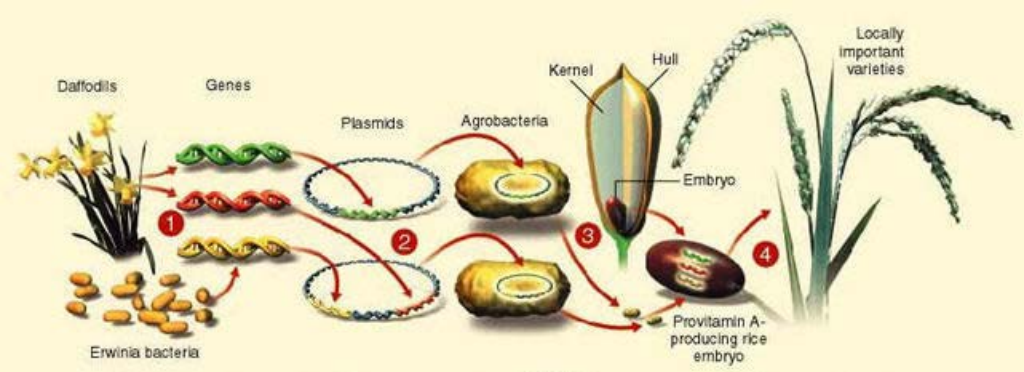


Figure 7.25 *–Steps showing formation of golden rice*

1-The genes that give golden rice it's ability to make β-carotene in its endosperm (the interior of the kernel) come from daffodils and a bacterium called Erwinia uredovora.

2. These genes, along with promoters (segments of DNA that activate genes), are inserted into plasmids of Agrobacterium tumifaciens.

3.These agrobacteria are then added to a petridish containing rice embryos. As they "infect" the embryos, they also transfer the genes that encode the instructions for making β-carotene.

4.The transgenic rice plants must now be crossed with strains of rice that are grown locally and are suited to a particular region's climate and growing conditions.

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Source:https://www.isaaa.org/kc/inforesources/biotechcrops/The_Golden_Rice_Technology.htm

Potato is an important food crop. The nutritive value of potato protein is diminished due to deficiency in essential amino acids lysine, tyrosine and sulphur containing amino acids methionine and cysteine. To improve the nutritive value of potato an Amaranthus seed albumin gene AmA I has been expressed in transgenic potato tubers. The protein thus produced is non-allergenic and rich in all essential amino acids.

High Lysine Corn: Corn is one of the major crops in the world, but its low lysine content is often problematic for animal consumption. While exogenous lysine supplementation is still the most common solution for today's feed corn, high-lysine corn has been developed. Reducing the lysine-poor seed storage proteins, zeins, or expressing a deregulated lysine biosynthetic enzyme,

CordapA, has shown increased total lysine or free lysine content in the grains of modified corn plants (Huang et al 2005).

Proteins of therapeutic importance, like those used in the treatment, diagnosis of human diseases can be produced in plants, using recombinant DNA technology. The proteins produced in transgenic plants for therapeutic use, are of three types – (i) antibodies, (b) proteins and (iii) vaccines. Antibodies directed against various diseases i.e., dental caries, rheumatoid arthritis, cholera, *E.coli* diarrhea, malaria, certain cancers, HIV, rhinovirus, influenza, hepatitis B virus and herpes simplex virus are known to be produced in transgenic plants. Vaccines against infectious diseases of the gastro intestinal tract have been produced in plants like potato and bananas. An anti cancer antibody has recently expressed in rice and wheat seed that recognizes cells of lung, breast and colon cancer and hence could be useful in both diagnosis and therapy in the future.

- Golden rice is genetically modified rice that now contains a large amount of Vitamin A.
- More correctly, the rice contains the element β-carotene which is converted in the body into Vitamin-A. So when you eat golden rice, you get more vitamin A.
- β-carotene gives carrots their orange colour and is the reason why genetically modified rice is golden.
- For the golden rice to make β-carotene three new genes are implanted: two from daffodils and the third from a bacterium.

Insect resistant plants

Bacillus thuringiensis is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin it produces for which **Bt gene** is responsible. Through recombinant DNA methods, its toxin gene can be introduced directly into the genome of the plant, where it is expressed and provides protection against insect pests of the plant. So transgene used for insect pest control is Bt gene**,** isolated from bacteria *B. thuringiensis* which produces a toxin protein. When insect larvae ingest these bacteria with their food, protein present in bacteria kills larvae and this toxic protein is encoded by the Bt gene.

Few strains of *B. thuringiensis* produce proteins that kill certain insects like lepidopterans (tobacco budworm, armyworm, butterflies and moths), coleopterans (beetles and weevils), dipterans (flies, mosquitoes). *B. thuringiensis* forms protein crystals during a particular phase of their growth. These crystals contain a toxic **insecticidal protein.** This Bt toxin (insecticidal) protein exist as **inactive protoxins** and when an insect ingest the inactive toxin, it is converted into an active form of toxin. **How this happens?** As soon as the inactive toxin comes in the gut of insect, alkaline pH (7.5-8.0) of the gut and specific digestive proteases solublise the crystals and inactive form changes into active form. proteases

inactive toxin $\xrightarrow[\text{proteases}]{\text{alkaline pH (7.5-8.0) \&}}$ active toxin

This activated toxin binds to the surface of midgut epithelial cells and create pores that cause cell swelling and lysis and eventually cause death of the insect. But this toxin does not kill the *Bacillus* itself. The reason is -Bt toxin protein exist as **inactive protoxins** and it become active

only after reaching in alkaline pH of the gut of insect in the presence of specific proteases so *Bacillus* remain protected.

Therefore keeping this quality of *B. thuringiensis* in mind, specific Bt toxin genes (cry) were isolated from *B. thuringiensis* and incorporated into the several crop plants like cotton.

Bt toxin gene has been cloned from the bacteria and been expressed in plants to provide resistance to insects without the need for insecticides.

Transgenic cotton, potato, tomato, corn, tobacco, canola, brinjal have been produced so far containing Bt gene (insecticidal gene).

Herbicide resistant plants

Plants that can tolerate herbicides are called Herbicide Resistant Plants.

1. Glyphosate tolerant plants- Glyphosate is a broad-spectrum herbicide that controls broadleaf, sedge, and grass weeds with minimal residual toxicity to crops or non-target vegetation.

Glyphosate (N-(phosphonomethyl)glycine), commercially introduced in 1974 is a effective broad spectrum post emergent herbicide and is among the most widely used agricultural chemicals globally. It is very toxicologically and environmentally safe. Glyphosate is the only herbicide that targets EPSP (5-enolpyruvyl-shikimate-3-phosphate) synthase enzyme and inhibits it in an amino acid pathway. So plants die because they lack the key amino acids. Glyphosate is absorbed across the leaves and stems of plants and is translocated throughout the plant. It concentrates in meristematic tissue. Initially developed to control the growth of weed species in agriculture, this herbicide also plays an important role in both modern silviculture and domestic weed control. The use of this virtually ideal herbicide is now being threatened by the evolution of glyphosate-resistant weeds. A **resistant EPSP synthase gene** allows crops to survive spraying of glyphosate. Glyphosate resistant transgenic tomato, potato, tobacco, cotton, petunia etc. have been developed by transferring aro A gene into a glyphosate EPSP synthetase from *Salmonella typhimurium* and *E. coli*.

2. Sulphonylurea tolerant plants- Sulphonylurea inhibits acetolactate synthetase (ALS), the enzyme involved in the biosynthesis of branched chain amino acids. Sulphonylurea resistant tobacco plants are produced by transforming the mutant ALS (acetolactate synthetase) gene from *Arabidopsis*.

3. Atrazine tolerant plants- Atrazine inhibits QB protein of photo system II in chloroplasts. QB protein of photo system II from mutant Amaranthus hybrids is transferred into tobacco and other crops to produce atrazine resistant transgenic plants.

4. Bromoxynil tolerant plants- Genes for resistance to some of the newer herbicides have been introduced into some crop plants and enable them to thrive even when exposed to the weed killer.

Pest resistant plants

Several nematodes parasites a wide variety of plants and animals including human beings. For example a nematode *Meloidegyne incognitia* infects the roots of tobacco plants and causes a great reduction in yield. A process of RNA interference (RNAi) was adopted to prevent this infestation.

Using *Agrobacterium* vectors, nematode-specific genes were introduces into the host plant. The introduction of DNA was such that it produced both sense and anti-sense RNA in the host cells. These two RNA's being complementary to each other formed a double stranded (dsRNA) that initiated RNAi and thus, silenced the specific mRNA of the nematode. The consequence was that the parasite could not survive in a transgenic host expressing specific interfering RNA. So the transgenic plant got itself protected from the parasite.

Flavr savr

The tomato fruit enzyme polygalacturonase (PG), because of its ability to dissolve cell-wall pectin, is key to fruit softening. The FLAVR SAVR tomato was developed through the use of antisense RNA to regulate the expression of the enzyme polygalacturonase (PG) in ripening tomato fruit. This enzyme as discussed is one of the most abundant proteins in ripe tomato fruit. So the genetically modified tomato went to U.S. market on May 21, 1994 known as the Flavr Savr, was no longer able to produce polygalacturonase (PG), due to a deactivated gene.

But high production costs mixed with the company's inexperience in tomato growing, handling, and transport led to financial troubles for the Flavr Savr. Eventually Calgene was bought out by Monsanto, a multinational agricultural company, and the Flavr Savr disappeared from shelves for good in 1997, just three years after its introduction.

Edible vaccine

Edible vaccines are nothing but transgenic plants that contain agents that trigger an animal's immune response. In simple terms, edible vaccines are plant or animal made pharmaceuticals. The concept of edible vaccines was developed by Arntzen (www.genomenewsnetwork.org) in the 1990s. The genes for proteins to be used in human (and animal) medicine can be inserted into plants and expressed by them. Corn is the most popular plant for these purposes, but tobacco, tomatoes, potatoes, rice and carrot cells grown in tissue culture are also being used. The earliest demonstration of an edible vaccine was the expression of a surface antigen from the bacterium *Streptococcus* mutants in tobacco.

Edible vaccines contain DNA fragments from the original pathogen. These fragments code for a protein that is usually a surface protein of the pathogen. This is responsible for eliciting the body's immune response.

Transgenic Potatoes for Diarrhea

The first human trial for an edible vaccine took place in 1997. Volunteers ate transgenic potatoes that contained the b-subunit of the *E. coli* heat-labile toxin, which causes diarrhea.

The disadvantage of using potato-based edible vaccine is that it has to be consumed raw; when cooked the protein may get denatured or in some cases less effective. Research has shown that by partial boiling at least half the vaccine remained alive.

Transgenic Banana for Hepatitis B

Genes for protein coat of hepatitis B virus was isolated and introduced into banana. The transgenic banana is rich in the antigenic protein of hepatitis B virus.

Transgenic Tomatoes against Diarrhea

At Cornell University, US researchers have developed transgenic tomatoes against the norwalk virus, which causes severe diarrhea. The tomatoes produced a surface protein specific to the virus. Recently, banana has been explored as an alternative source.

Protein, human growth hormone has been produced by transgenic crop plants with the gene inserted into the chloroplast DNA of tobacco plants.

Salt Tolerance

A large fraction of the world's irrigated crop land is so laden with salt that it cannot be used to grow most important crops. However, researchers at the University of California Davis campus have created **transgenic tomatoes** that grow well in saline soils. The transgene was a highly-expressed sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells. There was no sodium buildup in the fruit.

Virus resistant plants

Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes.TMV resistant tobacco and tomato plants are produced by introducing viral coat proteins. Other viral resistant transgenic plants are Potato virus resistant potato plants, RSV resistant rice, YMV resistant black gram and YMV resistant green gram etc.

Terminator Genes

This term is used (by opponents of the practice) for transgenes introduced into crop plants to make them produce sterile seeds (and thus force the farmer to buy fresh seeds for the following season rather than saving seeds from the current crop). The process involves introducing three transgenes into the plant: 1. A gene encoding a **toxin** which is lethal to developing seeds but not to mature seeds or the plant. This gene is normally inactive because of a stretch of DNA inserted between it and its promoter. 2. A gene encoding a **recombinase** - an enzyme that can remove the spacer in the toxin gene thus allowing to be expressed. 3. A **repressor** gene whose protein product binds to the promoter of the recombinase thus keeping it inactive.

When the seeds are soaked (before their sale) in a solution of tetracycline synthesis of the repressor is blocked so the recombinase gene becomes active and the spacer is removed from the toxin gene and it can now be turned on. Because the toxin does not harm the growing plant but only its developing seeds get affected so the crop can be grown normally except that its seeds are sterile.

Genetically engineered insulin

Insulin is a protein hormone produced in the pancreas which has an important function in the regulation of blood sugar levels. Insulin facilitates the transport of glucose into cells. A deficiency in insulin is one of the causes of the disease *Diabetes mellitus* or sugar diabetes resulting in harmful consequences. *D. mellitus*, describes a group a metabolic diseases in which the person has a high blood glucose (blood sugar), either because insulin production is inadequate or because the body's cells do not respond properly to insulin or both. Insulin is a hormone that helps the glucose get into the cells to give them energy.

Diabetes-
Body does not make insulin-**type 1 diabetes**
Body does not make or use insulin well-**type 2 diabetes**.
Without enough insulin, the glucose stays in blood.

Insulin consists of two short polypeptide chains: Chain A and chain B, linked together by disulphide bridges. In mammals, including humans, insulin is synthesized as prohormone which contains an extra stretch called the **C peptide**. This prohormone needs to be processed before it becomes a fully mature and functional hormone. The C peptide is not present in the mature insulin and is removed during processing.

The main challenge for production of insulin using rDNA techniques was getting insulin assembled in a mature form. In 1983, Eli Lilly, an American company prepared two DNA sequences corresponding to A and B chains of human insulin and introduced them in plasmids of *E.coli* to produce insulin chains. Chain A and B produced separately, extracted and combined by creating disulphide bonds to form human insulin (Figure 7.26).

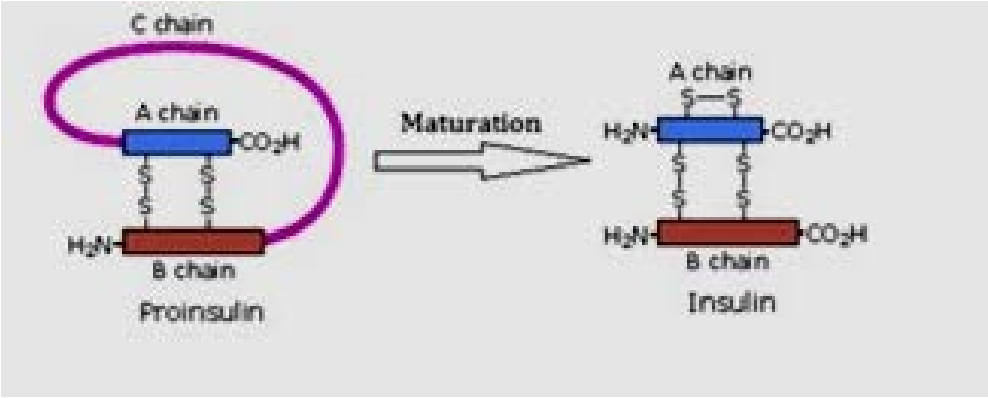


Figure 7.26*- Genetically engineered insulin*

Recombinant human insulin first entered clinical trials in humans in 1980. At that time, the A and B chains of the insulin molecule were produced separately and then combined by chemical techniques. Since 1986, a different recombinant process has been used. The human genetic coding for proinsulin is inserted into *E.coli* cells, which are then grown by fermentation to

produce proinsulin. The connecting peptide is cleaved enzymatically from proinsulin to produce human insulin (Figure 7.27).

Management of adult-onset diabetes is possible by taking insulin at regular time intervals. Insulin used for diabetes was earlier extracted from pancreas of slaughtered cattle and pigs. Insulin from animal source, though caused some patients to develop allergy or other types of reactions (being a foreign protein). Therefore genetically engineered insulin was prepared.

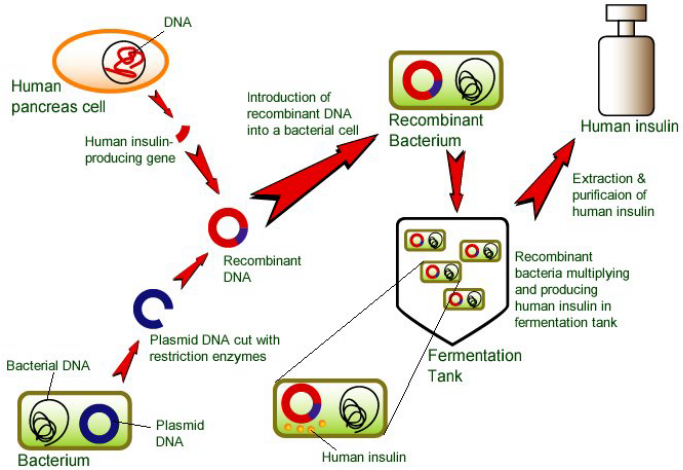


Figure 7.27*- Human insulin production*

7.7 DEVELOPMENT AND USE OF MOLECULAR MARKERS IN PLANT BREDD ING

A genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify cells, individuals or species. A marker must be polymorphic, that is, it must exist in different forms so that chromosome carrying the mutant gene can be distinguished from the chromosome with normal gene by the form of marker it also carries. This polymorphism in the marker can be detected at following levels:-

1. Phenotype (morphological)
2. Difference in chromosome characters (cytological)
3. Difference in proteins (biochemical)
4. Difference in the nucleotide sequence of DNA (molecular)

Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Morphological, cytological and biochemical markers are known as Classical markers.

Morphological markers: Those markers which can visually distinguish qualities on the basis of external appearance (morphology) like seed structure, flower colour, growth habit and other important agronomic traits, are known as morphological markers.

Cytological markers: Those markers which are related with chromosomes, like- variations present in their numbers, banding patterns, size, shape, order and position of chromosomes, are known as cytological markers.

Biochemical markers: Those markers which are related with biochemical properties (also known as isozymes). These are multi-molecular forms of enzymes which are coded by various genes, but have the same functions.

Molecular markers A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be monitored. Examples- RFLP, AFLP, SSRs etc.

A molecular marker should have some features:-

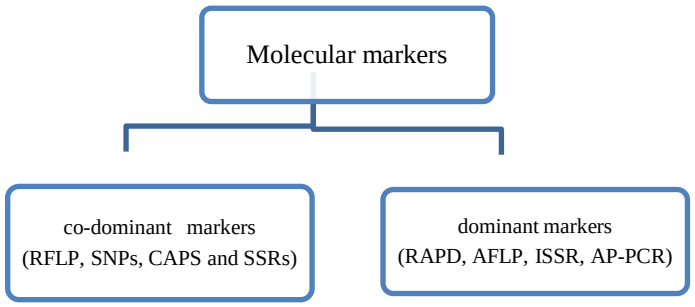
1. It must be polymorphic, having ability to detect higher level of polymorphism.
2. Co-dominant inheritance -The different forms of a marker should be detectable in diploid organism to allow discrimination of homozygous and heterozygous.
3. It should be evenly and frequently distributed throughout the genome.
4. It should be easy to use, fast and cost effective.
5. It should be reproducible.
6. Genetic differences should be clearly visible.

Unfortunately, no single molecular marker meet all these requirements. A wide range of molecular techniques is available that detect polymorphism at the DNA level. Molecular markers are classified into various groups:

I. On the basis of mode of gene action molecular markers may be co-dominant or dominant, means whether markers can discriminate between homozygotes and heterozygotes.

i) co-dominant markers- Those markers which clearly discriminate between homozygotes and heterozygotes are co-dominant markers or we can say that codominant markers are markers for which both alleles are expressed when co-occurring in an individual. Analyze one locus at one time. Example-RFLP, SNPs, CAPS and SSRs.

ii) dominant markers- Those markers which do not tell whether the allele is homozygous or heterozygous are dominant markers, they only depicts the presence or absence of allele. Allow for analyzing many loci at one time. Example-RAPD, AFLP, ISSR, SCAR, AP-PCR



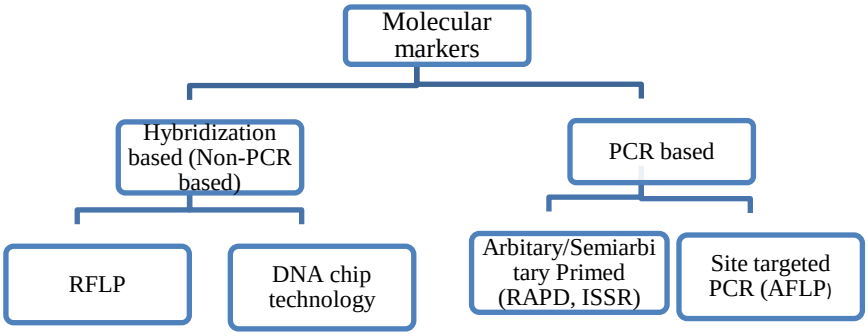
II. On the basis of mode of method of detection molecular markers may be

i) hybridization-based molecular markers or

ii) polymerase chain reaction (PCR)-based markers

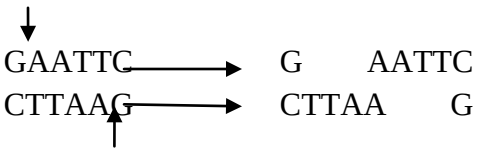
i. Hybridization-based molecular markers

1. Restriction fragment length polymorphism (RFLP):- RFLP is a non –PCR or hybridization based marker developed by Grodzicke et al (1974). RFLP was the **first technology** that enables the detection of polymorphism at the DNA sequence level. Variation in this DNA sequence is the basis for the genetic diversity within a species. Principle of RFLP is restriction digestion.



In this method, DNA is digested with restriction enzyme (s), which cut the DNA at any specific sequences; electrophoresed, blotted on a membrane and probed with a labeled clone. Polymorphism in the hybridization pattern is revealed and attributed to sequence difference between individuals. The DNA sequence variation detected by this method was termed RFLP (Botstein et al. 1980) (Figure 7.28).

RFLP variation is environmentally independent. Variation in **one DNA fragment** obtained with a specific enzyme is treated as one RFLP. When genomic DNA is digested with one of the restriction enzymes, a series of fragments are produced of varying length because the recognition sites are not associated in any type of gene and are distributed randomly throughout the genome. These fragments upon hybridization yield a characteristic pattern. Variation in this pattern can be caused by base pair deletion, mutation, inversion or by translocation which result in loss or gain of a recognition site resulting in a fragment of different length and polymorphism. Genomic restriction fragments of different lengths between genotypes can be detected on southern blots and by a suitable probe. For eg.:- Restriction endonuclease *Eco* R1 cuts DNA at a specific sequence as shown below-



When one or more nucleotides in the endonuclease recognition sequence are altered, *Eco*R1 will fail to cut the DNA strand at the altered site and a longer DNA fragment is produced. When a given restriction enzyme site is present in the DNA molecule of one chromosome but absent from the DNA molecule of the other homologous chromosome a shorter fragment is produced from the chromosome with the site, and a longer fragment from the other chromosome. It is now possible to distinguish the two chromosomes in such an individual on the basis of this RFLP. The individual is heterozygous for this RFLP and is said to be informative at that locus. Because the RFLP is inherited just like a gene, one can follow the individual chromosomes as they pass from generation to generation by tracing the inheritance of the marker fragments.

So **RFLP** is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction endonuclease or variation in restriction fragment lengths between individuals of a species when cut by a restriction enzyme. Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific. Probe size:- 0.5-2.0 Kb

The overall method can also be explained by going through Figure 7.29 and as follows:

Step I: Restriction digestion: Extraction of desired fragments of DNA using restriction endonuclease (RE). The enzyme RE has specific restriction site on the DNA, so it cut DNA into fragments. Different size fragments are generated along with the specific desired fragments.

Step II: Gel electrophoresis: The digested fragments are run in polyacrylamide gel or Agarose gel to separate the fragments on the basis of length or size or molecular weight. Fragments of different size form different bands.

Step III: Denaturation: The gel is placed in sodium hydroxide (NaOH) solution for denaturation to get single stranded DNA fragments.

Step IV: Blotting: The single stranded DNA fragments obtained are transferred into charge membrane i.e. Nitrocellulose paper by blotting (called capillary blotting or electro-blotting, as the case may be).

Step V: Baking and blocking: The nitrocellulose paper having transferred DNA is fixed by autoclaving. Then the membrane is blocked by using bovine serum albumin or casein to prevent binding of labelled probe non-specifically to the charged membrane.

Step VI: Hybridization and visualization: The labelled RFLP probe is hybridized with DNA on the nitrocellulose paper. The RFLP probes are complimentary as well as labelled with radioactive isotopes so they form colour band under visualization by autoradiography.

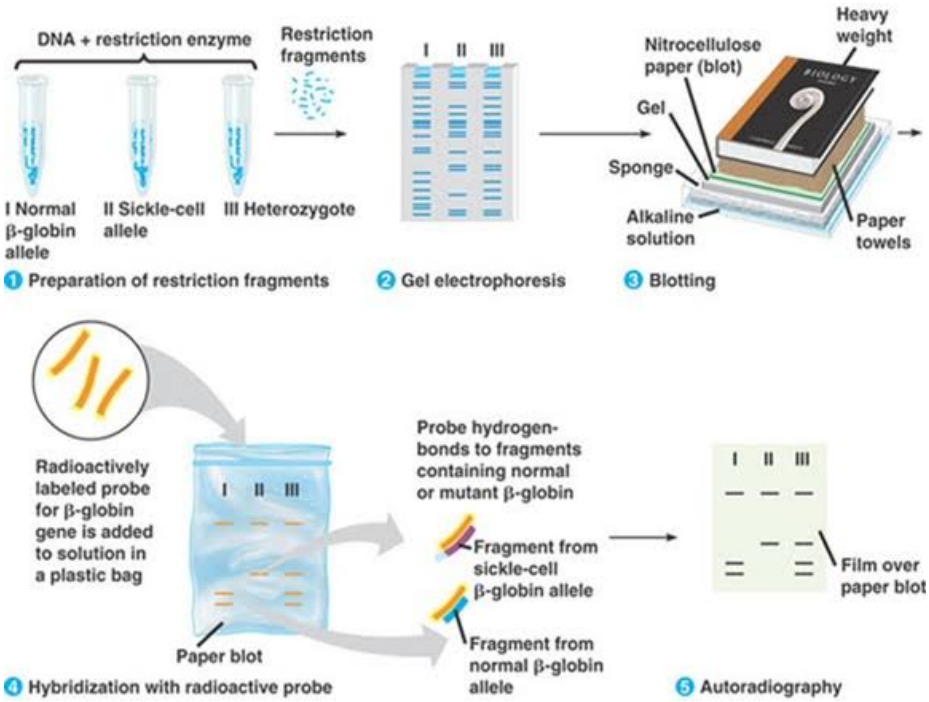


Figure 7.28*- Procedures of RFLP technique*

Uses of RFLP:-

1. RFLPs are co-dominant markers, enabling heterozygote to be distinguished from homozygote.
2. It permits direct identification of a gene type or cultivar in any tissue at any developmental stage in an environment independent manner.
3. After identification of gene for particular genetic or hereditary disease, that gene can be analyzed among other family members
4. It detect mutated gene.
5. It is the basis of DNA finger printing for paternity test, criminal identification etc.(forensic test).
6. The method is simple as no sequence- specific information is required

Problems:-

1. Labor intensive and expensive.
2. Requires relatively large amount of highly purified DNA.
3. Constant good supplies of probe that can reliably detect variation are needed.
4. Time consuming.
5. Require expertise in autoradiography.

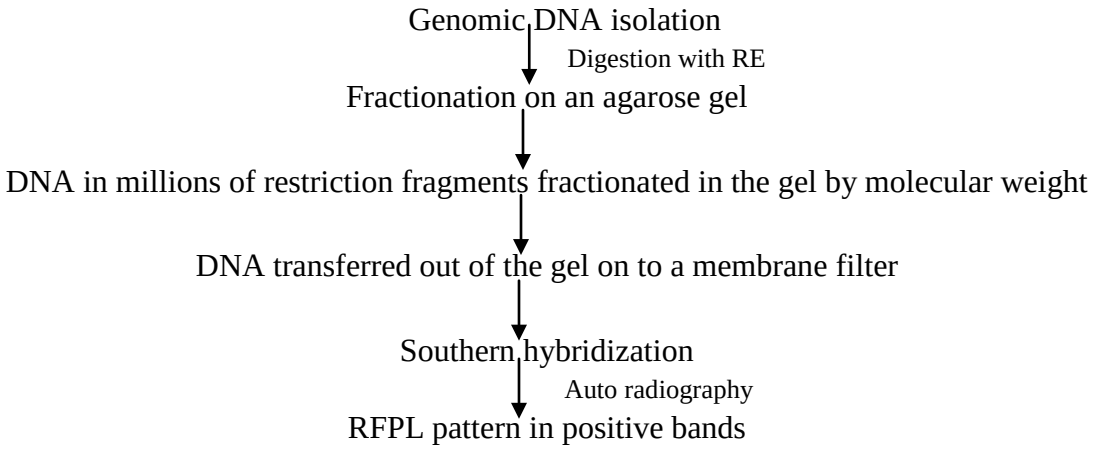


Figure 7.29*-Flow diagram of RFLP marker*

2. DNA chip technology-

DNA chip technology utilizes microscopic arrays (microarrays) of molecules immobilized on solid surfaces for biochemical analysis. The microarray technique is based on hybridisation of nucleic acids. In this technique, sequence complementarity leads to the hybridisation between two single-stranded nucleic acid molecules, one of which is immobilised on a matrix (Sourthern et al. 1999). So we can say that in the procedure of genomic analysis, microarrays are exposed to a labelled sample, hybridised, and complementary sequences are determined (Figure 7.30).

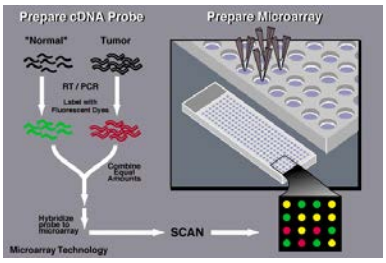


Figure 7.30*-Microarray technology*
Source: National Human Genome Research Institute

The basic principal behind DNA chips is the hybridization of samples to immobilized DNA molecules. There are different names for the microarrays, like DNA/RNA Chips, BioChips or Gene Chips. The array can be defined as an ordered collection of microspots (microscopic DNA spots attached to a solid surface), each spot containing a single defined species of a nucleic acid. As shown in figure 7.31, a **DNA chip** is a small piece of silicon glass (~1 cm^2) to which a large number of synthetic, single-stranded DNA oligonucleotides (**oligos**) have been chemically bonded [left]. Oligos function as DNA probes: they stick (anneal) selectively only to those DNA molecules whose nucleotide sequences are exactly complementary: T pairs with A, and G with C. They can therefore be used to identify the presence of specific DNA sequences in a heterogeneous mixture of genes, for example the presence of a particular allele against the background of a complete genome. In effect, oligos act like molecular **velcro**. A computer reads the pattern of annealing and reports which alleles are present.

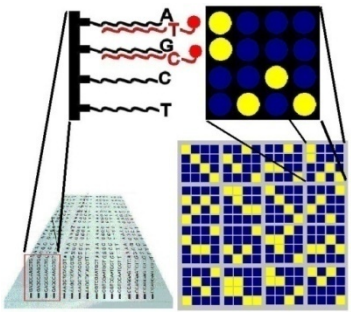


Figure 7.31*-Principle of a DNA microarray chip: use as Variant Detector Arrays (VDAs)*
(after SM Carr et al. 2008. Comp Biochem Physiol D, 3:11)

DNA chips can be used as Variant Detector Arrays (VDAs) to look for DNA sequences that differ by single nucleotide polymorphisms (**SNPs**). Single nucleotide polymorphisms (SNPs) are a type of polymorphism involving variation of a single base pair occurred at the specific location into the genome. In this example (Figure 7.31), the DNA sequences of the four oligos highlighted in the first bloc differ only at the last position. To determine which alleles are present, genomic DNA from an individual is isolated, fragmented, tagged with a fluorescent dye, and applied to the chip. The genomic DNA fragments anneal only to those oligos to which they are perfectly complementary. In this case, the allele with the **~T~** SNP allele binds to the **~~A** oligo, and the allele with the **~C~** SNP allele binds to

the **~~G** oligo. A computer reads the position of the two fluorescent tags and identifies the individual as a C/T heterozygote. [The single spots in the other three columns indicate that the individual is homozygous at the three corresponding SNP positions].

To determine whether an individual possesses a mutation for a particular disease-A sample of DNA from the patient's blood as well as a control sample - one that does not contain a mutation in the gene of interest, are taken. After denaturing the DNA in the samples - a process that separates the two complementary strands of DNA into single-stranded molecules, manageable fragments are generated by restriction digestion of the long strands of DNA. Labelling of each fragment is done by attaching a fluorescent dye. The individual's DNA is labelled with green dye and the control - or normal - DNA is labelled with red dye. Both sets of labelled DNA are then inserted into the chip and allowed to hybridize - or bind - to the synthetic DNA on the chip. If the individual does not have a mutation for the gene, both the red and green samples will bind to the sequences on the chip that represent the sequence without the mutation (the "normal" sequence). If the individual does possess a mutation, the individual's DNA will not bind properly to the DNA sequences on the chip that represent the "normal" sequence but instead will bind to the sequence on the chip that represents the mutated DNA.

Uses:

- The DNA microarray is a tool used to determine whether the DNA from a particular individual contains a mutation in genes.
- Scientists use **DNA microarrays** to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome
- The Gene Chip technology may be employed in diagnostics (mutation detection), in clinical diagnostic tests for some diseases.
- Microarrays can be used for gene discovery, mapping, polymorphism detection, DNA resequencing, genotyping on a genomic scale, and gene expression analysis,

Advantages:

High flexibility, which allows creation of a chip with any necessary sequences.

Disadvantages:

DNA chip is simple in concept, but generating probes on a solid array surface requires considerable expertise and technical tricks.

Time and resources consuming to synthesize the needed number of different oligonucleotides.

ii. PCR based markers:

With the discovery of polymerase chain reaction (PCR) technique, the new generation of PCR-based DNA markers was developed.

PCR-based markers may be divided into two types:

1. Locus non-specific markers e.g. RAPD, AFLP.
2. Locus specific markers e.g. SSRs, SNPs.

1. Random amplified polymorphic DNA (RAPD):-

RAPD is a molecular marker based on PCR amplification. **RAPD markers** are decamer (10 nucleotides long) DNA fragments which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. Principle of it is DNA amplification. RAPD used as the first PCR based molecular marker technique developed by Welsh & Mcckelland (1990) and Williams et al. (1990). RAPD amplification is performed in conditions of PCR. The DNA isolated from the genome of the species of interest is denatured, the template molecules are annealed with primers, and amplified by PCR. Single short oligonucleotide primers (usually a 10-base primer) can be arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. The amplified products are separated by electrophoresis and identified. Based on the nucleotide alterations in the genome, the polymorphisms of amplified DNA sequences differ which can be identified as bands on gel (Figure 7.32 and 7.33).

Genomic DNA from two different individuals often produces different amplification patterns (RAPDs). A particular fragment generated for one individual but not for other represents DNA polymorphism and can be used as a genetic marker. Since each random primer will anneal to a different region of the DNA, theoretically many different loci can be analyzed. PCR reaction requires cycling among three temperatures, the first to denature the template DNA strands (94^oC), the second to anneal the primer (35^o-40^oC) and the third for extension at temperature optimal for *Taq* Polymerase (72^oC).This cycle is usually repeated 25 to 45 times. Presence of RAPD band corresponds to a dominant allele, absence of band corresponds to a recessive allele. Thus heterozygous and homozygous dominant individuals cannot be differentiated with RAPD markers. DNA polymorphism among individuals can be due to -mismatches at the primer site, appearance of a new primer site or length of the amplified region between primer sites.

Advantages

- It is fast, simple.
- Relatively inexpensive and easy to perform.
- Need a small amount of DNA (15-25mg).
- Non radioactive assay.
- Does not require species probe libraries

Therefore, RADP gained popularity due to its simplicity, relative ease to perform, non requirement of prior sequence information and economical in terms of time and money.

Applications

- Construction of genetic maps
- It is useful for assessment of genetic fidelity, hybrid purity, fingerprinting of plant genomes, cultivar identification and genetic diversity.
- Mapping of traits
- Analysis of the genetic structure of populations
- For identification of somatic hybrids

Disadvantages

- Markers are dominant.

- Band could not differentiate homozygous or heterozygous individual

3. **Amplified fragment length polymorphism (AFLP)**

AFLP is a novel technique involving a combination of RFLP and RAPD. The AFLP technique is based on the selective amplification of subsets of genomic restriction fragments using the PCR (Figure 7.34). So the principle of it is DNA amplification. Thus, this technique combines the usefulness of restriction digestion and PCR. Multiple polymorphic markers are simultaneously produced and can be tested in one PCR. No prior information on genomic DNA sequences is needed.

Steps:-

1. After isolation genomic DNA is digested with two restriction enzymes to generate variable genomic DNA fragments.
2. Following heat inactivation of the REs the genomic DNA fragments are ligated to double- stranded oligonucleotide adaptors to generate template DNA for amplification.
3. Sequences in these adaptors (flanking the genomic DNA fragments) serve as primer binding sites on the restriction fragments. So it is possible to amplify many DNA fragments without having prior sequence knowledge.
4. PCR products (Amplicons) are separated on a gel and the resulting band pattern is visualized by autoradiography.

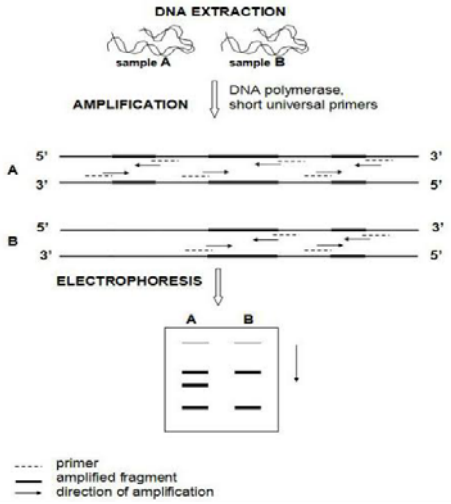


Figure 7.32*- Procedures of RAPD technique*

Source: Cultivar identification and traceability, a molecular approach.(2013) by Antonella Pasqualone In book: Cultivars: chemical properties, antioxidant activities and health benefits. Publisher: Nova Science Publisher Inc., Editors: K. Carbone

Isolation of DNA

↓ Taq DNA polymerase, primers, dNTPs and buffer in

Denature DNA (94 ^{o}C, 1 min)

↓

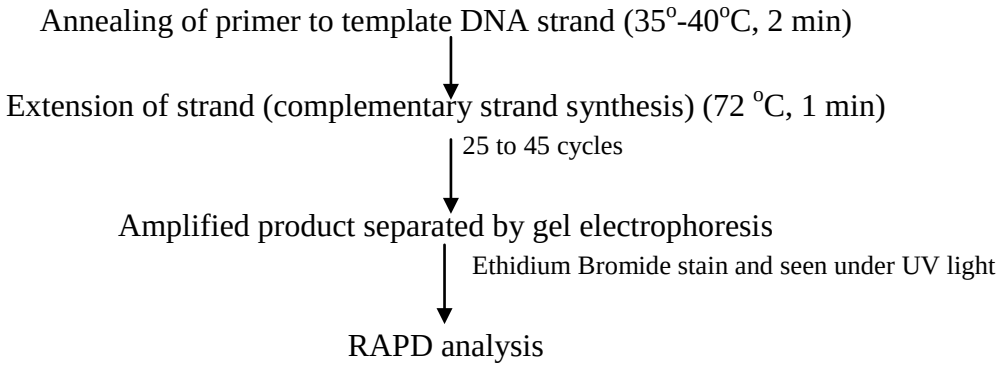


Figure 7.33*-An outline of RAPD procedure*

AFLP

- Highly sensitive method for detecting polymorphism throughout the genome.
- It is a combination of RFLP and RAPD methods.
- Universally applicable and highly reproducible.

Applications: AFLP can be used for mapping, fingerprinting and genetic distance calculation between genotypes.

Advantages:

- Highly reproducible.
- Can discriminate heterozygote from homozygotes.
- Extremely sensitive method

Disadvantages

- Very expensive.
- Extremely sensitive method.

2. Inter simple sequence repeat (ISSR)

ISSR is another PCR based molecular marker developed by Ziet Kiewwicz et al (1994). ISSR markers, also popularly known as random amplified microsatellites (RAMs) are present in the genomes of all eukaryotes. Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. The term microsatellite was coined by Litt and Lutty (1989). These are tandemly arranged repeats of mono, di, tri, tetra and penta nucleotides in different lengths of repeat motifs (eg. A,T,AT,GA,AGG,AAAC etc.). The ISSR marker are multilocus, mostly dominant genetic markers. Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences.

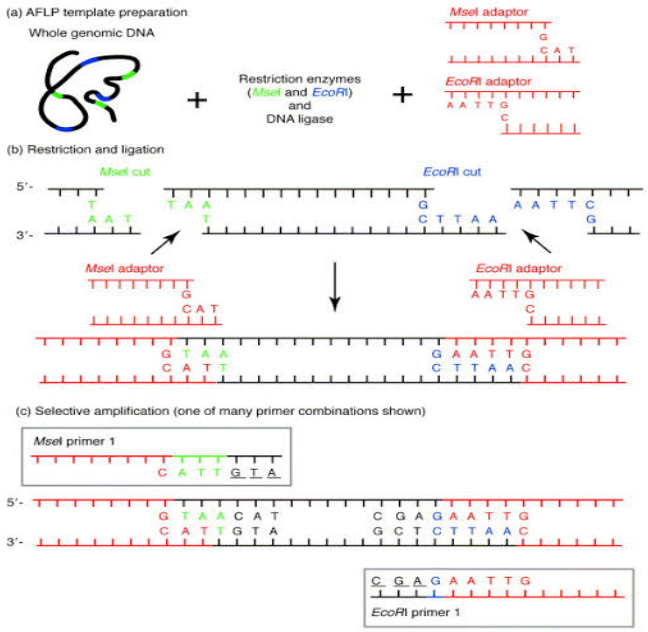


Figure 7.34*- AFPL method*

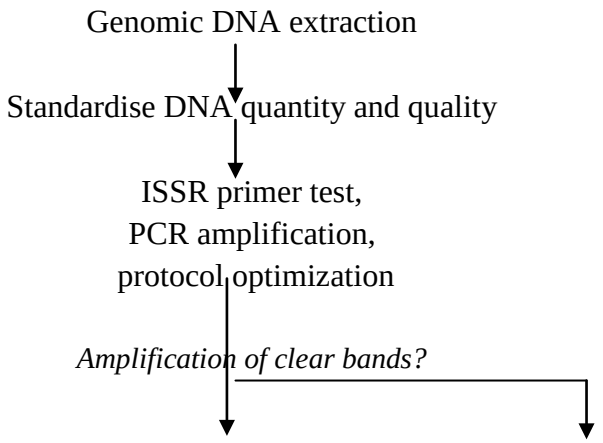
Source: Mueller and Wolfenbarger (1999)

Here genomic DNA is commonly used as the template for ISSR-PCR (Figure 7.35 A and B). An ISSR primer is usually 16–25 base pairs (bp) in length, and comprises mainly, or solely, of repeated DNA motifs (2–4 bp each) meant to be complementary to microsatellite regions in the genome. ISSR-PCR involves only one primer in each reaction, e.g. single-primer PCR amplification. However, this single primer actually acts as both the 'forward' and 'reverse' primers which are essential for an amplification to take place.

Important components of an ISSR experiment:

- gDNA as template for ISSR-PCR
- ISSR primer design
- PCR amplification with ISSR primers (ISSR-PCR)
- Gel electrophoresis
- Scoring of bands
- Estimation of basic parameters

-Simple sequence repeats, also known as microsatellites.
-This term is coined by Litt and Lutty (1989).
-Ideal DNA markers for genetic mapping and population studies because of their abundance.



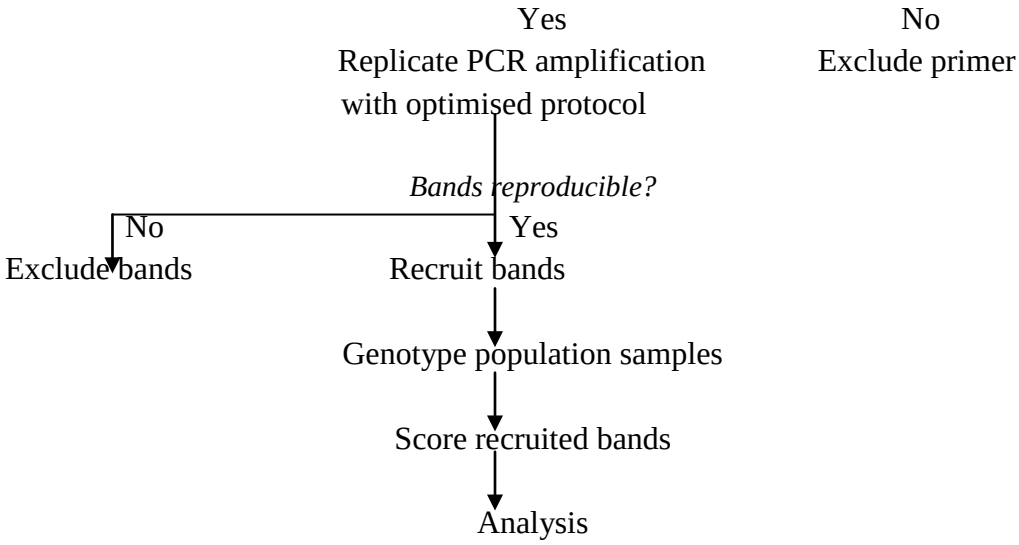
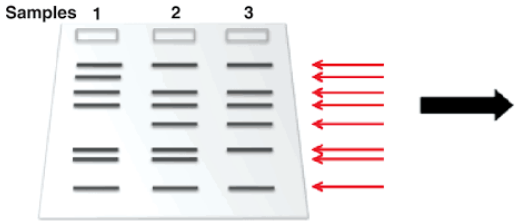


Figure 7.35A*. Recommended process flow chart for ISSR genotyping experiments (Ng and Tan, 2015)*



Locus	Sample 1	Sample 2	Sample 3
L1	1	1	1
L2	1	0	0
L3	1	1	1
L4	1	1	1
L5	0	1	1
L6	1	1	1
L7	1	1	0
L8	1	1	1

Figure 7.35B*- Scoring ISSR bands (Ng and Tan, 2015)*

Applications:

- They are easy to use.
- They are low-cost markers and
- They are methodologically less demanding compared to other dominant markers,
- ISSR is an ideal genetic marker for beginners and for organisms whose genetic information is lacking.

PCR based approach of microarrays

Microarrays have been widely used as single-nucleotide-polymorphism (SNP) genotyping platforms. Several alternative approaches have been used to detect **SNP's** but the most commonly used are allele discrimination **by hybridization**. Allelic discrimination by hybridization suffers background due to non-specific hybridization in complex genomes. In order to reduce this background, Affymetrix developed a **PCR based approach** to reduce genomic complexity.

In brief, SNPs for their assay are selected to be between restriction sites that are <1kb apart. Genomic DNA is fragmented with a restriction enzyme, end repaired and adapters for PCR are

ligated to the fragments. PCR is performed under conditions that selectively amplify products of <1kb in size. This method reduces genomic complexity by approximately 50-fold and results in a corresponding increase in signal to noise on the array (Matsuzaki et al., 2004). Today SNP arrays capable of detecting >1M different human SNPs are available.

Microarrays can be used for expression analysis, polymorphism detection, DNA resequencing, and genotyping on a genomic scale.

Table 7.5–Comparison of different dominant DNA markers for genetic variation studies (Ng and Tan, 2015)

Marker	Simplified procedure	Amount of genomic DNA required as starting material	Reproducibility of results	Source of variation	Overall cost
Amplified Fragment Length Polymorphism (AFLP)	gDNA is fragmented using REs, ligated with adapter sequences before PCR amplification, then subjected to gel electrophoresis	Moderate (>100 ng); involves PCR amplification	High	1. Mutation at restriction sites 2. Indels within restricted DNA fragments	Moderate
Random Amplified Polymorphic DNA (RAPD)	Fragments of DNA are randomly PCR-amplified using short random primers, before gel electrophoresis	Low (<50 ng); involves PCR amplification	Low	1. Mutation at PCR priming sites 2. Indels within amplified DNA fragments	Low
Long primer-RAPD	Fragments of DNA are randomly PCR-amplified using long random primers, before gel electrophoresis	Low (<50 ng); involves PCR amplification	High	1. Mutation at PCR priming sites 2. Indels within amplified DNA fragments	Low
Inter-Simple Sequence Repeat (ISSR)	Fragments of DNA are randomly PCR-amplified using primers containing microsatellite sequences, before gel electrophoresis	Low (<50 ng); involves PCR amplification	High	1. Mutation at PCR priming sites, including changes in the repeat number of the SSR motif 2. Indels within amplified DNA fragments	Low

Note: Comparisons made are relative among the listed DNA markers, given good DNA sample preparation and proper handling.

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7.8 SUMMARY

In this unit we have discussed about the genetic engineering or recombinant DNA technology, tools used in genetic engineering including different types of enzymes and vectors which are necessary elements for genetic transformation. This unit includes different methods of transfer of genes like direct and indirect methods. This section is followed by discussion about protoplast, isolation and fusion of protoplasts for production of somatic hybrids and somatic hybridization. After that, unit covers a very important topic i.e., transgenics. It includes introduction of transgenics and their benefits with examples of transgenics developed so far. Last section of this unit covers development and use of molecular markers in plant breeding. The whole unit is summarized in the following key points:

- The basis of recombinant DNA technology is the ability to manipulate DNA molecules in the test tube
- Enzymes and vectors are main tools of genetic engineering.
- DNA polymerases, are enzymes that synthesize new polynucleotides
- Nucleases are those enzymes which cleave or cut genetic material (DNA or RNA) by breaking the phosphodiester bonds
- Ligases are those enzymes which join DNA molecules together by synthesizing phosphodiester bonds between nucleotides

- Different vectors like plasmids, lambda phage, cosmids, phasmids, BACs, YACs etc are used as cloning vectors.
- There are various methods of gene transfer for development of transgenics like physical (electroporation, micro injection, particle bombardment, silicon carbide whiskers), chemical (PEG mediated, calcium phosphate co-precipitation, DEAE dextran procedure, lipofection) and *Agrobacterium* mediated gene transfer,
- Somatic hybridization is a technique which allows the manipulation of cellular genomes by protoplast fusion.
- Transgenics (plants) are the ones, whose DNA is modified by means of recombinant DNA technology.
- Transgenic plants have been developed to improve the nutritional value (Goldenrice), to provide resistance against insects (Bt cotton), herbicides, pests, etc. to increase self life (Flavr Savr) etc.
- A wide range of molecular markers are available that detect polymorphism at the DNA level like RAPD, RFLP, AFLP etc.

7.9 GLOSSARY

Cell: Structural and functional unit of life.

Animal cell: An organised unit of protoplasm usually enclosing a central nucleus and surrounded by a plasma membrane.

Cell: Structural and functional unit of life.

Cloning: Making many identical copies of a cell or DNA molecule or an organism.

Cosmid: A plasmid having cos sites.

***E*.coli**: Rod like bacterium normally found in the colon of humuna and other mammals and widely used in biomedical research.

Fosmid: vector having the F plasmid origin of replication and a ʎ cos site.

Fusogens: Fusion inducing agents are called fusogens.

Gene: Region of DNA, that controls a discrete hereditary character of an organism.

Hybrid: The progeny (F_1, heterozygote) of a cross between two individuals different in one or more heritable characters (genes).

Hybridization: The mating or crossing of two plants or lines of dissimilar genotypes.

Insertional inactivation- Inactivation of any gene by inserting new DNA somewhere in-between that gene.

Ligases: Ligases are those enzymes which join DNA molecules together by synthesizing phosphodiester bonds between nucleotides.

Molecular markers: A DNA sequence that is readily detected and whose inheritance can easily be monitored.

Nucleases: Nucleases are those enzymes which cleave or cut genetic material (DNA or RNA) by breaking the phosphodiester bonds that link one nucleotide to the next.

Phasmids: Those vectors which have combined properties of phage and plasmid.

Plant cell: An organised unit of protoplasm usually enclosing a central nucleus and surrounded by a plasma membrane outside of which it possess a rigid cell wall.

Primer: A short oligonucleotide that is attached to a single-stranded DNA molecule in order to provide a start point for strand synthesis.

Protoplast: Naked plant cell having all the components of plant cell excluding the cell wall or The entire content of the cell bounded by the plasma membrane, except the cellulosic cell wall.

Sexual hybridization: Here male gametes or microspores contribute only haploid nuclear genome and no cytoplasm while the female gamete contributes both cytoplasm and haploid nuclear genome.

Shuttle vector- a vector (usually a plasmid) that can propagate in two different host species.

Somatic hybrid: A hybrid produced by fusion of somatic cells of two varieties or species is called somatic hybrids**.**

Somatic hybridization: The process of producing somatic hybrids /The technique of hybrid production through the fusion of isolated somatic protoplasts under *in vitro* conditions and subsequent development of their product into a hybrid plant

Totipotency: The potential of plant cell or tissue to develop into an entire plant if suitably stimulated.

Transgenics: Transgenics (plants) are the ones, whose DNA is modified by means of recombinant DNA technology.

Vector: An agent that can carry a DNA fragment into a host cell.

7.10- SELF ASSESSMENT QUESTIONS

Short answer type questions

Q. What is Genetic engineering?

Ans. Genetic engineering or recombinant DNA technology is a process in which the alteration of the genetic makeup of cells is done by deliberate and artificial means.

Q.What is the role of Primer?

Ans.For initiation of DNA synthesis there must be a short, double- stranded region to provide a 3’ end onto which the enzyme will add new nucleotide. This short strand is called as primer. So we can say that a DNA polymerase requires a primer to initiate the synthesis of a new polynucleotide.

Q. Name the enzymes necessary for DNA manipulation.

Ans. DNA polymerase, Restriction endonuclease, Ligase, Alkaline phosphatase, etc.

Q. What is Homopolymer tailing?

Ans. Homopolymer tailing is a method of adding similar nucleotides to the 3 prime end of the DNA strand (blunt end strand) with the help of terminal **d**eoxynucleotidyl transferase.

Q. Why an optimum reaction temperature is required for proper functioning of *E.coli* DNA polymerase I?

Ans. *E.coli* DNA polymerase I enzyme has an optimum reaction temperature of 37°C, a usual temperature of the natural environment of the bacterium, inside the intestine of mammals such as

humans. Therefore , for its proper functioning , the test tube reactions are incubated at 37 °C and terminated by raising the temperature to 75 °C or above, destroying its enzymatic activity.

Q.What are palindromic sequences?

Ans. The nucleic acid sequence in a double strand wherein reading in a certain direction on one strand matches the sequence reading in the same direction on the complementary strand.

Example- 5'-GAATTC-3'
3'-CTTAAG-5'

Q.What is *Taq* DNA polymerase?

Ans. Heat-stable DNA polymerase isolated from a thermostable microbe (*Thermus aquaticus*).

Q. What is vector?

Ans. Vector is an agent that can carry a DNA fragment into a host cell.

Q. Differentiate between cloning vector and expression vector.

Ans. If vector is used for reproducing the DNA fragment, it is called a **cloning vector**. If used for expressing certain gene in the DNA fragment, it is called an **expression vector**.

Q. Mention the features required to facilitate cloning into a vector.

Ans. Features required to facilitate cloning into a vector are origin of replication, selectable marker and cloning sites.

Q. What is plasmid?

Ans. Plasmids are naturally occurring, self replicating extra chromosomal material present in a bacteria and in the nuclei of some eukaryotic cells.

Q. Explain natural plasmids**.**

Ans. Natural plasmids are those plasmids which occur naturally in bacteria and are not constructed *in vitro* for the sole purpose of cloning.

Q. Name few natural plasmids.

Ans. pSC, Col E1 and RSF 2124.

Q. What is the full form of pBR322?

Ans. In plasmid pBR322 p stands for plasmid, B for Bolivar, R for Rodriguez and 322 is the number used by them to designate the plasmid (strain number).

Q. What is the full form of pUC?

Ans. p means plasmid, U means University and C means California. It was named so because it was produced at the University of California.

Q. What is lac selection/Blue white screening?

Ans. Lac selection/Blue white screening is a method for selection of recombinants**.** New recombinant can be identifies by using insertional inactivation. Identifying recombinant is important because manipulation (process of formation of recombinants) results a variety of ligation products, including plasmids that have recircularized without insertion of new DNA.

Q.Why different types of cloning vectors other than plasmids are required?

Ans. The reason for developing cloning vectors based on *E.coli* bacteriophage genome and others was the inability of plasmid vectors to handle DNA fragments greater than about 10 kb in size. When insert size was large, either it interfere with the plasmid replication system or rearranged

itself in such a way that the recombinant DNA molecule become lost from the host cell. In addition to this, for preparing a genomic library for a eukaryote, the cloned fragment should be large enough to contain a whole gene

Q. What is Lambda (λ) phage?

Ans. . Lambda (λ) phage is a viruses that can infect bacteria.

Q. Name the plasmid of eukaryotic cell, Yeast.

Ans. Yeast has a natural plasmid, called the 2µm circle.

Q.Why wild type λ DNA not itself suitable as a vector?

Ans. Wild type λ DNA contains several target sites for most of the restriction endonucleases, so not itself suitable as a vector.

Q. What is Phage M13?

Ans. Phage M13 is a filamentous phage of *E.coli* with a single stranded circular DNA genome. The genomes are enclosed in a protein coat forming a long filamentous form.

Q. Name the components responsible for vector's stable replication like a chromosome.

Ans. Origin of replication, the centromere and the telomere.

Q.What do you understand by shuttle vector?

Ans. A vector (usually a plasmid) that can propagate in two different host species is known as shuttle vector. Therefore, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types

Q.What is the insert size of different vectors?

Ans. Insert size for plasmids is ~10kb, for ʎ phage it is 5-25 kb, for ʎ cosmids it is 35–45 kb, for P1 phage it is 70–100 kb, for PACs it is 100–300 kb, for BACs it is ≤300 kb and for YACs it is 200–2000 kb.

Q. What are direct methods of gene transfer or DNA uptake?

Ans. Physical gene transfer method and chemical gene transfer method

Q. Name the physical gene transfer methods.

Ans. Electroporation, micro injection, biolistics/ particle bombardment/microprojectile, and silicon carbide whiskers.

Q. What is microprojectile bombardment?

Ans. Shooting foreign DNA into plant cells or tissue at a very high speed. This technique is also known as particle bombardment, particle gun method, biolistic process, microprojectile bombardment or particle acceleration.

Q. Name the chemical gene transfer methods.

Ans. PEG (Polyethylene glycol) mediated gene transfer/ DNA uptake, calcium phosphate co-precipitation method, DEAE dextran procedure and liposome-mediated transformation.

Q.What are fusogens?

Ans: Fusion inducing agents are called fusogens.

Q. What is the other name for indirect methods of gene transfer or DNA uptake?

Ans. Vector mediated gene transfer.

Q. Why wild-type Ti-plasmids are not suitable as general gene vectors?

Ans. Wild-type Ti-plasmids are not suitable as general gene vectors because the T-DNA contains oncogenes that cause disorganized growth of the recipient plant cells. To be able to regenerate plants efficiently we must use vectors in which the T-DNA has been disarmed by making it non-oncogenic.

Q. Define protoplast.

Ans. Protoplast can be defined as a plant cell without cell wall

Q. Why osmoticum is required in enzymatic isolation of protoplast?

Ans. Osmoticum prevents the plasma membrane from rupturing. During enzymatic isolation process an osmoticum is required which prevents the protoplasts from bursting as the mechanical barrier of cell wall for support is absent.

Q. What do you mean by somatic hybridization?

Ans. A hybrid produced by fusion of somatic cells of two varieties or species is called somatic hybrids and the process of producing somatic hybrids is known as somatic hybridization

Q. What is cybridization?

Ans. When the nucleus of only one parent and cytoplasm of both the parents are fused in the hybrid cell, then instead of hybrid, it is known as cybrid and process is known as cybridization.

Q. What are transgenics?

Ans. Transgenics (plants) are the ones, whose DNA is modified by means of recombinant DNA technology.

Q.Why need of Transgenics (genetically modified crops)?

Ans. Food supply in the earth according to demand is still not enough to feed the growing human population, even after the Green Revolution which succeeded in tripling the food supply. Increased yields have been due to improved crop varieties, use of agrochemicals (fertilizers and pesticides). For most of the farmers agrochemicals are too expensive and conventional breeding is not helping in increasing the yield as per requirements. So there must be an alternative path for the farmers to obtain maximum yield from their field and to minimize the use of agrochemicals so their harmful effects on the environment are reduced.

Q. Why golden rice is important?

Ans. The transgenic rice (Golden rice) exhibits an increased production of β-carotene as a precursor to vitamin A and the seed is yellow in colour. Such yellow or golden rice may be a useful tool to treat the problem of vitamin A deficiency in young children.

Q. Name the source of genes inserted in golden rice.

Ans. The genes that give golden rice it's ability to make β-carotene in its endosperm (the interior of the kernel) come from daffodils and a bacterium called *Erwinia uredovora*.

Q. Why Bt gene is important?

Ans. When insect larvae ingest *B. thuringiensis* bacteria with their food, protein present in bacteria kills larvae and this toxic protein is encoded by the Bt gene.

Q. Why does this toxin not kill the *Bacillus*?

Ans. Bt toxin protein exist as **inactive protoxins** and it become active only after reaching in alkaline pH of the gut of insect in the presence of specific proteases so *Bacillus* remain protected.

Q. What is FLAVR SAVR

Ans. It is transgenic tomato. The tomato fruit enzyme polygalacturonase (PG) is responsible for fruit softening as it is able to dissolve cell-wall pectin. The transgenic tomato due to a deactivated gene was no longer able to produce polygalacturonase so remain fresh for long time (increased self life).

Q. What is RNAi?

Ans.A method which involve silencing of a specific mRNA due to a complementary dsRNA molecule that bind to and prevents translation of the mRNA (silencing). The source of this complementary RNA could be from an infection by viruses having an RNA genome or mobile genetic elements (transposons) that replicate via an RNA intermediate.

Q. How do edible vaccines work?

Ans. Edible vaccines contain DNA fragments from the original pathogen. These fragments code for a protein that is usually a surface protein of the pathogen. This is responsible for eliciting the body's immune response.

Q. Why molecular markers are important?

Ans. Because they are able to detect polymorphism at the level of small DNA fragments.

Q. RFLP stand for what**?**

Ans. Restriction fragment length polymorphism.

Q. What is RAPD?

Ans. Random amplified polymorphic DNA **(**RAPD) is a molecular marker based on PCR amplification.

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7.12- SUGGESTED READINGS

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7.13-TERMINAL QUESTIONS

Q. Name the enzymes used in genetic engineering and discuss about their role in cloning.

Q. What do you mean by cloning vectors? How they are different from expression vectors?

Q. Write an essay about cloning vectors.

Q. What are the different methods of gene transfer for plants? Explain them with their advantages and disadvantages.

Q. Write in detail about protoplast and somatic hybridization.

Q. What are GMOs? Write about golden rice, Bt cotton, and genetically engineered insulin.

Q. Describe methods of isolation of protoplast.

Q. Explain Somatic hybridization technique.

Q. Discuss electrofusion.

Q. Write an essay on transgenisc.

Q. Describe various types of molecular markers.

BLOCK-3

BIOSAFETY AND IPR

UNIT-8: BIO- AND FOOD-SAFETY, INTELLECTUAL PROPERTY RIGHTS AND ETHICAL ISSUES

Contents:

8.1-OBJECTIVES

After studying this unit, you will be able to—

- Define Bio- and Food-safety.
- Understand the process of patents and their use.
- Understand and define various terms like Trade Secrets, Copyright, Trademarks etc.
- Know the ethical issues involved.
- List and understand various Plant Genetic Resources.
- Get to know Intellectual Property Rights and understand trade agreements related to them.
- Comprehend the Bio-safety and containment practices and Food-safety of Genetically Modified (GM) crops.

8.2-INTRODUCTION

Food is the primary need of every organism and human beings are no different. Beside other necessities, it is paramount. Next comes the Environment in which we all live. With the help of recombinant DNA technology, scientists have combined DNA sequences from different sources to create functional DNA molecules with novel properties. These molecules are introduced in various organisms which may be micro-organisms, plants or certain animals and such organisms are known as Genetically Modified Organisms (GMOs). The never-ending demand of food for an ever-increasing population and various factors deteriorating our efforts to grow more grains have led to various scientific discoveries and inventions which have further led us to a world engaged in developing high yield Genetically Modified crops commonly known as GM crops. The development in this regard is a result of our endeavour to grow more with lesser available resources and get rid of low-quality grains, vegetables and animal products which are partially eaten by maggots and pests.

Biotechnology is safe when practiced properly. You might have heard of the Genetically Modified vegetable crops such as *Bt* Cotton, *Bt* Brinjal etc. *Bt*Brinjal was developed by Maharashtra Hybrid Seeds Company (Mahyco), by inserting a gene from the soil Bacterium *Bacillus thuringiensis* into the genome of various brinjal cultivars, in order to develop resistance against lepidopteron insects - the Brinjal Fruit and shoot borer. Brinjal is otherwise an extremely pest-prone crop and is highly susceptible to Fruit & Shoot Borer (FSB) pest. But this crop was banned in 2010 following concerns raised on biodiversity and public health. Hence, besides the various benefits of GM crops, the major concern that strikes our environmental scientists is the Bio- and Food-safety which is believed to be hampered by this technology.

In this chapter, we will learn and understand the Bio- and Food-safety concerns that affect our efforts to develop Genetically Modified Organisms (GMOs) for providing sufficient food and a healthier environment for a sustainable life on earth.

8.3 BIO- AND FOOD-SAFETY

8.3.1 Bio-Safety

In the present scenario, where the whole world is under Covid-19 pandemic, you must be aware of the safe practices to avoid infection from Corona Virus. Such practice, so as to avoid infections and prevention of pathogens, toxins etc. into our bodies is a small part of the process of Biosafety. Biosafety refers to "the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release".[1]

With the expansion in the field of genetic engineering and commercialization of transgenic organisms, scientists have expressed fear about the use of such genetically engineered microorganisms, transgenic plants and animals that they might disturb the environment:

i. By multiplying rapidly and replacing the native microbes.

ii. By transferring genes related to virulence or pathogenesis into native microbial populations and increasing their virulence or changing harmless microbes into pathogenic microbes.

The transgenic plants could pose biological and ecological risks:

i. By the production of toxic or allergic metabolites.

ii. By introducing unexpected new susceptibilities to pathogens.

iii. By transmission of new traits to sexually compatible weeds.

iv. By disturbing ecosystems through persistence or altered reactions to parasites, symbionts and competitors.

v. By the escape of transgenes providing resistance against antibiotics into other neighbouring plants.

vi. By providing resistance in insects against pesticides and in weeds against herbicides.

Realizing the possible hazards of cloning recombinant DNA technology, National Institutes of Health (NIH), USA established the Recombinant Advisory Committee (RAC) in 1974, which recommended various guidelines from time to time. The United Nations Environment Programme (UNEP) and World Health Organization (WHO) have also issued guidelines for safe use of GMOs.

8.3.2 Biosafety Guidelines in India

In India, Ministry of Environment, Forest and Climate Change (MoEF& CC) in 1989, has laid down rules and procedures for the manufacture, import, use and release of GMOs and also the products obtained from such organisms. This was done under the Environment Protection Act (EPA). The Recombinant DNA Advisory Committee (RDAC), Department of Biotechnology (DBT), New Delhi had laid down Recombinant DNA Safety Guidelines and Regulations in 1990, which were revised in 1994. While DBT implements the research and development utilizing GMOs and recombinant products, MoEF& CC implements the large-scale commercial use of these.

The essential features of these guidelines and regulations are:

i. Every organization associated with research and development using recombinant DNA technology has to set up an Institutional Biosafety Committee (IBC). All experiments concerned with the genetic manipulation in plants and likely to have biohazard potential are to be reported to IBC.

ii. Review Committee on Genetic Manipulation (RCGM) of DBT is the review committee which reviews all the approvals of ongoing projects on GMOs. Trial permits are issued only on the recommendation of RCGM.

iii. For the inspection of monitoring of field experiments, each state has a State Biotechnology Coordination Committee (SBCC) and a District Level Committee (DLC).

iv. There is a Genetic Engineering Appraisal Committee (GEAC) responsible for the appraisal of activities involving large scale use of hazardous microorganisms and recombinants in research and industrial production from the environmental angle. It is also responsible for appraisal of proposals relating to release of genetically engineered (GE) organisms and products into the environment including experimental field trials. *(REF: https://geacindia.gov.in/about-geac-india.aspx)*

v. The biosafety guidelines recognize three levels of risks in case of experiments with micro-organisms. These are pathogenicity of micro-organisms, local prevalence of concerned disease and epidemic causing strains in India.

vi. Experiments with micro-organisms, plants and animals are grouped into following 3 categories—

a. Exempt category for self-cloning experiments.

b. Category requiring intimation of initiation to competent authority i.e. experiments involving non-pathogenic DNA vector systems.

c. Category requiring review and approval by competent authority i.e. cloning of genes for toxins, antibiotic resistance etc.

vii. Four biosafety levels (BL-1, BL-2, BL-3 & BL-4) have been recognized, each one for one particular risk group (RG-1, RG-2, RG-3 & RG-4).

viii. Biological safety in the laboratory is achieved by standard laboratory practices and containment strategies.

ix. NIH has recommended separate containment facilities for each biosafety level.

x. Biological containment consists of use of vectors and hosts in such a way that it limits the infective ability of a vector to a specific host and the host-vector survival in the environment is controlled.

xi. Physical containment aims to limit the spread of dangerous micro-organisms. It involves good laboratory practices, use of safety equipment and laboratory design.

xii. Experiments with GM plants are carried out under a special environment that is achieved by using glass house containment.

xiii. To carry out genetic manipulation, permission from the Department of Environment is needed.

xiv. All products of recombinant DNA technology are subjected to general regulations.

xv. GMOs are to be released under appropriate containment to ensure safety.

xvi. Planned field experiments with transgenic plants can be carried out only after a stepwise evaluation inside the green house and data collected for promoter sequence, target gene sequence, regulatory mechanism, cell line used for **shuttling** and amplification.

8.3.3 Objectives of Biosafety Guidelines

Biosafety guidelines are developed to ensure protection and regulate research and development activities using recombinant DNA technology so that the adverse effects of this technique are minimized while encouraging the research activities. These aim for

i. The safe transfer of 'insert DNA' into the vector and of recombinant DNA into the host organism.

ii. Safe handling and use of living modified organisms resulting from recombinant DNA technology.

iii. Conservation and sustainable use of biological diversity for using them as a source of good genes.

iv. Reduce the risk of adverse effects of recombinant DNA on human health.

8.4 FOOD SAFETY

Access to sufficient amounts of safe and nutritious food is key to sustaining life and promoting good health. Unsafe food containing harmful bacteria, viruses, parasites or chemical substances can cause more than 200 different diseases – ranging from diarrhoea to cancers. Around the world, an estimated 600 million – almost 1 in 10 people – fall ill after eating contaminated food each year.[3]

Food safety refers to the practice of routines in the preparation, handling and storage of food so as to prevent foodborne illness and hazards. In their journey from farm to factory to fork, food products encounter many health hazards; and during this journey, the safe food handling practices are to be implemented and practiced at every stage to prevent risks and harms to the consumers of such food products.

In India, the Food Safety and Standards Authority of India (FSSAI) has been established under Food Safety and Standards, 2006, which is a consolidating statute related to food safety and regulation. FSSAI has been created for laying down scientific standards for articles of food and to regulate their manufacture, storage, distribution, sale and import to ensure availability of safe and wholesome food for human consumption.[2]

8.4.1 Food Safety Guidelines

The World Health Organization has published five keys to safer food, which are as under

i. **Keep clean—** Washing hands before preparation and during handling of food items is a must. Sanitizing and protecting the kitchen surface from insects and pests is another important measure because dangerous microorganisms are widely found in soil, water, animals and people. These microorganisms are carried on hands, wiping cloths and utensils, especially cutting boards, and the slightest contact can transfer them to food and cause foodborne diseases.

ii. **Separate raw & cooked—**Raw meat, poultry and seafood must be kept separate from other foods. Separate utensils, knives etc. should be used for handling such raw foods and separate containers must be used to separate them from other foods while in storage. Raw meat, poultry and seafood and their juices may contain harmful micro-organisms which may be transferred to other foods while in storage and transportation.

iii. **Cook thoroughly—**foods—especially meat, poultry, eggs and seafood must be cooked thoroughly as proper cooking kills almost all dangerous micro-organisms. Foods like soups etc. should be boiled at 70^{o}C at least for one minute.

iv. **Keep food at safe temperatures—**cooked food must not be left at room temperature for more than 2 hours as micro-organisms can multiply very quickly if food is so stored. The cooked food should be refrigerated, preferably below 5^{o}C and should not be stored for long even in the refrigerator. The food should be kept piping hot (more than 60^{o}C) before serving.

v. **Use safe water and raw materials—**Raw materials, including water and ice may be contaminated with dangerous micro-organisms and chemicals, hence safe water must be used for washing raw material and cooking food. If safe water is not available, it must be made safe by chlorination, physical filtration. Raw materials can be made safe by washing, peeling etc.

8.4.2 Food Safety Regulations in India

As mentioned earlier, Food Safety and Standards Authority of India (FSSAI) has been established under the Food Safety and Standards Act, 2006 which consolidates various Acts and Orders that have hitherto handled food related issues in various Ministries and Departments.[2]

The primary tasks that FSSAI performs[2] can be summarized as under—

i. Framing of Regulations to lay down the Standards and guidelines in relation to articles of food and specifying appropriate system of enforcing various standards thus notified.
ii. Laying down mechanisms and guidelines for accreditation of certification bodies engaged in certification of food safety management system for food businesses.
iii. Laying down procedure and guidelines for accreditation of laboratories and notification of the accredited laboratories.
iv. To provide scientific advice and technical support to Central Government and State Governments in the matters of framing the policy and rules in areas which have a direct or indirect bearing of food safety and nutrition.
v. Collect and collate data regarding food consumption, incidence and prevalence of biological risk, contaminants in food, residues of various contaminants in foods products, identification of emerging risks and introduction of rapid alert systems.
vi. Creating an information network across the country so that the public, consumers, Panchayats etc receive rapid, reliable and objective information about food safety and issues of concern.
vii. Provide training programmes for persons who are involved or intend to get involved in food businesses.

viii. Contribute to the development of international technical standards for food, sanitary and phyto-sanitary standards.

ix. Promote general awareness about food safety and food standards.

8.4.3 Food Safety Guidelines for Food Business Operators by FSSAI—

While at home, we can maintain hygiene to prevent food contamination. But for the foods that we eat out or the food material that comes to us cooked and packaged i.e. the foods that are supplied by Food Business Operators (FBOs), FSSAI has set up guidelines[5] which can be summed up as under:

8.4.3.1 Location and Surroundings

- Food Establishment shall ideally be located away from environmental pollution and industrial activities, to avoid contamination.

8.4.3.2 Layout and Design of Food Establishment Premises

- Layout of the food establishment shall be such that food preparation / manufacturing processes are not amenable to cross-contamination from other pre and post manufacturing operations like goods receiving, pre-processing (viz. packaging, washing / portioning of ready-to-eat food etc).
- Surfaces of floors, ceiling, walls, doors etc. must be smooth and non-absorbent so as to prevent growth of undesirable moulds.
- Windows, doors etc. should have proper screening to avoid infestation of rodents and insects. Floors should have proper drainage.
- Drains must be designed so as to prevent entry of rodents from them.

8.4.3.3 Equipment and Containers

- Equipment and containers coming in direct contact with food must be non-corrosive which do not impart toxicity to the food.
- Utensils and containers must be such as to prevent any toxic gases, odours, dust, dirt, flies and insects.
- Appropriate facilities for cleaning and disinfection of utensils and equipment must be arranged.
- Containers and equipment used to hold waste material, cleaning chemicals and other dangerous substances shall be identified and stored separately to prevent malicious or accidental contamination of food.
- The fittings / equipment coming in contact with food must be kept in good condition. Chipped enamelled containers should not be used.

8.4.3.4 Facilities

a. Water Supply- Only potable water, with appropriate facilities of its storage and distribution, should be used as an ingredient in processing and cooking. Water storage tanks should be cleaned periodically and non-potable water pipes should be clearly distinguished from those used for potable water.

b. **Cleaning Utensils / Equipments-** adequate facilities like hot and cold water supply, for cleaning and disinfecting utensils and equipments should be in place.

c. Washing of raw materials-every sink for washing raw materials must have hot / cold water supply and it must not be used for washing utensils and equipments.

d. **Ice and Steam-** Ice and steam used in direct contact with food shall be prepared from potable water in a manner so as to prevent contamination.

e. Drainage and Waste Disposal- food waste and other wastes must be removed from the place where food is being handled or cooked. The disposal of sewage and effluents shall be in conformity with requirements of Factory / Environment Pollution Control Board. Drainage system should be such as to eliminate the contamination of food and water supply.

f. Personnel Facilities and Toilets- Separate toilets for males and females, separate washing areas with hot / cold water supply should be in place. Number of toilets must be adequate depending upon the number of employees. Display boards mentioning Do's and Dont's must be put up inside the premises of the food processing facility.

g. Air Quality and Ventilation- Ventilation systems should be installed in a way so that air does not flow from contaminated areas to clean areas.

h. Lighting- In order to maintain hygiene there should be proper lighting and it should be ensured that food is not contaminated by breakage of electrical fittings.

i. Food Operations and Controls—

(i) Procurement of raw materials- No raw material or ingredient thereof shall be accepted by an establishment if it is known to contain parasites, undesirable microorganisms, pesticides, veterinary drugs or toxic items, decomposed or extraneous substances. Raw materials must be purchased in quantities that correspond to storage / preservation capacity. All raw materials must be checked and cleaned physically and thoroughly. Receiving temperature of potentially high risk food should be 5ºC or below and that of frozen food should be -18ºC or below.

(ii) Storage of Raw Material and Food- Storage facilities should be designed so as to protect raw materials and food from contamination, allow maintenance and cleaning and avoid pest access and accumulation. Cold storage facilities should be provided wherever needed and raw food especially meat, poultry and seafood shall be cold stored separately.

Storage of raw materials, ingredients, work-in-progress and processed / cooked or packaged food products shall be subject to FIFO (First in, First Out), FEFO (First Expire First Out) stock rotation system as applicable.

Containers made of non-toxic material should be used for storage of raw materials, processed food etc.

(iii) Food Processing / Preparation, Packaging and Distribution / Service- The Food Business shall develop and maintain the systems such as time and temperature

of receiving, processing, cooking, cooling, storage, packaging, distribution and food service upto the consumer, to ensure the safety and suitability of food.

Whenever frozen food / raw materials are being used / handled / transported, proper care should be taken so that defrosted / thawed materials are not stored back after opening for future use.

If thawing is required then only the required portion of food should be thawed at a time.

(iv) Food Packaging- Packaging materials shall provide protection for all food products to prevent contamination, damage and shall accommodate required labelling as laid down under the FSS Act & the Regulations there under. Where food comes in direct contact with the packaging material, only food grade packaging material should be used where food comes in direct contact with the packaging material.

(v) Food Distribution / Service- Processed / packaged and ready-to-eat food shall be protected as per the required storage conditions during transportation and service. Temperatures and humidity which are necessary for sustaining food safety and quality shall be maintained. The conveyances and containers shall be designed, constructed and maintained in such manner that they can effectively maintain the requisite temperature, humidity, atmosphere and other conditions necessary to protect food. Containers used for transporting / serving foodstuffs shall be non-toxic, kept clean and maintained in good condition in order to protect foodstuffs from any contamination.

Where the same conveyance or container is used for transportation of different foods, or high-risk foods such as fish, meat, poultry, eggs etc., effective cleaning and disinfections shall be carried out between loads to avoid the risk of cross-contamination.

j. Management and Supervision— A detailed Standard Operating Procedure (SOP) for the processing of food as well as its packing, despatch and storage will be developed for proper management which in turn would help in identifying any problem and the exact point, so that damage control would be faster.

The Food Business shall ensure that technical managers and supervisors have appropriate qualifications, knowledge and skills on food hygiene principles and practices to be able to ensure food safety and quality of its products, judge food hazards, take appropriate preventive and corrective action, and to ensure effective monitoring and supervision.

k. Food Testing Facilities— A well-equipped laboratory for testing of food materials / food for physical, microbiological and chemical analysis in accordance with the specification/standards laid down under the rules and regulations shall be in place inside the premises for regular / periodic testing and whenever required.

In case of any suspicion or possible contamination, food materials / food shall be tested before dispatch from the factory. If there is no in-house laboratory facility, then regular testing shall be done through an accredited lab notified by FSSAI.

In case of complaints received and if so required, the company shall voluntarily do the testing either in the in-house laboratory or an accredited lab or lab notified by FSSAI.

l. Audit, Documentation and Records— Appropriate records of food processing / preparation, production / cooking, storage, distribution, service, food quality, laboratory test results, cleaning and sanitation, pest control and product recall shall be kept and retained for a period of one year or the shelf-life of the product, whichever is more. A periodic Audit of the whole system shall be done.

m. Sanitation and Maintenance of Establishment Premises—

(i) Cleaning & Maintenance - A cleaning and sanitation programme shall be in place and the record thereof shall be properly maintained, which shall indicate specific areas to be cleaned, cleaning frequency and cleaning procedure to be followed, including equipment and materials to be used for cleaning. Equipment used in manufacturing will be cleaned and sterilized at set frequencies.

Cleaning chemicals shall be handled and used carefully in accordance with the instructions of the manufacturer and shall be stored separately away from food materials, in clearly identified containers, to avoid any risk of contaminating food.

(ii) Pest Control System- Food establishment, including equipment and building shall be kept in good repair to prevent pest access and to eliminate potential breeding sites. Holes, drains and other places where pests are likely to gain access shall be kept in sealed condition or fitted with mesh / grills / claddings or any other suitable means as required and animals, birds and pets shall not be allowed to enter into the food establishment areas/ premises.

Food materials shall be stored in pest-proof containers stacked above the ground and away from walls. Pest infestations shall be dealt with immediately and without adversely affecting the food safety or suitability. Treatment with permissible chemical, physical or biological agents, within the appropriate limits, shall be carried out without posing a threat to the safety or suitability of food. Records of pesticides / insecticides used along with dates and frequency shall be maintained.

n. Personal Hygiene-

(i) Health Status-Personnel known, or believed, to be suffering from, or to be a carrier of a disease or illness likely to be transmitted through food, shall not be allowed to enter into any food handling area . The Food Business shall develop a system whereby any person so affected, shall immediately report illness or symptoms of illness to the management and medical examination of a food handler shall be carried out apart from the periodic checkups, if clinically or epidemiologically indicated.

Arrangements shall be made to get the food handlers / employees of the establishment medically examined once in a year to ensure that they are free from any infectious, contagious and other communicable diseases. A record of these examinations signed by a registered medical practitioner shall be maintained for inspection purposes.

The factory staff shall be compulsorily inoculated against the enteric group of diseases as per recommended schedule of the vaccine and a record shall be kept for inspection.

In case of an epidemic, all workers are to be vaccinated irrespective of the scheduled vaccination.

(ii) Personal Cleanliness- Food handlers shall maintain a high degree of personal cleanliness. All food handlers should be provided with adequate and suitable clean protective clothing, head covering, face masks, gloves and footwear and the food business shall ensure that the food handlers at work should wear only clean clothing and gear.

Food handlers shall always wash their hands with soap and clean potable water, disinfect their hands and then dry with hand drier or clean cloth towel or disposable paper at the beginning of food handling activities immediately after handling raw food or any contaminated material, tools, equipment or work surface, where this could result in contamination of other food items or after using the toilet.

Food handlers engaged in food handling activities shall refrain from smoking, spitting, chewing, sneezing or coughing over any food whether protected or unprotected and eating in food preparation and food service areas.

The food handlers should trim their nails and hair periodically, do not encourage or practice unhygienic habits while handling food.

Persons working directly with and handling raw materials or food products shall maintain high standards of personal cleanliness at all times. In particular, they shall not smoke, spit, eat or drink in areas or rooms where raw materials and food products are handled or stored; wash their hands at least each time work is resumed and whenever contamination of their hands has occurred; e.g. after coughing / sneezing, visiting toilet, using telephone, smoking etc.; avoid certain hand habits - e.g. scratching nose, running finger through hair, rubbing eyes, ears and mouth, scratching beard, scratching parts of bodies etc.- that are potentially hazardous when associated with handling food products, and might lead to food contamination through the transfer of bacteria from the employee to product during its preparation. When unavoidable, hands should be effectively washed before resuming work after such actions.

o. Visitors- Generally visitors should be discouraged from going inside the food handling areas. The Food Business shall ensure that visitors to its food manufacturing area must wear protective clothing, footwear and adhere to the other personal hygiene provisions.

p. Product Information and Consumer Awareness— All packaged food products shall carry a label and requisite information as per provisions of Food Safety and Standards Act, 2006 and Regulations made there under so as to ensure that adequate and accessible information is available to each person in the food chain to enable them to handle, store, process, prepare and display the food products safely and correctly and that the lot or batch can be easily traced and recalled if necessary.

q. Training— The Food Business shall ensure that all food handlers are aware of their role and responsibility in protecting food from contamination or deterioration. Food handlers shall have the necessary knowledge and skills which are relevant to food processing / manufacturing, packing, storing and serving so as to ensure the food safety and food quality.

The Food Business shall ensure that all the food handlers are instructed and trained in food hygiene and food safety aspects along with personal hygiene requirements.

Periodic assessments of the effectiveness of training, awareness of safety requirements and competency level shall be made, as well as routine supervision and checks to ensure that food hygiene and food safety procedures are being carried out effectively.
Training programmes shall be routinely reviewed and updated wherever necessary.

8.4 PATENTS

Patent is a special right to the inventor that has been granted by the Government through legislation for trading new articles. It is a personal property which can be licenced or sold by the person / organisation just like any other property. The patents give the inventor the rights to inhibit others from making, using or selling his invention. Hence, a patent is the right granted by the State to an inventor to exclude others from commercially exploiting the invention for a limited period, in return for the disclosure of the invention, so that others may gain the benefit of the invention. The disclosure of the invention is thus an important consideration in any patent granting procedure.[6]
The procedure for granting patents, extent of the exclusive rights vary widely between countries according to national laws and international agreements. The TRIPS (Trade-Related Aspects of Intellectual Property Rights) Agreement under the World Trade Organization (WTO) requires Member countries to make patents available for any inventions, whether products or processes, in all fields of technology without discrimination, subject to the normal tests of novelty, inventiveness and industrial applicability. It is also required that patents be available and patent rights enjoyable without discrimination as to the place of invention and whether products are imported or locally produced (Article 27.1).[7]

There are three permissible exceptions to the basic rule of patentability:

First - the inventions contrary to *ordre public* or morality which explicitly includes inventions dangerous to the human, animal or plant life or health (Article 27.2).[7]

Second exception is that Members may exclude from patentability diagnostics, therapeutic and surgical methods for the treatment of humans or animals (Article 27.3(a)).[7]

Third is that Members may exclude plants and animals other than micro-organisms and essentially biological processes for the production of plants or animals other than non-biological processes. However, any country excluding plant varieties from patent protection must provide an effective *sui generis* (unique) system of protection (Article 27.3(b)).[7]
In India, the Indian Patent Act (1970) allows the 'process patents' but not the 'product patent', and the maximum duration of patent is for 5 years from the date of grant, and 7 years from the date of filing the patent application.[8]

A patent consists of three parts- the grant, specifications and claims.

i. Grant is a signed document and is an agreement that grants patent right to the inventor. It is filled at the patent office which is not published.

ii. Specification is a narrative in which the subject matter of invention is described as to how the invention was carried out.

iii. Claim specifically defines the scope of the invention to be protected by the patent to which the others may not practice.

The Specification and Claims sections are published as a single document which is made public at a minimum charge from the patent office.

8.5 TRADE SECRETS

Normally, any confidential business information which gives a competitive edge to an enterprise may be protected as a trade secret.[9] In other words, the private proprietary information that benefits the owners is called a trade secret.[10] Trade secrets are intellectual property (IP) rights on confidential information which may be sold or licensed as defined by the World Intellectual Property Organization (WIPO) of the United Nations.

In order to qualify as a trade secret, the information must be commercially valuable, known only to a limited group of persons and the rightful holder of the information must take reasonable steps to keep it secret, including the use of confidentiality agreements for business partners and employees.[9] The unauthorized acquisition, use or disclosure of such secret information is considered dishonest commercial practice, hence it is regarded as a violation of the trade secret protection.

8.5.1 Types of Information protected by Trade Secrets-

Trade Secrets can be of two types-

- Technical information- such as related to manufacturing processes, pharmaceutical test data etc.[9]
- Commercial information- such as distribution methods, list of suppliers and clients etc.[9]

Financial information, recipes, formulas, source codes etc. are other examples of information that may be protected by trade secrets.[9] One of the most popular examples of trade secrets is Coca Cola that has kept the formulae of its products a secret, hence it has a competitive edge over other companies.

8.5.2 Trade Secrets in India

There is no specific legislation in India to protect trade secrets. However, at times Indian Courts have upheld trade secret protection on the basis of principles of equity, breach of confidence etc. which comes under the Indian Contracts Act, 1872.

8.6 COPYRIGHT

Copyright is a legal term used to describe the rights that creators have over their literary and artistic works such as books, music, paintings, computer programs, databases, maps, technical drawings etc.[13] Copyright protection extends only to expressions, and not to ideas, procedures, methods of operation or mathematical concepts as such.[13] The material under copyright cannot be reprinted or reproduced without written permission of the copyright holders. While patents and trade secrets provide protection of only basic knowhow, copyrights protect the expressed materials viz., materials in printed, video-recorded or taped forms.

8.6.1 Copyrights in India-

The Copyright Act, 1957 has been amended five times since then, i.e., in 1983, 1984, 1992, 1994, 1999 and 2012. The main reasons for amendments to the Copyright Act, 1957 include to bring the Act in conformity with two WIPO internet treaties concluded in 1996 namely, the WIPO Copyright Treaty ("WCT") and WIPO Performances and Phonograms Treaty ("WPPT"). [14]

Section 9 of the Copyright Act requires for establishment of an office to be called the Copyright Office for the purpose of the Act.[14]

8.7 TRADEMARKS

A trademark is an identification symbol which is used in the course of trade to enable the public to distinguish one trader's goods from the similar goods of other traders. The public makes use of the trademarks to choose from a plethora of products and then repeat their orders by using the trademarks.[15]

8.7.1 Trade-Mark Registry in India-

The Trade Marks Registry was established in India in 1940 and presently it administers the Trade Marks Act, 1999 and the rules made thereunder. It acts as a resource and information Centre and is a facilitator in matters relating to trademarks in the country.[16]

8.8 ETHICAL ISSUES

Ethics is the discipline concerned with what is good or bad, right or wrong.[17] The widespread development of Biotechnology, life sciences, genetic engineering and exploitation of genetic resources have brought the focus on the ethical angle of these techniques. The misuse of Biotechnology and related technologies may result in loss of human life, threat to human health, environmental hazards, political & financial losses etc. to institutions and Governments. To avoid and minimize such harms, the ethical dimension of life sciences needs to be taken care of. Beside others, the most talked about aspect is the ethical implication of protecting biotechnological inventions through the Intellectual Property System.

Some general principles involved in ethical concerns in context of protecting Intellectual Property are[17]-

- transparency and access to information.
- prior informed consent.
- access to fruits of technology.
- pluralism, or accommodation of different value systems.

There may arise some ethical questions, examples of which may be such as - Should research on human cloning be permitted? Should patents be granted for DNA sequences? Should genetically modified organisms (GMOs) be allowed?, and so on.

8.9 PLANT GENETIC RESOURCES

According to the National Bureau of Plant Genetic Resources (NBPGR), Plant Genetic Resources (PGR) are any living material of present and potential value for humans. PGR includes all our agricultural crops and some of their wild relatives because they possess some valuable traits. Occasionally, genes, DNA fragments and RNA are also included under the purview of Genetic Resources.

8.9.1 Conservation of Plant Genetic Resources-

According to the Food & Agricultural Organization (FAO) of the United Nations, plant diversity is being eroded due to use of modern varieties and reduction in the number of cultivars. Modern agriculture, which is intensive and focussed on increased production, has resulted in a narrow genetic base for the crops grown. While, traditional agriculture had a large number of diverse crop varieties. Plant Genetic Resources provide valuable traits needed to overcome the dangers of extinction of important plant species, being the only source of plant genetic diversity.

So, to conserve the diversity found within species of cultivated plants, experts employ a strategy that combines *ex-situ* conservation (storing diversity in genebanks) with *in-situ* on-farm conservation.

Examples-

The Thrissur centre of NBPGR in Kerala is responsible for collection and evaluation of germplasm of southern peninsular region with particular emphasis on spices and plantation crops.

8.10 PLANT VARIETAL PROTECTION AND REGISTRATION

As per World Intellectual Property Organization, Plant variety protection, also called a “plant breeder’s right,” is a form of intellectual property right granted to the breeder of a new plant variety. The International Union for the Protection of New Varieties of Plants, known as “UPOV” was established by the International Convention for the Protection of New Varieties of Plants. It was adopted in Paris in 1961. In that way, the intellectual property rights of plant breeders were recognized for their crop varieties on an international basis. In order to accelerate agricultural development, it is necessary to protect the rights of farmers and plant breeders, who conserve & improve old varieties and develop new varieties of important crop plants.

8.11 GATT and TRIPS

8.11.1 GATT

GATT stands for General Agreement on Tariffs & Trade and in a way it promotes trade liberalization. For the first time it was agreed upon in 1948 between 23 countries. It remained in effect till 1995 when its members were 128 countries. It was replaced by the World Trade Organization (WTO). It was framed by the developed countries to get rid of conflicts that arise among the countries for getting a share in world trade. However, it is considered that, for a long time, the benefits from GATT were achieved only by the developed countries.

The GATT agreement functions through the Goods Council (Council for Trade in Goods) which comprises representatives from all WTO members.[18] GATT covers international trade of goods through multilateral agreements between the member countries. The primary purpose of GATT was to promote international trade by reducing or removing trade barriers such as tariffs and practices of trade protectionism.

8.11.2 TRIPS

TRIPS (Trade-Related Aspects of Intellectual Property Rights) Agreement was negotiated during the Uruguay Multilateral Trade Negotiations (MTN) Round, which introduced the intellectual property rules into the multilateral trading system for the first time. It is administered by the WTO and considered the most comprehensive multilateral agreement on intellectual property (IP). As mentioned earlier, TRIPS agreement facilitates the availability of patents for inventions, whether products or processes, in all fields of technology without discrimination.

8.12 PATENTING AND BIOLOGICAL MATERIAL

Like other inventions, an invention in the field of biology can also be patented as per prevalent law. The processing of patents of biological materials varies across different jurisdictions (of different countries). Generally, biological inventions are patented like chemical ones. These include biological processes like processes for producing useful products like proteins, enzymes etc., products like genetically modified organisms (GMOs), composition or formulations, methods etc.

Patenting of biological material and liveforms was earlier not permitted, however, the laws were amended later on. For example- Oncomouse, which is a genetically engineered mouse, containing human cancer mouse was developed in the United States. Patent was issued in favour of this liveform and cleared the way for future patents for inventions in biology. In the wake of this patent, a superbug was allowed patent, which was developed by Dr. Anand Mohan Chakrabarty, an Indian born American scientist. Dr. Chakrabarty was allowed to treat oil spills by using the superbug that was developed by using a bacterium, *Pseudomonas* that was capable of degrading hydrocarbons (present in the oil). This superbug was developed by inserting into the bacteria multiple circular DNA molecules (known as plasmids), each with genes encoding different enzymatic functions in hydrocarbon degradation, he and his team were able to create a new variety of *Pseudomonas* that could degrade crude oil in Petri dishes.[19]

Other examples of patents in biology are [20, 21]

i. Genetically engineered *E. coli* in which human genes for insulin, growth hormone etc. were introduced.

ii. Recombinant Mouse-Human Chimeric fab Against Hepatitis B Surface Antigen.

iii. Recombinant Mouse-Human Chimeric fab Against Hepatitis B Surface Antigen.

iv. Bollworm-resistant cotton, insect resistant tobacco etc.

8.13 BIO-SAFETY AND CONTAINMENT PRACTICES

Modern biotechnology has evolved for the benefit of humankind. If handled properly, biotechnology can be the most important technological tool to eradicate all the problems related to human health, environment, food production etc. But if the protocols of biosafety are not followed and policies and procedures are not set in place, the boon of biotechnology may turn into the most destructive and harmful inventions of man. We have recently seen this in the form of Covid-19 pandemic, where it is believed that the Corona virus has spread from a laboratory in Wuhan, China.

Biosafety includes a range of measures, policies and procedures so as to minimize potential hazards that Biotechnology may pose to the Human health and environment. The **Cartagena Protocol** on biosafety to the Convention on Biological Diversity (CBD) is an international agreement which aims to ensure the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology that may have adverse effects on biodiversity, taking also into account risks to human health.[22]

The protocol primarily deals with GMOs that are to be introduced into the environment (such as seeds, trees or fish) and does not cover pharmaceuticals for humans addressed by other international agreements and organizations.[23]

8.14 FOOD SAFETY OF GM CROPS

From the discussion done so far, you are now aware that the foods that have been derived from organisms whose genetic material (DNA) has been modified artificially are Genetically Modified (GM) foods e.g. genetically engineered papaya is 'ring spot virus resistant', thus with high productivity. And, the purpose of GM crops has been to increase productivity and the major concern has been to mitigate the health-related worries involved. Despite many concerns, we already have many drugs, foods and other consumables that have been derived from genetic modification of organisms. Also, before any food product is launched into the market, it undergoes rigorous tests and animal feeding trials. You are now aware that there is a set of regulations and legislations put in place by our governments and authorities which allow only the tested and approved GM foods on our plates.

An example regarding GM foods is well known and worth a quote here.

- In 1998, a Scottish scientist Dr. Pusztai published his research findings based on his lab experiments with GM potatoes and rats. His experiments were to find out whether the protein, known as lectin-found in plants, was harmful to rats. Lectin was investigated so as to introduce pest resistance in crops.
- His findings involved three groups of rats. One group ate non-GM crops laced with lectins, another group ate the potatoes that were genetically modified to produce their own lectins and the third group ate non-GM i.e. conventional potatoes.
- Based on his experiments, Dr. Pusztai claimed that the rats eating GM potatoes had smaller livers and brains, larger spleens and had suppressed immune systems. And, the rats that ate non-GM potatoes showed no such side-effects.

- These conclusions of Dr. Pusztai were, however, declined by a group of four independent bodies including the Royal Society.
- The Royal Society, London claims that, so far there is no evidence that a crop is dangerous because it is GM and since widespread commercialization of GM products, there has been no evidence of ill effects of consumption of any approved GM crop.[24]
- Moreover, an animal feeding trial of GM tomatoes modified to produce high levels of antioxidants showed the GM tomatoes reduced the levels of cancer. This is not because the tomatoes are GM, but rather because they produce antioxidants, which are known to reduce cancer.[24]

8.15 SUMMARY

Food is the primary need for sustenance of a healthy life of all the living organisms. Today's world order is rapid development and the major obstacle in poverty and ever-increasing demand of food grain. The development of life sciences and particularly biotechnology has helped us to satiate the hunger of millions to a large extent. Today we have high yield, pest resistant and nutrient rich superfoods just because of genetic engineering. Many cross bred vegetable crops have added to our delicacies. The bio-safety and food-safety guidelines of nations are set in place which regulates the introduction of GM foods in our food chain. Intellectual Property Rights including patents, copyrights, trademarks etc. are there to lessen the trade related arguments and to mitigate the losses to the worthy. GATT (now WTO) and TRIPS have provided trade liberalization by providing equal share in trade of products. For the conservation of genetic diversity, the National Bureau of Plant Genetic Resources (NBPGRs) have been set up all over India, which are responsible for the *in-situ* and *ex-situ* conservation and preservation of genetic resources.

8.16 GLOSSARY

1. **Bt -** *Bacillus thuringiensis* (or *Bt*) is a Gram-positive, soil-dwelling bacterium, the most commonly used biological pesticide worldwide. *Bt* Brinjal is an example where gene from this bacterium has been inserted.
2. **Transgenic organisms -** organisms that have been developed or modified by the use or Recombinant DNA technology.
3. **Virulence -** the severity or harmfulness of a disease or poison.
4. **Pathogenesis -** the manner of development of a disease.
5. ***Sui generis*-** constituting a class alone:Unique, Peculiar
6. ***Ordre public*-** The term '*ordre public*', derived from French law, expresses concerns about matters threatening the social structures which tie a society together, i.e., matters that threaten the structure of civil society as such.
7. ***in-situ* conservation-**conservation of species in their natural habitat.
8. ***ex-situ* conservation-**An approach to the conservation of biodiversity that is based on keeping organisms and species alive by the deliberate removal of biological resources (seed, pollen, sperm, individual organisms) from their original habitat or natural environment, and protecting them elsewhere under controlled conditions.

8.17 SELF ASSESSMENT QUESTIONS

8.17.1 Objective Type

1. In the phrase 'GM crops', GM stands for—
   (i) Geographically Modified (ii) Geologically Modified
   (iii) Genetically Modified (iv) None of the above.
2. In GM cropssuch as *Bt*Cotton, *Bt* Brinjal etc., *Bt*stands for—
   (i) Bacterium - *Bacillus thuringiensis* (ii) Virus - *Bacillus thuringiensis*
   (iii) Fungus - *Bacillus thuringiensis* (iv) All of the above.
3. With regard to consumption of food produce from GM crops, our scientists are mainly concerned about—
   (i) Bio- and Food-safety of food (ii) Market value of food produce,
   (iii) Nutritional value of food (iv) Scientists are not at all concerned.
4. In India, which Ministry / agency had laid down rules and procedures,in 1989, for the manufacture, import, use and release of GMOs and the products obtained from such organisms—
   (i) Ministry of Environment, Forest and Climate Change (MoEF& CC)
   (ii) Recombinant DNA Advisory Committee (RDAC)
   (iii) Department of Biotechnology (DBT).
   (iv) Ministry of Law & Justice.
5. Which Government Agency has been created for laying down scientific standards for articles of food and to regulate their manufacture, storage, distribution, sale and import to ensure availability of safe and wholesome food for human consumption, in India—
   (i) Review Committee on Genetic Manipulation (RCGM)
   (ii) Genetic Engineering Appraisal Committee (GEAC)
   (iii) Recombinant DNA Advisory Committee (RDAC)
   (iv) Food Safety and Standards Authority of India (FSSAI)
6. Which international agreement requires Member countries to make patents available for any inventions, whether products or processes, in all fields of technology without discrimination, subject to the normal tests of novelty, inventiveness and industrial applicability—
   (i) General Agreement on Tariffs and Trade (GATT)
   (ii) Agreement on Trade-Related Aspects of Intellectual Property Rights
   (iii) Trade-Related Aspects of Intellectual Property Rights (TRIPS)
   (iv) All of the above.
7. A patent consists of which three parts—
   (i) Grant, specifications and claims (ii) Cost, speciality and effects
   (iii) Subject, material and awareness (iv) None of the above.
8. With regard to Trademark, choose the appropriate option–
   (i) It is an identification symbol which is used in the course of trade to enable the public to distinguish one trader's goods from the similar goods of other traders.

(ii) The public makes use of the trademarks to choose from a plethora of products and then repeat their orders by using the trademarks.
(iii) Both of the above are correct.
(iv) None of the above is correct.

9. Any confidential business information which gives a competitive edge to an enterprise is known as –
(i) Patent (ii) Trade Secret
(ii) Intellectual Property (IP) (iv) Copyright
10. By inserting a gene from the soil Bacterium *Bacillus thuringiensis* into the genome of various brinjal cultivars,*Bt*Brinjal was developed by—
(i) Ministry of Environment, Forest and Climate Change (MoEF& CC)
(ii) Maharashtra Hybrid Seeds Company (Mahyco)
(iii) National Bureau of Plant Genetic Resources (NBPGR)
(iv) Govind Ballabh Pant University of Agriculture & Technology, Pantnagar.

8.17.2 Subjective Type

1. Why do we need Genetically Modified organisms and crops?
2. What are the concerns of some scientists about the use of genetically engineered organisms?
3. Summarize the recommendations of Recombinant Advisory Committee (RAC) established by the National Institutes of Health (NIH), USA.
4. What is Bio-safety. Elaborate the objectives of Biosafety guidelines?
5. How the Bio-safety guidelines are being implemented in India?
6. What do you mean by Food-safety?
7. Discuss the Food-safety guidelines as issued by World Health Organization (WHO).
8. Describe the functions and role of Food Safety and Standards Authority of India (FSSAI).
9. What are patents. Explain different parts of patent?
10. Define – (i) Copyright (ii) Trademark (iii) Trade Secrets.
11. What ethical issues are involved in the use of Biotechnology?
12. Why Plant Genetic Resources are important. How Plant Genetic Resources are conserved through National Bureau of Plant Genetic Resources (NBPGR).
13. What is the significance of GATT and TRIPS in promoting trade among nations?
14. Describe the food-safety concerns related to use of GM crops?

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8.20 TERMINAL QUESTIONS

1. Is it possible in practice to avoid the bio-hazards and food-hazards in the present polluted environment?
2. Will more pandemics, like Covid-19, hit the human race in future?
3. How to implement the principles of Bio-ethics effectively so as to reduce the hazards to human health and environment?

8.21- ANSWERS TO OBJECTIVE-TYPE QUESTIONS

1. (iii) Genetically Modified.
2. (i) Bacterium - *Bacillus thuringiensis.*
3. (i) Bio- and Food-safety of food.
4. (i) Ministry of Environment, Forest and Climate Change (MoEF& CC).
5. (iv) Food Safety and Standards Authority of India (FSSAI).
6. (iii) Trade-Related Aspects of Intellectual Property Rights (TRIPS).
7. (i) Grant, specifications and claims.
8. (iii) Both of the above are correct.
9. (ii) Trade Secret.
10. (ii) Maharashtra Hybrid Seeds Company (Mahyco).

Abbreviations—

1. GE – Genetically Engineered.
2. GM- Genetically Modified
3. GMOs - Genetically Modified Organisms.
4. Mahyco - Maharashtra Hybrid Seeds Company.
5. FSB - Fruit & Shoot Borer pest.
6. NIH - National Institutes of Health, USA.
7. RAC - Recombinant Advisory Committee.
8. UNEP - United Nations Environment Programme.
9. WHO – World Health Organization.
10. MoEF& CC - Ministry of Environment, Forest and Climate Change.
11. RDAC - Recombinant DNA Advisory Committee.
12. EPA - Environment Protection Act.
13. IBC - Institutional Biosafety Committee.
14. RCGM - Review Committee on Genetic Manipulation.
15. DBT – Department of Biotechnology.
16. SBCC - State Biotechnology Coordination Committee.
17. DLC - District Level Committee.
18. GEAC - Genetic Engineering Appraisal Committee.
19. FSSAI - Food Safety and Standards Authority of India.
20. FBOs - Food Business Operators.
21. FIFO - First in, First Out.
22. FEFO - First Expire First Out.
23. SOP - Standard Operating Procedure.
24. TRIPS - Trade-Related Aspects of Intellectual Property Rights.
25. WTO - World Trade Organization.
26. IPR - Intellectual Property Rights.
27. WIPO - World Intellectual Property Organization.
28. WPPT - WIPO Performances and Phonograms Treaty.
29. WCT - WIPO Copyright Treaty.
30. NBPGR - National Bureau of Plant Genetic Resources.

31. PGR - Plant Genetic Resources.
32. FAO - Food & Agricultural Organization.
33. UPOV - International Union for the Protection of New Varieties of Plants.
34. GATT - General Agreement on Tariffs & Trade.
35. CBD - Convention on Biological Diversity.

UNIT-9 BIOTECHNOLOGICAL APPLICATIONS

Contents

9.1 OBJECTIVES

The introduction of the unit will discuss biotechnological applications. The unit describes the detailed applications of biotechnology in agriculture, horticulture, forestry, medicines, and industries. The unit will be beneficial for the teaching and, learning of undergraduate and postgraduate courses.

9.2 INTRODUCTION

Biotechnology refers to the combination of biological sciences and technology. The term *biotechnology* was first given by Karoly Erekyin 1919,meaning the production of products from raw materials with the aid of living organisms. The concept of biotechnology encompasses a wide range of procedures for modifying living organisms according to human purposes, going back to the domestication of animals, cultivation of plants, and "improvements" to these through breeding programs that employ artificial selection and hybridization. Modern usage also includes genetic engineering as well as cell and animal and plant tissue culture technologies. The American Chemical Society defines biotechnology as the application of biological organisms, systems, or processes by various industries to learning about the science of life and the improvement of the value of materials and organisms such as pharmaceuticals, crops, and livestock. According to the European Federation of Biotechnology (EFB), biotechnology is the integration of natural science and organisms, cells, parts thereof, and molecular analogs for products and services. Biotechnology is based on the basic biological sciences (e.g., molecular biology, biochemistry, cell biology, embryology, genetics, microbiology) and conversely provides methods to support and perform basic research in biology.

Biotechnology is the research and development in the laboratory using bioinformatics for exploration, extraction, exploitation, and production from any living organisms and any source of biomass by means of biochemical engineering where high value-added products could be planned (reproduced by biosynthesis, for example), forecasted, formulated, developed, manufactured, and marketed for the purpose of sustainable operations (for the return from bottomless initial investment on R & D) and gaining durable patents rights (for exclusives rights for sales, and prior to this to receive national and international approval from the results on animal experiment and human experiment, especially on the pharmaceutical branch of biotechnology to prevent any undetected side-effects or safety concerns by using the products).The utilization of biological processes, organisms or systems to produce products that are anticipated to improve human lives is termed biotechnology.

By contrast, bioengineering is generally thought of as a related field that more heavily emphasizes higher systems approaches (not necessarily the altering or using of biological materials *directly*) for interfacing with and utilizing living things. Bioengineering is the application of the principles of engineering and natural sciences to tissues, cells, and molecules. This can be considered as the use of knowledge from working with and manipulating biology to achieve a result that can improve functions in plants and animals. Relatedly, biomedical engineering is an overlapping field that often draws upon and applies *biotechnology* (by various definitions), especially in certain sub-fields of biomedical or

chemical engineering such as tissue engineering, biopharmaceutical engineering, and genetic engineering.

9.3 GENETIC ENGINEERING

Genetic engineering, recombinant DNA technology and biotechnology – the buzz words you may have heard often on radio or TV, or read about in featured articles in newspapers or popular magazines. It is a set of techniques that are used to achieve one or more of three goals: to reveal the complex processes of how genes are inherited and expressed, to provide better understanding and effective treatment for various diseases, (particularly genetic disorders) and to generate economic benefits which include improved plants and animals for agriculture, and efficient production of valuable biopharmaceuticals. The characteristics of genetic engineering possess both vast promise and potential threat to human kind. It is an understatement to say that genetic engineering will revolutionize the medicine and agriculture in the 21st future. As this technology unleashes its power to impact our daily life, it will also bring challenges to our ethical system and religious beliefs.

Genetic Engineering and Human Health Soon after the publication of the short essay by Crick and Watson on DNA structure (1953), research began to uncover the way by which DNA molecules can be cut and "spliced" back together. With the discovery of the first restriction endonuclease by Hamilton Smith et al. (1970), the real story of genetic engineering began to unfold. The creation of the first engineered DNA molecule through splicing DNA fragments of two unrelated species together was made public in 1972. Soon followed were a whole array of recombinant DNA molecules, genetically modified bacteria, viruses, fungi, plants and 2 animals. The debate over the issues of "tinkering with God" heated up and public outcry over genetic engineering was wide-spread. The birth of "Dolly", the first mammal ever cloned from an adult body cell, has elevated the debate over the impact of biological research to a new level. Furthermore, a number of genetically modified organisms (GMOs) have been commercially released since 1996. Today, it is estimated that over 70% of US foods contain some ingredients from GMOs. Obviously, genetic engineering holds tremendous promise for medicine and human well-being. Medical applications of genetic engineering include diagnosis for genetic and other diseases; treatment for genetic disorders; regenerative medicine using pluripotent (stem) cells; production of safer and more effective vaccines, and pharmaceuticals; the prospect of curing genetic disorders through gene therapy; the list goes on... Owing to its potential to give humanity unprecedented power over life itself, the research and application of genetic engineering has generated much debate and controversy. Many human diseases, such as cystic fibrosis, Downs syndrome, fragile X syndrome, Huntington's disease, muscular dystrophy, sickle-cell anemia, Tay-Sachs disease, etc. are inherited. There are usually no conventional treatments for these disorders because they don't respond to antibiotics or other conventional drugs. Another area is the commercial production of vaccines and pharmaceuticals through genetic engineering, which has emerged as a rapidly developing field. The potential of embryonic stem cells to become any cell/tissue/organ under adequate conditions holds enormous promise for regenerative medicine.

9.4 PREVENTION OF GENETIC DISORDERS

Although prevention may be achieved by avoiding these environmental factors that cause the abnormality, the most effective prevention, when possible, is to reduce the frequency of or eliminate entirely the harmful genes (mutations) from the general population. As more precise tools and procedures for manipulating individual genes are optimized, this will eventually become a reality. The prevention of genetic disorders at present is usually achieved by ascertaining those individuals in the population who are at risk of passing a

serious genetic disorder to their offspring, offering them genetic counselling and prenatal screening followed with the selective abortion of affected foetuses. Genetic counselling is the processes of communicating information gained through classic genetic studies and contemporary research to those individuals who are themselves at risk or have a high likelihood of passing defects to their offspring. During counselling, information about the disease itself - its severity and prognosis, whether or not there are effective therapies, and the risk of recurrence is generally presented. For those couples who find the risks unacceptably high, counselling may also include discussions ofcontraceptive methods, adoption, prenatal diagnosis, possible abortion and artificial insemination by a donor, etc. Even though the final decision must still rest with the couple themselves, the significant increase in the accuracy of risk assessment made possible with genetic technology makes it easier for parents to make well-informed decisions. To these couples who find the burden of having an affected child unbearable, prenatal diagnosis may solve their dilemma. Prenatal screening could be performed for a varietyof genetic disorders. It requires samples of foetal cells or chemicals produced by the foetus through either amniocentesis or chorionic villus sampling. After sampling, severalanalyses could be performed. First, biochemical analysis is used to determine theconcentration of chemicals in the sample and therefore diagnose whether a particular foetus is deficient or low in enzymes that facilitate specific biological reactions. Next, analysis of the chromosomes of the foetal cells can show if all the chromosomes arepresent, and whether or not there are any structural abnormalities in any of them. Finally, the most effective means is to detect the defective genes through recombinant DNA techniques. This has become possible with the rapid increase of DNA copies through atechnique called PCR, which can produce virtually unlimited copies of a specific gene orDNA fragment, starting with as little as a single copy. Routine prenatal diagnosis isbeing performed to screen foetus for Down syndrome, Huntington's disease, sickle-cell anaemia and Tay-Sachs disease. Procedures are being developed for prenatal diagnosis of more and more severe genetic disorders. Thus, an effective roadblock to the passing ofdefective genes from one generation to another in the population is possible.

9.5 TREATMENT OF DISEASES AND GENETIC DISORDERS

Genetic engineering may be used for direct treatments of diseases or genetic disorders through various means, including the production of possible vaccines for AIDS, treatment for various cancers, synthesis of biopharmaceuticals for a variety of metabolic, growth and development diseases, etc. In general, biosynthesis is a process where gene coding for a particular product is isolated, cloned into another organism (mostly bacteria), and later expressed in that organism (host). By cultivating host organism, large quantities of the gene products can be harvested and purified. A few examples will illustrate the useful features of biosynthesis. Insulin is essential for the treatment of insulin-dependent diabetes, the most severe form of diabetes. Historically, insulin was obtained from a beef or pig pancreas. Two problems exist for the traditional supply of insulin. First, large quantities of the pancreas are needed to extract enough insulin for continuous treatment of one patient. Second, insulin so obtained is not chemically identical to human insulin, hence some patients may produce antibodies which can seriously interfere with the treatment. Human insulin produced through

genetic engineering is quite effective yet without any side-effects. It has been produced commercially and made available to patients since 1982. Another successful story in biosynthesis is the production of human growth hormone (HGH), which is used in the treatment of children with growth retardation called pituitary dwarfism. The successful biosynthesis of HGH is important due to several reasons. The conventional source of HGH was human pituitary glands removed at autopsy, which only exist in brain and liver. Each child afflicted with pituitary dwarfism needs twice-a-week injections until the age of 20. Such a treatment regime requires over a thousand pituitaries. It's obvious that autopsy supply could hardly keep up with the demand. Furthermore, due to a small amount of virus contamination in the extracted HGH, many children receiving treatment developed virus related diseases. Other biopharmaceuticals under development or in pre-clinical or clinical trials through genetic engineering include anti-cancer drugs, anti-aging agents and a possible vaccine for AIDS, malaria, etc. Broadly speaking, three types of gene therapy exist, germ line therapy, enhancement gene therapy and somatic gene therapy. All gene therapy trials currently underway or in the pipeline are restricted to the somatic cells as targets for gene transfer. The germ line therapy involves the introduction of novel genes into germ cells such as egg/early.

9.6 BIOTECHNOLOGICAL APPLICATIONS IN AGRICULTURE

The agricultural biotechnology sector shares a common scientific foundation with the therapeutic biotechnology sector, including similar characteristics of a lengthy time to market for emerging products. But the challenges, goals, and opportunities for agricultural applications of biotechnology provide a very different context for innovation and entrepreneurs. Now just 30 years old, the agriculture biotechnology sector is entering its third cycle of innovation, with the first wave being agricultural biotechnology trait creation followed by a second wave of agriculture biotechnology trait commercialization. The agricultural trait segment continues to focus on two product categories, herbicide tolerance and insect resistance, in spite of few entrepreneurial opportunities available. However, agriculturebiotechnology applications to chemistries, biopesticides, microbials, and natural products offer new opportunities. These sectors offer new business opportunities that, like the first wave of agriculture biotechnology, enable entrepreneurs to think about creating disruptive businesses, not just disruptive technologies for today's business models.

Agriculture biotechnology uses the bacterial, fungal and algal cultures for the crop improvement and yield enhancement. The microbial strains present into the soil and water environment is called the indigenous cultures due to their natural presence. These indigenous microbial cultures present in soil as well as in plant parts. In soil they are known as free living microorganism, whereas in plant root regain called as rhizospheric microbes. Rhizospheric microbes are specific because they are attracting due to the plant root exudates. In this way microbes make a special type of interaction with plants is known as plant microbe interactions. The PGP microbial strains help to enhance the crops yield and agricultural productivity. Plant rhizosphere provide the mechanical support and facilitating the water and nutrient uptake.

These compounds secreted by plant roots act as chemical attractants for a vast number of heterogeneous, diverse and actively metabolizing soil microbial communities. The chemicals

which are secreted by roots into the soils are generally called as root exudates. The exudation of a wide range of chemical compounds modifies the chemical and physical properties of the soil and thus, regulates the structure of soil microbial community in the immediate vicinity of root surface. In fact, some of the exudates act as repellants against microorganisms while others act as attractants to lodge the microbes. The composition of these exudates is dependent upon the physiological status and species of plants and microorganisms. Moreover, these exudates also promote the plant-beneficial symbiotic interactions and inhibit the growth of the competing plant species. Also, microbial activity in the rhizosphere affects rooting patterns and the supply of available nutrients to plants, thereby modifying the quality and quantity of root exudates. A fraction of these plant-derived small organic molecules is further metabolized by microorganisms in the vicinity as carbon and nitrogen sources, and some microbe-oriented molecules are subsequently re-taken up by plants for growth and development. Indeed, carbon fluxes are critical determinants of rhizosphere function. It is reported that approximately 5–21% of photosynthetically fixed carbon is transported to the rhizosphere through root exudation. Thus, the rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/or in association with roots hairs, and plant-produced materials. Largely, three separates but interacting components are recognized in the rhizosphere: the rhizosphere (soil), the rhizoplane, and the root itself. Of these, the rhizosphere is the zone of soil influenced by roots through the release of substrates that affect microbial activity. The rhizoplane, on the other hand, is the root surface including the strongly adhering soil particles while the root itself is a component of the system, because many micro-organisms (like endophytes) also colonize the root tissues. Microbial colonization of the rhizoplane and/or root tissues is known as root colonization, whereas the colonization of the adjacent volume of soil under the influence of the root is known as rhizosphere colonization.

9.6.1 Biofertilizers

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants. Use of biofertilizers is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers. They can be grouped in different ways based on their nature and function.

9.6.2 N2 fixers

(a) Free living: Aerobic- *Azotobacter, Beijerinckia, Anabaena*; Anaerobic- *Clostridium* Faultative anaerobic- *Klebsiella*

(b) Symbiotic: *Rhizobium, Frankia, Anabaena azollae*

(c) Associative symbiotic: *Azospirillum*

(d) Endophytic: *Gluconacetobacter, Burkholdria*

9.6.3 Phosphate solubilizers

(a) Bacteria: *Bacillus megaterium* var. *phosphaticum; B. subtilis, B. circulans; Pseudomonas striata*

(b) **Fungi:** *Penicillium* spp. *Aspergillus awamori*

9.6.4 Phosphate Mobilizer

The Arbuscular Mycorrhizae (AM) fungi, Ectomycorrhizal fungi, ericoid mycorrhiza and orchid mycorrhizae can play an important role in phosphate mobilization.

9.6.5 Importance of biofertilizers

Biofertilizers are known to make a number of positive contributions in agriculture.

• Supplement fertilizer supplies for meeting the nutrient needs of crops.

• Add 20 – 200 kg N/ha (by fixation) under optimum conditions and solubilise/mobilise 30-50 kg P2O5/ha.

• They liberate growth promoting substances and vitamins and help to maintain soil fertility.

• They suppress the incidence of pathogens and control diseases.

• Increase the crop yield by 10-50%. N2 fixers reduce depletion of soil nutrients and provide sustainability to the farming system.

• Cheaper, pollution free and based on renewable energy sources.

• They improve soil physical properties, tilth and soil health.

9.6.6 Rhizobium

Rhizobia are soil bacteria, live freely in soil and in the root region of both leguminous and non-leguminous plants. However, they enter into symbiosis only with leguminous plants, by infesting their roots and forming nodules on them. Non legume nodulated by Rhizobia is *Trema* or *Parasponia* sp. The nodulated legumes contribute a good deal to the amount of N_2 fixed in

the biosphere, (50-200 kg N/ha) varied with crops. The nitrogen fixation by clover and cowpea is 130 kg N/ha and 62-128 kg N/ha respectively. Beijerinck first isolated and cultivate a microorganism from the roots of legumes in 1888 and he named this as *Bacillus radicola* and latter modified as Rhizobium. Legume plants fix and utilise this N by working symbiotically with Rhizobium in nodules on their roots. The host plants provide a home for bacteria and energy to fix atmospheric N2 and in turn the plant receives fixed N2 (as protein).

9.6.7 Biotechnological applications for horticulture

The requirement of fruits and vegetables is increasing proportionally with the increasing population in the country. How do we keep horticultural production on par with the burgeoning population? Although conventional plant breeding techniques have made considerable progress in the development of improved varieties, they have not been able to keep pace with the increasing demand for vegetables and fruits in the developing countries. Therefore, an immediate need is felt to integrate biotechnology to speed up the crop improvement programmes. Biotechnological tools have revolutionized the entire crop improvement programmes by providing new strains of plants, supply of planting material,

more efficient and selective pesticides and improved fertilizers. Many genetically modified fruits and vegetables are already in the market in developed countries. Modern biotechnology encompasses broad areas of biology from utilization of living organisms or substances from those organisms to make or to modify a product, to improve plant or animal or to develop micro-organisms for specific use. It is a new aspect of biological and agricultural science which provides new tools and strategies in the struggle against world's food production problem. The major areas of biotechnology which can be adopted for improvement of horticultural crops are; tissue culture, genetic engineering, molecular diagnostics, molecular markers and development

9.6.8 Biotechnological applications for Forestry

At present, most of the planting material for different afforestation programscomes from sources that are genetically diverse and that give poor yields. Therefore,in order to enhance productivity, it is essential to have a highly efficient productiontechnology including high yielding certified planting material. In this context, tissueculture and other biotechnological tools can play an important role in boosting productivity. Tissue culture technology, together with mycorrhizae, nitrogen fixing microorganisms and institutional support, etc. have been used to enhance the productivity offorest trees in the United States, Brazil, Japan and some West European countries.Success has also been obtained in the case of orchids, ornamentals and a number ofvegetable and fruit species. Species of strawberry and apple are now produced commercially by tissue culture. In India, the technique of raising plants through tissueculture has been perfected for a few tree species, e.g., Bamboo, Dalbergia, Eucalyptus, Leucaena, Prosopis, Santalum, Sesbania and elites of Tectona. In order to achieve the quantum jump in the production of biomass for fuel, fodder, timber and industrial woods using the new tools of biotechnology, the following species have been identifiedfor mass propagation through tissue culture: *Acacia nilotica, Alnus nepalensis, Hardwickia binata, Madhuca latifolia, Prosopis cineraria, Tamarindus indica, Dendrocalamus strictus, Bambusa arundinacea, Bambusa vulgaris, Tectolla grandis, Shorea robusta, Dalbergia latifolia, Santalum album, Populus deltoides.*

It is necessary that greater emphasis be given to the following three areas foroverall applications of biotechnology towards the enhancement of biomass production, (1). Improvement ill overall growth of forests. This involves improvement in regeneration techniques, ensuring better survival of young saplings, development of improved genetic stocks and reduction in the damage caused by fire, pests, etc. Plantingwith genetically improved stock alone can increase the timber yield per hectare byabout 25 percent over current rates of growth, (2). Advances ill the products manufactured from forest resources. Here, the potential is limited only by the researchers' vision. A substantial reduction in the susceptibility of wood to decay could improve the useful life of products ranging from thoseused in construction to utility poles.

(3). Understanding the impacts of non-forestry related activities on forest. More research is required to understand the effect of acid rain on thegrowth and productivity of the forests. This work will lead to solutions to mitigate theimpact of this by-product of industrial growth.Organisational integration at the level of the individual scientist, department and organisation must be taken into account if biotechnology is to have any realisticapplication in

enhancing biomass production. Optimally efficient research integrationis determined, in part, by the organisational setting involved and the technologies. employed. If research and product development capabilities arc not contained withinthe same organisation, they must be established through licensing arrangements,collaborative research or research network.The integration of new technologies can be achieved through multi-disciplinaryteams whose objectives are in agreement with those of conventional plant breeders.Collaborative research also necessitates the support of plant breeders for evaluationand testing. In the following areas, both applied as well as the basic aspects of biotechnology need to be integrated with conventional methods, in order to make an impacton forestry:

(i) Mass propagation of elite plants through tissue culture

(ii) Scaling up the production of artificial seeds

(iii) Cryopreservation of the gene pools of elite trees

(iv) Exploiting somaclonal variants for tree improvement

(v) Microspore and another culture for fixation of heterosis

(vi) Development, in some important tree species of a genetic transformation system using agrobacterium and regeneration of transformed plantlets by tissueculture. Genetic manipulation of trees through protoplast fusion or somatic hybridisation

(vii) Large-scale field evaluation of tissue culture raised plants.

9.6.9 Biotechnological applications for medicines

Biotechnology has a variety of applications in the field of medicine. Some of the biotechnology applications in medicine include the following:

9.6.9.1 Recombinant Insulin

Insulin is required by diabetic patients to remove excess sugar from the blood. Diabetic patients have a very low level of insulin or no insulin produced by the body. Therefore, they need external insulin to control blood glucose levels. Later it was discovered that the insulin produced by the pancreas of the pigs can be used by humans. But there were not enough pigs to provide the quantities of insulin required. This led to the cloning of the human insulin gene.The specific gene sequence that codes for human insulin were introduced in *E.coli* bacteria. The gene sequence altered the genetic composition of the *E.coli* cells. Within 24 hours several *E.coli* bacteria containing the recombinant human insulin gene were produced. The recombinant human insulin was isolated from *E.coli* cells.

9.6.9.2 Gene Therapy

Gene Therapy holds the most promising answer to the problem of genetic diseases. Gene therapy is used to treat genetic disorders usually by the insertion of a normal gene or correct gene for the defective or inactive gene into an individual with the help of vectors such as retrovirus, adenovirus, and herpes simplex virus.The normal gene replaces the defective or inactive gene and carries out its functions. The therapy has the highest chances of developing a permanent cure if introduced in the earliest stages of life.

9.6.9.3 Molecular Diagnosis

Medical diagnosis is another application of biotechnology in the health sector. Many times, the pathogen concentration increases by the time the disease is diagnosed. Hence, early diagnosis and knowledge of pathophysiology are essential for an effective cure. This can be

achieved with the help of techniques such as Recombinant DNA Technology, Polymerase Chain Reaction (PCR) and Enzyme-Linked Immunosorbent Assay (ELISA), etc.

9.6.9.4 Pharmacogenomics

Pharmacogenomics has led to the production of drugs that are best suited to an individual's genetic makeup. It can be applied in diseases such as cancer, depression, HIV, asthma, etc.

9.6.9.5 Edible Vaccines

Vaccines are obtained by animals and cell cultures. These vaccines contain inactivated pathogens.The transgenic plants can produce antigens that can be used as edible vaccines. Antigenic proteins from several pathogens can be expressed in plants such as tomato and banana. Transgenic sugarbeet can treat foot and mouth disease of animals, transgenic banana and tomato can cure diseases such as cholera and hepatitis B.

9.7 BIOTECHNOLOGICAL APPLICATIONS FOR INDUSTRY

Industrial biotechnology includes modern application of biotechnology for sustainable processing and production of chemical products, materials and fuels. Biotechnological processing uses enzymes and microorganisms to produce products that are useful to a broad range of industrial sectors, including chemical and pharmaceutical, human and animal nutrition, pulp and paper, textiles, energy, materials and polymers, using renewable raw materials.Use of biotechnology to substitute existing processes makes many of these industries more efficient and environmentally friendly, contributing to industrial sustainability in various ways. This paradigm change involves various areas, ranging from the most known ones, such as pharmaceutical and agricultural, to production of materials such as biopolymers and also bioplastics.Industrial biotechnology can produce the same results as the petrochemical industry, but using biological catalysts instead. Application of the state of the art of a vast range of scientific disciplines to industrial biotechnology, namely biochemistry, microbiology, genomics, proteomics, bioinformatics, systems biology and process engineering is the foundation for leveraging the rapid, specialized and competitive growth of the sector, based on biocatalysts that enable high productivity, performance and stability.With the adoption of industrial processes based on biotechnology, metabolic engineering has become an increasingly important subject. The goal of metabolic engineering is to maximize the production of compounds that are of industrial interest in microorganisms that act within this context as cell factories through their genetic manipulation.According to a recent OECD study (The Bioeconomy to 2030: designing a policy agenda, http://www.oecd.org/futures/bioeconomy/2030), the industrial applications of biotechnology in 2030 will be responsible for 39% of the economic value generated by Biotechnology, which illustrates the healthy investment in research and development expected in this area.

9.8 FERMENTATION

Fermentation is a metabolic process that converts sugar to acids, gases or alcohol. It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation. Fermentation is also used more broadly to refer to the bulk growth of microorganisms on a growth medium, often with the goal of producing a specific chemical product like enzyme, vaccines, antibiotics, food product/additive etc. French microbiologist Louis Pasteur is often remembered for

his insights into fermentation and its microbial causes. The science of fermentation is known as zymology

Fermentation takes place in the lack of oxygen (when the electron transport chain is unusable) and becomes the cell's primary means of ATP (energy) production. It turns NADH and pyruvate produced in the glycolysis step into NAD^+ and various small molecules depending on the type of fermentation. In the presence of O2, NADH and pyruvate are used to generate ATP in respiration. This is called oxidative phosphorylation, and it generates much more ATP than glycolysis alone. For that reason, cells generally benefit from avoiding fermentation when oxygen is available, the exception being obligate anaerobes which cannot tolerate oxygen.

The first step, glycolysis, is common to all fermentation pathways:

$C_6H_{12}O_6 + 2NAD+ 2ADP^+ + 2Pi \rightarrow 2\ CH_3COCOO^- + 2\ NADH + 2\ ATP + 2\ H_2O + 2H^+$

The pyruvate is CH3COCOO$^-$. Pi is inorganic phosphate. Two ADP molecules and two Pi are converted to two ATP and two water molecules via substrate-level phosphorylation. Two molecules of NAD^+ are also reduced to NADH. In oxidative phosphorylation the energy for ATP formation is derived from an electrochemical proton gradient generated across the inner mitochondrial membrane (or, in the case of bacteria, the plasma membrane) via the electron transport chain. Glycolysis has substrate-level phosphorylation (ATP generated directly at the point of reaction).

Humans have used fermentation to produce food and beverages since the Neolithic age. For example, fermentation is used for preservation in a process that produces lactic acid as found in such sour foods as pickled cucumbers, kimchi and yogurt, as well as for producing alcoholic beverages such as wine and beer. Fermentation can even occur within the stomachs of animals, such as humans.

9.8.1 Definitions of Fermentation

To many people, fermentation simply means the production of alcohol: grains and fruits are fermented to produce beer and wine. If a food soured, one might say it was 'off' or fermented. Here are some definitions of fermentation. They range from informal, general usage to more scientific definitions.

1. Preservation methods for food via microorganisms (general use).
2. Any process that produces alcoholic beverages or acidic dairy products (general use).
3. Any large-scale microbial process occurring with or without air (common definition used in industry).
4. Any energy-releasing metabolic process that takes place only under anaerobic conditions (becoming more scientific).
5. Any metabolic process that releases energy from a sugar or other organic molecules, does not require oxygen or an electron transport system, and uses an organic molecule as the final electron acceptor (most scientific).

9.8.2 Examples of Fermentation

Fermentation does not necessarily have to be carried out in an anaerobic environment. For example, even in the presence of abundant oxygen, yeast cells greatly prefer fermentation to aerobic respiration, as long as sugars are readily available for consumption (a phenomenon

known as the Crabtree effect). The antibiotic activity of hops also inhibits aerobic metabolism in yeast. Fermentation react NADH with an endogenous, organic electron acceptor. Usually this is pyruvate formed from the sugar during the glycolysis step. During fermentation, pyruvate is metabolized to various compounds through several processes:

1. Ethanol fermentation, aka alcoholic fermentation, is the production of ethanol and carbon dioxide

2. Lactic acid fermentation refers to two means of producing lactic acid:

(a) Homolactic fermentation is the production of lactic acid exclusively

(b) Heterolactic fermentation is the production of lactic acid as well as other acids and alcohols.

Sugars are the most common substrate of fermentation, and typical examples of fermentation products are ethanol, lactic acid, carbon dioxide, and hydrogen gas (H2). However, more exotic compounds can be produced by fermentation, such as butyric acid and acetone. Yeast carries out fermentation in the production of ethanol in beers, wines, and other alcoholic drinks, along with the production of large quantities of carbon dioxide. Fermentation occurs in mammalian muscle during periods of intense exercise where oxygen supply becomes limited, resulting in the creation of lactic acid.

9.9 SUMMARY

The biotechnology is an interdisciplinary branch of science that deals with biochemistry, microbiology, genetic engineering for mankind. Biotechnology offers various aspect of application in agriculture, horticulture, forestry, medical and industries. The trained biotechnologist can help the society by developing biofertilizers in agriculture, development of genetical modified plants and production of value-added products using industrial microbiology. In due to the development of the next generation high-throughput technologies new branch emerged as bioinformatics. Bioinformatics mainly deals with the genomic, transcriptomics and proteomics datasets generated from the living systems.

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9.11 TERMINAL QUESTIONS

Q1. Discuss the role of biotechnology in agriculture in detail

Q2. Write notes on following

i. Genetic Engineering

ii. Role of biotechnology in horticulture

iii. Prevention of genetic disorders

iv. Treatment of diseases and genetic disorders

Q3. What are the varieties of applications in the field of medicine?

Q4. What are the applications of biotechnology in Industry? Discuss in detail.

Q5. Define fermentation and explain its process along with the examples.

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