TWO-YEAR
POST GRADUATE DEGREE PROGRAMME (CBCS)
IN
BOTANY

SEMESTER - III

Course: BOTCOR T310

(Plant Molecular Biology and Biotechnology)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI – 741235, WEST BENGAL

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Director's Messeage

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distane Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personal involved in the process to overcome the challenges inherent to curriculam design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distane Learning (DODL) University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC-DEB Regulations, 2020 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of SLMs, making them useful to the lesrners, besides avoiding errors as far as practicable. Further, suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Amalendu Bhunia, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticisms to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every Members of PGBOS (DODL), University of Kalyani, Heartfelt thanks is also due to the Course Writers- faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learners friendly, flexible text that meets curriculum requirements of the Post Graduate Programme through distance mode.

All the Self Learning Materials are self-writing and collected from e-Book, journals and websites.

Director
Directorate of Open & Distance Learning
University of Kalyani

SYLLABUS
COURSE – BOTCOR T310
Plant Molecular Biology and Biotechnology
(Full Marks – 75)

Course	Group	Details Contents Structure		Study hour
BOTCOR T310	**Plant Molecular Biology**	**Unit 1. Recombinant DNA Technology**	1. Recombinant DNA Technology: Restriction enzymes, cloning vectors, construction of recombinant DNA.	1
		Unit 2. Biology of RNA types	2. Biology of RNA types: Ribosomal RNA, transfer RNA and messenger RNA; mRNA modifications: capping, splicing and tailing, Regulatory RNAs and their role in gene silencing, antisense RNA, Ribozyme, Riboswitch.	1
		Unit 3. Protein sorting	3. Protein sorting: Protein targeting in organelles.	1
		Unit 4. Genetic transformation	4. Genetic transformation: *Agrobacterium* mediated (co- culture, *in planta*, agroinfection); Direct method (PEG, electroporation, particle gun method); Reporter genes- screenable and selectable markers.	1
		Unit 5. Blotting techniques – I	5. Blotting techniques: Southern, Northern and Western Blot; DNA fingerprinting, DNA foot printing.	1
		Unit 6. Blotting techniques – II	6. Blotting techniques: Basic idea of proteomics and genomics, c-DNA and genomic library.	1
		Unit 7. DNA sequencing	7. DNA sequencing: manual and automated methods, application as Bioinformatics tool.	1
		Unit 8. Polymerase Chain reaction	8. Polymerase Chain reaction: Types and their application.	1
		Unit 9: DNA-protein interactions	9. DNA-protein interactions: Methods for detection and analysis.	

Course	Group	Details Contents Structure		Study hour
BOTCOR T310	Biotechnology	**Unit 10. Plant Tissue Culture**	1. Plant Tissue Culture: Cellular totipotency; organogenesis, somatic embryogenesis, Role of SERK and LEC genes during SE; haploidy and DH populations in crop improvement.	1
		Unit 11. Biotechnological Applications for Crop Management	2. Biotechnological Applications for Crop Management: Approaches to improve shelf life of fruits and vegetables; herbicide resistance; insect and pest management.	1
		Unit 12. Micropropagation	3. Micropropagation: Production of virus free plants, virus free assessment methods, assessment by RAPD and ISSR markers, certification for quality plants.	1
		Unit 13. Fermentation Technology– I	4. Fermentation Technology: Application of fermentation; batch, fed batch & continuous cultures of microbes.	1
		Unit 14. Fermentation Technology – II	5. Fermentation Technology: Bioreactors: Principles & their design; microbial strain improvement.	1
		Unit 15. Immobilization	6. Immobilization of microbial enzymes & whole cells and their applications in industries.	1
		Unit 16. Microbes as Food	7. Microbes as Food & in food processing, single cell protein.	1
		Unit 17. Biofertilizers and Biopesticides	8. Biofertilizers and Biopesticides in agriculture.	1
		Unit 18. Environmental Biotechnology	9. Environmental Biotechnology: Treatment of waste & waste water; bioremediation.	1

Content

COURSE – BOTCOR T310

Plant Molecular Biology and Biotechnology

Hard Core Theory Paper **Credits (A+B): = 3**

Group – A (Plant Molecular Biology)

Content Structure

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1. Introduction
2. Course Objective
3. Recombinant DNA Technology: Restriction enzymes, cloning vectors, construction of recombinant DNA.
4. Biology of RNA types: Ribosomal RNA, transfer RNA and messenger RNA; mRNA modifications: capping, splicing and tailing, Regulatory RNAs and their role in gene silencing, antisense RNA, Ribozyme, Riboswitch.
5. Protein sorting: Protein targeting in organelles.
6. Genetic transformation: *Agrobacterium* mediated (co- culture, *in planta*, agroinfection); Direct method (PEG, electroporation, particle gun method); Reporter genes- screenable and selectable markers.
7. Blotting techniques: Southern, Northern and Western Blot; DNA fingerprinting, DNA foot printing, basic idea of proteomics and genomics, c-DNA and genomic library.
8. DNA sequencing: manual and automated methods, application as Bioinformatics tool.
9. Polymerase Chain reaction: Types and their application.
10. DNA-protein interactions: Methods for detection and analysis.
11. Let's sum up
12. Suggested Readings
13. Assignments

1. Introduction

Molecular Biology is the branch of biology that studies the structure and function of macro molecules that encode and regulate the flow of genetic information used by living organisms. This course will focus on the structure and content of the three genomes found in plant cells, gene structure, expression, and regulation. Other topics addressed in this class are transposable elements, and plant transformation procedures. A brief introduction to bioinformatics is also included.

2. Course Objectives

You should gather knowledge after studying the course.

This course aims to:

- Provide students with a solid understanding of the relationship between structure and function ofprotein.
- Knowledge about the basic methods and approaches used in molecular biology, blootingtechniques, DNA fingerprinting, DNA foot printing
- Familiarize students with the utilization of bioinformatics resources

3. Recombinant DNA Technology: Restriction enzymes, cloning vectors, construction of recombinant DNA.

Recombinant DNA (rDNA) is a form of artificial DNA that is created by combining two or more sequences that would not normally occur together through the process of gene splicing.

Recombinant DNA technology is a technology which allows DNA to be produced via artificial means. The procedure has been used to change DNA in living organisms and may have even more practical uses in the future.

Stanley N. Cohen, who received the Nobel Prize in Medicine in 1986 for his work on discoveries of growth factors. **Stanley N. Cohen** and **Herbert Boyer** constructed the first recombinant DNA using bacterial DNA and plasmids.

Restriction enzymes:

These are also known as molecular scissors, used for cutting of DNA. The cutting of DNA at specific locations became possible with the discovery of molecular scissors, i.e., restriction enzymes. In the year 1963, the two enzymes responsible for restricting the growth of bacteriophage in E. coli were isolated. One of these added methyl groups to DNA, while the other cuts the DNA. Later was termed as restriction endonucleases.

The first restriction endonuclease was isolated by Smith Wilcox and Kelley in 1968 was Hind II. It was found that it always cuts DNA molecules at a particular point by recognising a specific sequence of six base pairs known as recognition sequence for Hind II. Today, more than 900 restriction enzymes have been isolated from over 230 bacterial strains each of which recognise different recognition sequences.

Restriction endonuclease enzymes occur naturally in bacteria as a chemical weapon against the invading viruses. They cut both strands of DNA when certain foreign nucleotides are introduced in the cell. Endonucleases break strands of DNA at internal positions in random manner.

The first observations on the existence of restriction enzymes was made by Arber and Dussoix in 1962, and proposed model to explain the restriction phenomenon. Their views on restriction enzymes affirmed that certain bacterial strains contained endonucleases able to cleave unprotected DNA. In addition, several other strains contained a modification system responsible for protecting their own DNA.

Some of the observations were made by W. Arber and his associates while studying the efficiency of plating of the bacteriophage lambda on different strains of *Escherichia coli*. They even demonstrated that restriction endonucleases were able to cleave DNA from other strains while exempting that of theoriginal strains.

They are also associated with modifying enzymes, which methylate the DNA. Methylated DNA escape cleavage by endonucleases, and prevents the cell from degrading its own DNA. Thus, invadingforeign DNA in bacteria that has not been correctly methylated will be degraded.

In 1970, Smith, Wilcox and Kelly have characterized and purified restriction enzymes and elucidated their recognition and cleavage site of a more useful restriction enzyme, Hind II.

Naming of Restriction Enzymes:

The convention for naming these enzymes proceeds in a way that the first letter of the name comes from the genes and the second two letters come from the species of prokaryotic cell, from which they were isolated, e.g., Eco RI comes from *E. coli* RY13. The letter 'R' is derived from the name of strain. Roman numbers following the names, indicate the order in which the enzymes were isolated from that strain of bacteria.

Naming exercise of RE enzymes is based on following rules:

1. Each RE enzyme is named by a three-letter code.
2. The first letter of this code is derived from the first epithet (first letter of name) of the genus name.It is printed in italics.
3. The second and third letters are from the first two letters of its species name. They are also printedin italics.
4. This is followed by the strain number. If a particular strain has more than one restriction enzyme,these will be identified by Roman numerals as I, II, III, etc.

For example, the enzyme Eco RI was isolated from the bacterium *Escherichia* (*E*) *coli* (*co*)

strain RY13 (R) and it was the first endonuclease (I). R also indicates antibiotic resistant plasmid of the bacterium. Likewise, Hind II from *Haemophilus influenzae* strain Rd and Bgl I from *Bacillus globigii*.A few restriction endonuclease enzymes, their sources and recognition sites are given below:

Table 55.3 A few restriction endonuclease enzymes, their sources and recognition sites.

Name of the restriction endonuclease enzyme	Source (Microorganisms)	Recognition sequence and cleavage site
1. *Aat* II	*Acetobacter aceti*	GACGT ↓ C
2. *Bcl* I	*Bacillus Caldoxyticus*	T ↓ GATCA
3. *Cvn* I	*Chromatium vinosum*	CC ↓ TNAGG
4. *Eco* RI	*Escherichia coli* RY13	G ↓ AATTC
5. *Eco* RII	*Escherichia coli R245*	↓ CCTGG
6. *Hind* II	*Haemophilus influenzae* Rd	GTP_y ↓ PuAC*
7. *Hind* III	*Haemophilus influenzae* Rd	A ↓ AGCTT
8. *Kpn* I	*Klebsiella pneumoniae* OK	GGTAC ↓ C
9. *Nop* I	*Nocardia opaca*	G ↓ TC GAC
10. *Nsp* B II	*Nostoc*	C(A/C)G ↓ C(T/G)G

Restriction enzymes belong to a larger class of enzymes called nucleases, which are of two types:

(a) Exonucleases remove nucleotides from the ends of the DNA either (5′ or 3′) in one strand of duplex.

(b) Endonucleases make cuts at specific positions within DNA. Each restriction endonucleasesfunction by 'inspecting' the length of a DNA sequence.

Once it finds its specific recognition sequence, it will bind to the DNA and cut each of the two strandsof the double helix at specific points in their sugar phosphate backbones.

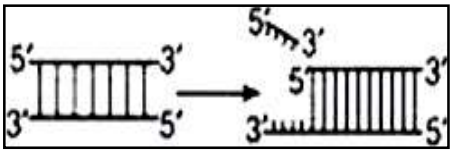
Differences between Exonucleases and Endonucleases

Exonucleases:

a) These nucleases cleave base pairs of DNA at their terminal ends.

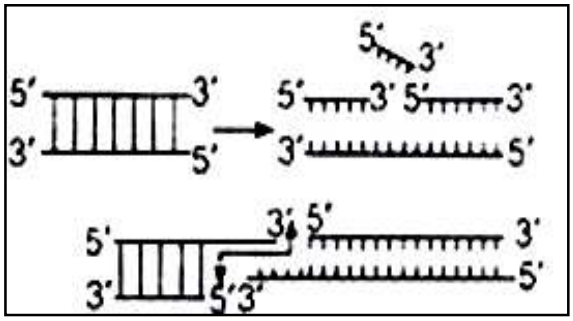
b) They act on single – strand of DNA or gaps in double –stranded DNA.

c) They do not cut RNA.



Endonucleases:

- They cleave DNA at any point except the terminal ends.
- They cleave one strand or both strands of double – stranded DNA.
- They may cut RNA.

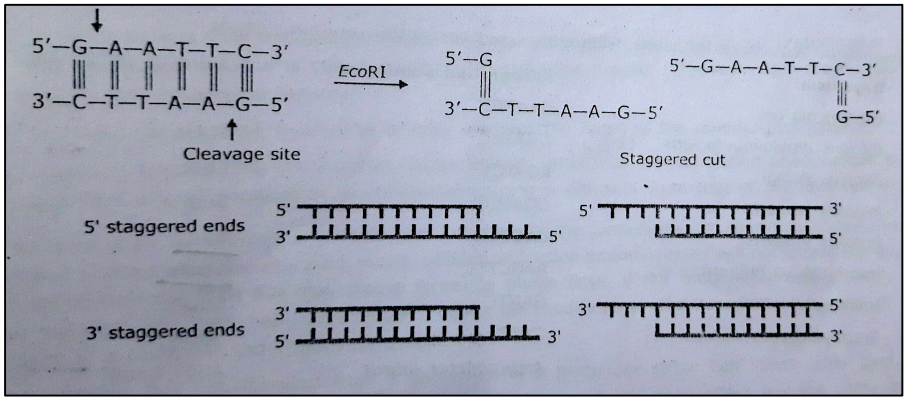


Each restriction endonuclease recognizes a specific palindromic nucleotide sequences in the DNA. Recognition sites of most restriction enzymes have a two fold rotational symmetry. Restriction enzymes have corresponding symmetry to facilitate recognition and usually cleave the DNA on the axis of symmetry.

Palindrome in the DNA is a groups of letters that forms the same words when read both forward and backward. For example, the following sequences read the same on the two strands in 5′ → 3′ direction as well as 3′ → 5′ direction.

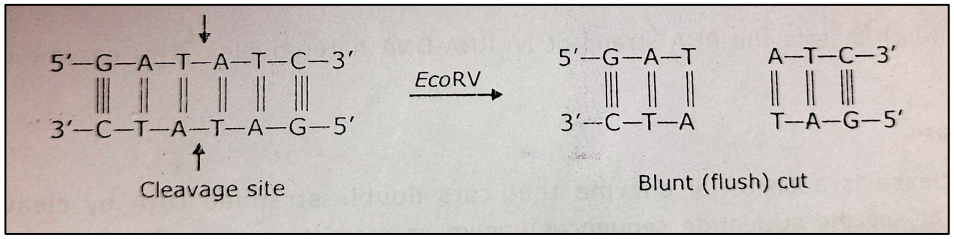
5' overhangs: The enzyme cuts asymmetrically within the recognition site such that a short single- stranded segment extends from the 5' ends. Bam HI cuts in this manner.

3' overhangs: Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3' ends. KpnI cuts in this manner.



Blunt cut ends:

Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. SmaI is an example of an enzyme that generates blunt ends.



Ischizomers, Neochischizomers and Isocaudomers:

- Restriction enzymes that have the same recognition sequence as well as the same cleavagesite are **Isoschizomers.** Eg: Sphl and Bbul.
- SphI (CGTAC/G) and BbuI (CGTAC/G)
- Restriction enzymes that have the same recognition sequence but cleave the DNA at adifferent site within that sequence are **Neochizomers.** Eg: SmaI and XmaI.
- Isocaudomers are pairs of restriction enzymes that have slightly different recognition sequences but upon cleavage generate identical termini. For example the enzymes **Mbo I andBamH I** are isocaudomers.

Types:

Naturally occurring restriction endonucleases are categorized into four groups (Types I, II III, and IV) based on their composition and enzyme cofactor requirements, the nature of their target sequence, andthe position of their DNA cleavage site relative to the target sequence. All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific fragments with terminal 5'-phosphates. They differ in their

recognition sequence, subunit composition, cleavage position, and cofactor requirements, as summarised below:

Type I enzymes (EC3.1.21.3)

- Capable of both restriction and modification activities
- The cofactors S-Adenosyl methionine (AdoMet), ATP, and Mg2+, are required for their fullactivity
- Contain two R (restriction) subunits, two M (methylation) subunits and one S (specificity) subunit
- Cleave DNA at random length from recognition site

Type II enzymes (EC3.1.21.4)

- Mostly used for gene analysis and cloning
- More than 3500 REs
- Recognize 4-8 bp sequences
- Need Mg^{2+} as cofactor
- Cut in close proximity of the recognition site
- Single function (restriction digestion) enzymes independent of methylase
- Homodimers
- ATP hydrolysis is not required
- Examples: *Eco*RI, *Eco*RII, *Bam*HI, *Hind*III

Type III enzymes (EC3.1.21.5)

- Large enzymes
- Combination restriction and modification
- Cleave outside of their recognition sequences
- Require two recognition sequences in opposite orientations within the same DNA molecule.
- Require ATP (but do not hydrolyse it); S-adenosyl-L-methionine stimulates the reaction but isnot required

Type IV enzymes

- Cleave only normal and modified DNA (methylated, hydroxymethylated and glucosyl-hydroxymethylated bases).
- Recognition sequences have not been well defined
- Cleavage takes place ~30 bp away from one of the sites

Artificial Restriction Enzymes

- Generated by fusing a natural or engineered DNA binding domain to a nuclease domain
- Can target large DNA sites (up to 36 bp)
- Can be engineered to bind to desired DNA sequences

Mechanism of Action of Restriction Enzymes:

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA. The enzyme makes two incisions, one through each of the sugar-phosphate backbones (i.e., each strand) of the double helix without damaging the nitrogenous bases.

The chemical bonds that the enzymes cleave can be reformed by other enzymes known as ligases, so that restriction fragments carved from different chromosomes or genes can be spliced together, provided their ends are complementary.

Restriction enzymes cut the strand of DNA a little away from the center of the palindrome sites, but between the same two bases on the opposite strands. This leaves single-stranded portion at the ends. There are overhanging stretches called sticky ends on each strand as given in above figure.

Restriction endonucleases are also used in genetic engineering to form recombinant molecules of DNA, which are composed of DNA from different sources or genomes. The resultant DNA fragmentshave the same sticky ends, which are complementary to each other, therefore can be joined together (end-to-end) using DNA ligases, when cut by the same restriction enzyme.

Many of the procedures of molecular biology and genetic engineering rely on restriction enzymes. The term restriction comes from the fact that these enzymes were discovered in *E.*

coli strains that appeared to be restricting the infection by certain bacteriophages.

Cloning vectors:

Vectors are those DNA molecules that can carry a foreign DNA fragment when inserted into it. A vector must possess certain minimum qualifications to be an efficient agent for the transfer, maintenance and amplification of the passenger DNA.

Criteria of an Ideal Vector are as follows –

- ✓ The vector should be small and easy to isolate.
- ✓ They must have one or more origins of replication so that they will stably maintain themselveswithin host cell.
- ✓ Vector should have one or more unique restriction sites into which the recombinant DNA can beinserted.
- ✓ They should have a selectable marker (antibiotic resistance gene) which allows recognition oftransformants.
- ✓ Vector DNA can be introduced into a cell.
- ✓ The vector should not be toxic to host cell.

History of Cloning Vectors:

Herbert Boyer, Keiichi Itakura, and Arthur Riggs were three scientists working in the Boyer's lab, University of California, where they recognized a general cloning vector. This cloning vector had restriction sites for cloning foreign DNA and also, the expression of antibiotic resistance genes for the screening of recombinant/ transformed cells. The first vector used for cloning purposes was pBR322, a plasmid. It was small in size, nearly 4kB, and had two selectable markers.

Features of Cloning Vectors:

1. Origin of Replication (ori)

- ✓ A specific set/ sequence of nucleotides where replication initiates.
- ✓ For autonomous replicationinside the host cell.
- ✓ Foreign DNA attached to ori also begins to replicate.

2. Cloning Site

- ✓ Point of entry or analysis for genetic engineering.
- ✓ Vector DNA at this site is digested and foreign DNA is inserted with the aid of restriction enzymes.
- ✓ Recent works have discovered plasmids with multiple cloning sites (MCS) which harbour upto 20 restriction sites.

3. Selectable Marker

- ✓ Gene that confers resistance to particular antibiotics or selective agent which, under normalconditions, is fatal for the host organism.
- ✓ Confers the host cell the property to survive and propagate in culture medium containing theparticular antibiotics.

4. Marker of Reporter Gene

- ✓ Permits the screening of successful clones or recombinant cells.
- ✓ Utilised extensively in blue-white selection.

5. Inability to Transfer via Conjugation

- ✓ Vectors must not enable recombinant DNA to escape to the natural population of bacterial cells.

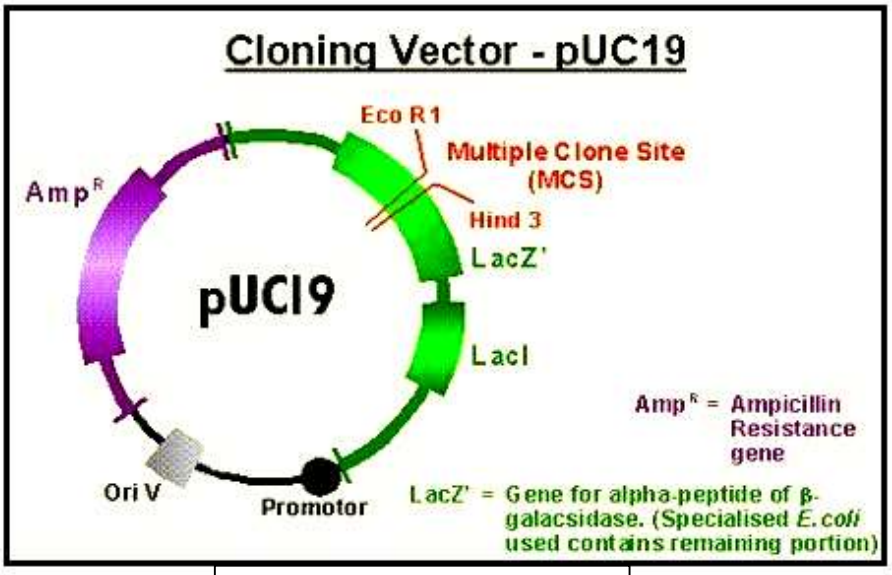


Fig: Cloning Vector

Types of Cloning Vectors:

1. **Plasmids**

- Plasmids were the first vectors to be used in gene cloning.
- They are naturally occurring and autonomously replicating extra-chromosomal double- stranded circular DNA molecules. However, not all plasmids are circular in origin.
- They are present in bacteria, archaea, and eukaryotes.
- The size of plasmids ranges from 1.0 kb to 250 kb.
- DNA insert of up to 10 kb can be cloned in the plasmids.
- The plasmids have high copy number which is useful for production of greater yield ofrecombinant plasmid for subsequent experiments.
- The low copy number plasmids are exploited under certain conditions like the cloned geneproduces the protein which is toxic to the cells.
- Plasmids only encode those proteins which are essential for their own replication. Theseprotein-encoding genes are located near the ori.
- Examples: pBR322, pUC18, F plasmid, Col plasmid.

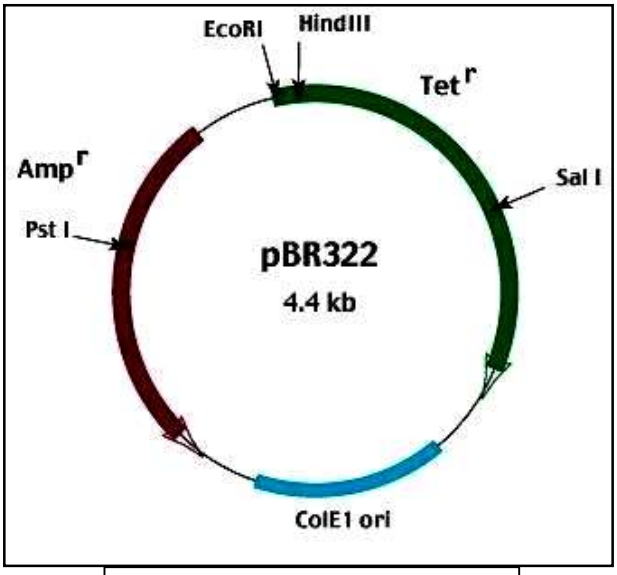


Fig: pBR322 plasmid

Nomenclature of plasmid cloning vector:

pBR322 cloningvector has the following elements –

p= plasmid

B= Bolivar (name of the scientist)

R= Rodriguez (name of the scientist)

322= number of plasmid discovered in the same lab

Advantages of using Plasmids as vectors:

- Easy to manipulate and isolate because of small size.
- More stable because of circular configuration.
- Replicate independent of the host.
- High copy number.
- Detection easy because of antibiotic-resistant genes.

Disadvantages of using Plasmids as vectors:

- Large fragments cannot be cloned.
- Size range is only 0 to 10kb.
- Standard methods of transformation are inefficient.

2. Bacteriophage:

- ❖ Bacteriophages or phages are viruses which infect bacterial cells.
- ❖ The most common bacteriophages utilized in gene cloning are Phage λ and M13 Phage.
- ❖ A maximum of 53 kb DNA can be packaged into the phage.

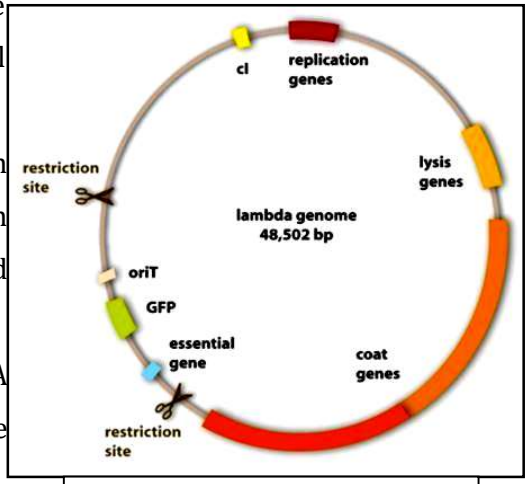


Fig: Bacteriophage λ

- ❖ If the vector DNA is too small, it cannot be packaged properly into the phage. Examples: Phage Lambda, M13 Phage, etc.

Phage Lambda λ:

- ❖ It has head, tail, and tail fibers.
- ❖ Its genome consists of 48.5 kb of DNA and 12 bp ss DNA which comprise of sticky ends at both the terminals. Since these ends are complementary, they are cohesive and also referred to as cos sites.
- ❖ Infection by λ phage requires adsorption of tail fibers on the cell surface, contraction of the tail, and injection of the DNA inside the cell.

M13 Phage:

- ❖ These vectors are used for obtaining single-stranded copies of the cloned DNA.
- ❖ They are utilized in DNA sequencing and in vitro mutagenesis.
- ❖ M13 phages are derived from filamentous bacteriophage M13. The genome of M13 is 6.4 kb.
- ❖ DNA inserts of large sizes can be cloned.
- ❖ From the double-stranded inserts, pure single-stranded DNA copies are obtained.

Advantages of using Phage vectors:

- They are way more efficient than plasmids for cloning large inserts.
- Screening of phage plaques is much easier than identification of recombinant bacterialcolonies.

3. Phagemids or Phasmid:

- ❖ They are prepared artificially.
- ❖ Phasmid contains the F1 origin of replication from F1 phage.
- ❖ They are generally used as a cloning vector in combination with M13 phage.
- ❖ It replicates as a plasmid and gets packaged in the form of single-stranded

DNA in viral particles.

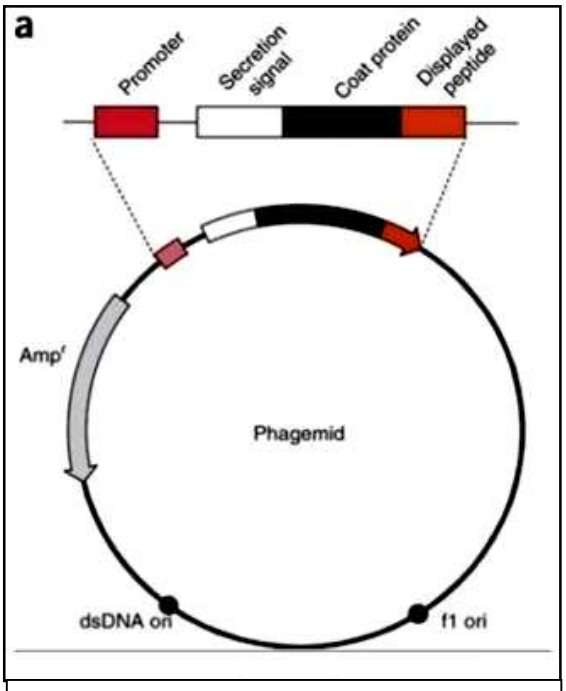


Fig: Phagemid

Advantages of using Phagemids:

- They contain multiple cloning sites.
- An inducible lac gene promoter is present.
- Blue-white colony selection is observed.

4. **Cosmids:**
    - ❖ Cosmids are plasmids.
    - ❖ They are capable of incorporating the bacteriophage λ DNA segment. This DNA segment contains cohesive terminal sites (cos sites).
    - ❖ Cos sites are necessary for efficient packaging of DNA into λ phage particles.
    - ❖ Large DNA fragments of size varying from25 to 45 kb can be cloned.
    - ❖ They are also packaged into λ This permits the foreign DNA fragment or genes to be introduced into the host organism by the mechanism of transduction.

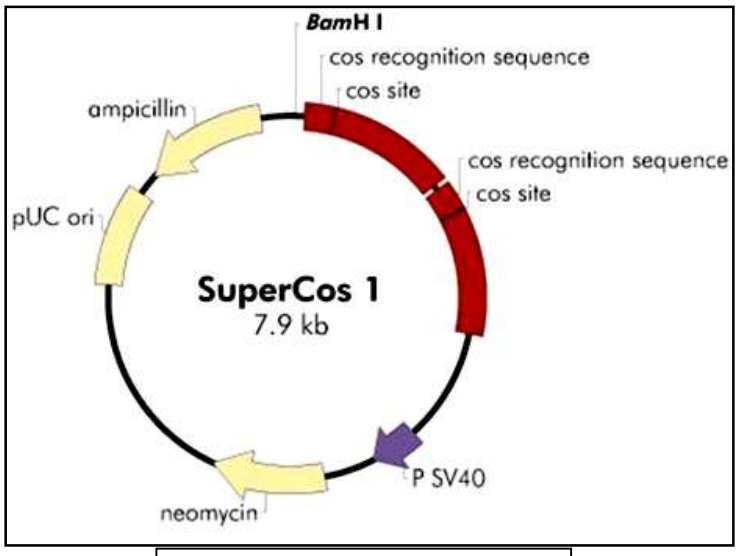


Fig: Cosmid Vector

Advantages of using Cosmids as vectors:

- They have high transformation efficiency and are capable of producing a large number of clones from a small quantity of DNA.
- Also, they can carry up to 45 kb of insert compared to 25 kb carried by plasmids and λ.

Disadvantages of using Cosmids as vectors:

- Cosmids cannot accept more than 50 kb of the insert.

5. **Bacterial Artificial Chromosomes (BACs):**
    - ❖ Bacterial artificial chromosomes are similar to *E. coli* plasmid vectors.
    - ❖ They contain ori and genes which encode ori binding proteins. These proteins are critical for BAC replication.
    - ❖ It is derived from naturally occurring F' plasmid.
    - ❖ The DNA insert size varies between 150 to 350 kb.

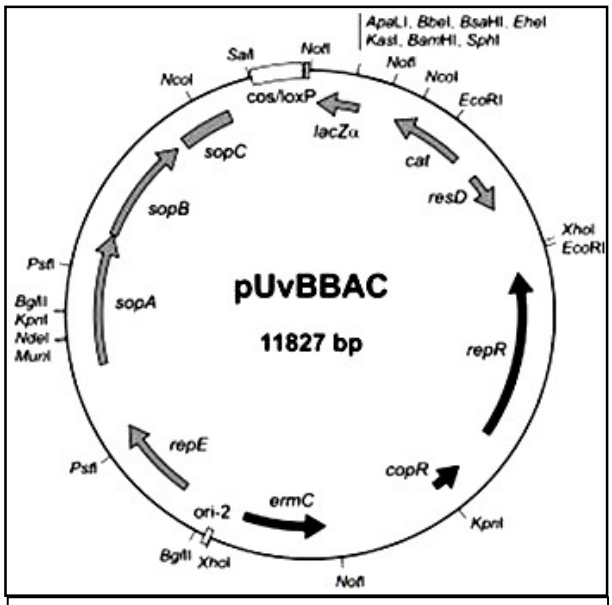


Fig: Bacterial Artificial Chromosome

Advantages of BACs:

- They are capable of accommodating large sequences without any risk of rearrangement.
- BACs are frequently used for studies of genetic or infectious disorders.
- High yield of DNA clones is obtained.

Disadvantages of BACs:

- They are present in low copy number.
- The eukaryotic DNA inserts with repetitive sequences are structurally unstable in BACs often resulting in deletion or rearrangement.

6. **Yeast Artificial Chromosomes (BACs):**
    - ❖ A large DNA insert of up to 200 kb can be cloned.
    - ❖ They are used for cloning inside eukaryotic cells. These act as eukaryotic chromosomes inside the host eukaryotic cell.
    - ❖ It possesses the yeast telomere at each end.

- ❖ A yeast centromere sequence (CEN) is present which allows proper segregation during meiosis.
- ❖ The ori is bacterial in origin.
- ❖ Both yeast and bacterial cells can be used as hosts.

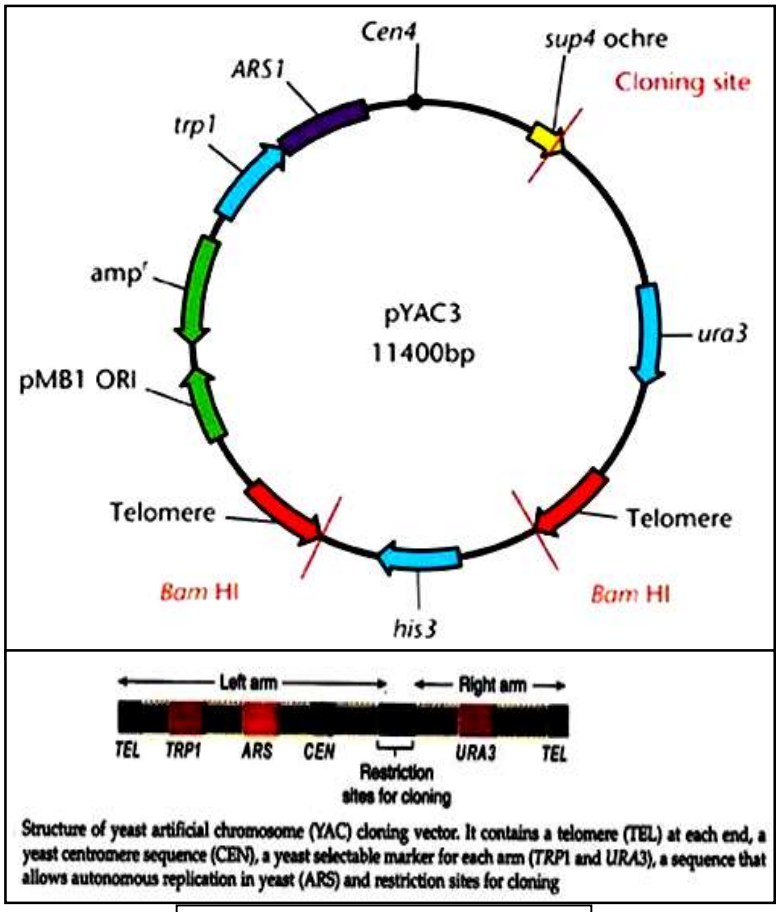


Fig: Yeast Artificial Chromosome

Advantages of using YACs:

- A large amount of DNA can be cloned.
- Physical maps of large genomes like the human genome can be constructed.

Disadvantages of using YACs:

- Overall transformation efficiency is low.

- The yield of cloned DNA is also low.

Advantages of BACs over YACs:

- Comparatively stable.
- Easy to transform.
- Simple purification required.
- User- friendly.
- Aid in the development of vaccines.

7. Expression Vectors:

- ❖ Express the DNA insert producing specific protein.
- ❖ They have prokaryotic promoter.
- ❖ Ribosome binding site.
- ❖ Origin of replication.
- ❖ Antibiotic resistance gene.
- ❖ Expression vectors with strong promoters.
- ❖ Inducible Expression Vectors.
- ❖ Eukaryotic expression vectors.

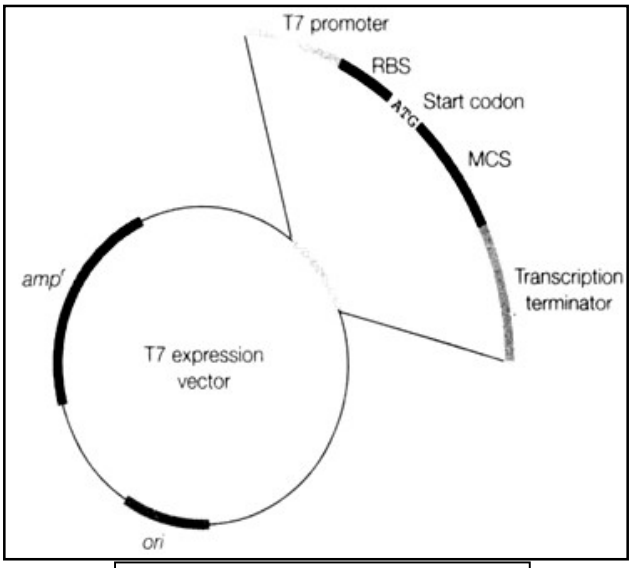


Fig: Expression Vector

8. **Human artificial Chromosome (HACs):**

- ❖ Human artificial chromosomes are artificially synthesized.
- ❖ They are utilized for gene transfer or gene delivery into human cells.
- ❖ It can carry large amounts of DNA inserts.
- ❖ They are used extensively in expression studies and determining the function of the human chromosomes.

Advantages of using HACs:

- No upper limit on DNA that can be cloned.
- It avoids the possibility of insertional mutagenesis.

Summary of vectors and what they can carry.

The size of DNA that vector can carry	Vector
0 – 10 kb	Standard plasmid
0 – 23 Kb	Lambda Bacteriophage
30 – 44 Kb	Cosmid
70 – 100 Kb	Bacteriophage P1
130 – 150 Kb	P1 Artificial chromosome PAC
Maximum 300 Kb	Bacterial Artificial Chromosome BAC
0.2 – 2 Mb	Yeast Artificial Chromosome YAC

9. **Shuttle vectors:**

Vectors contain sequences required for replication and selection in both *E. coli* and the desired host cells, so that the construction and many other manipulations of the recombinant plasmids can be completed in *E. coli*. Most of the eukaryotic vectors are constructed as shuttle vectors.

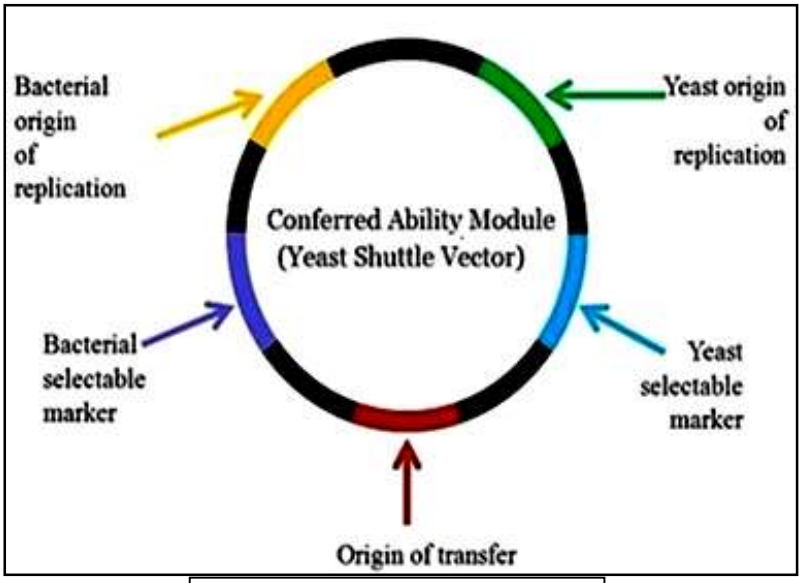
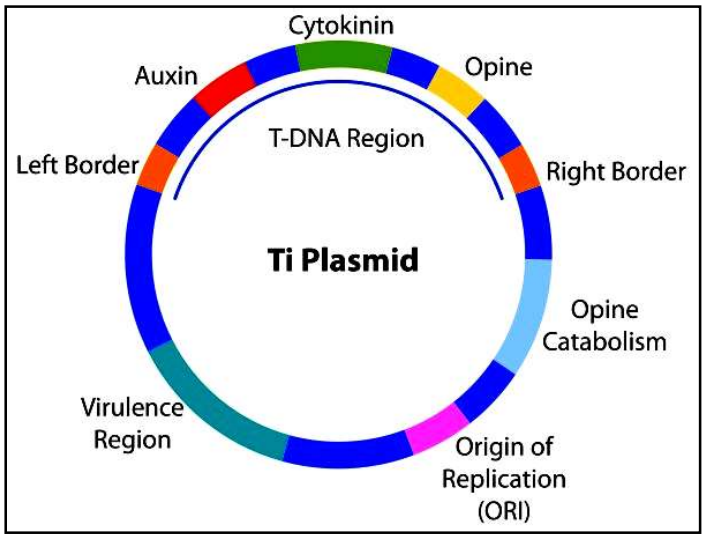


Fig: Shuttle Vector

10. Ti plasmid:

- Place the target gene in the T-DNA region of a Ti plasmid, then transform the recombinant Ti plasmid.
- Recombinant T-DNA transformed into the *A. tumefaciens* cell carrying a modified Ti plasmid without T-DNA (T-DNA that are responsible for crown gall formation. The deleted T- DNA is called disarmed T-DNA shuttle vector).



Construction of recombinant DNA:

Tools for Constructing Recombinant DNA:

Recombinant DNA technology utilizes a number of biological tools to achieve its objectives, most important of them being the enzymes. Important biological tools for rec DNA technology are -

1. Enzymes
    a) Restriction Endonucleases
    b) Restriction Exonucleases
    c) DNA ligases
    d) DNA polymerase
2. Cloning Vector
3. Host organism
4. DNA insert or foreign DNA
5. Linker and adaptor sequences

1. Enzymes:

A number of specific enzymes are utilized to achieve the objectives of rec DNA technology.

The enzymology of genetic engineering includes the following types of enzymes:

a) Restriction Endonucleases

These enzymes serve as important tools to cut DNA molecules at specific sites, which is the basic need for rec DNA technology.

These are the enzymes that produce internal cuts (cleavage) in the strands of DNA, only within or near some specific sites called recognition sites/recognition sequences/ restriction sites 01 target sites. Such recognition sequences are specific for each restriction enzyme. Restriction endonuclease enzymes are the first necessity for rec DNA technology.

The presence of restriction enzymes was first of all reported by W. Arber in the year 1962. He found that when the DNA of a phage was introduced into a host bacterium, it was fragmented into small pieces. This led him to postulate the presence of restriction enzymes. The first true restriction endonuclease was isolated in 1970s from the

bacterium E. coli by Meselson and Yuan.

Another important breakthrough was the discovery of restriction enzyme Hind-II in 1970s by Kelly, Smith and Nathans. They isolated it from -the bacterium Haemophilus influenza. In the year 1978, the Nobel Prize for Physiology and Medicine was given to Smith, Arber and Nathans for the discovery of endonucleases.

b) Restriction Exonucleases

Exonuclease is an enzyme that removes nucleotides from the ends of a nucleic acid molecule. An exonuclease removes nucleotide from the 5′ or 3′ end of a DNA molecule. An exonuclease never produces internal cuts in DNA.

In rec DNA technology, various types of exonucleases are employed like Exonuclease Bal31, *E. coli* exonuclease III, Lambda exonuclease, etc.

Exonculease Bal31 is employed for making the DNA fragment with blunt ends shorter from both its ends.

c) DNA ligases

The function of these enzymes is to join two fragments of DNA by synthesizing the phosphodiester bond. They function to repair the single stranded nicks in DNA double helix and in rec DNA technology. They are employed for sealing the nicks between adjacent nucleotides. This enzyme is alsotermed as molecular glue.

d) DNA Polymerases

These are the enzymes which synthesize a new complementary DNA strand of an existing DNA or RNA template. A few important types of DNA polymerases are used routinely in genetic engineering. One such enzyme is DNA polymerase, which prepared from *E coli*. The Klenow fragment of DNA polymerase-I is employed to make the protruding ends double-stranded by extension of the shorter strand.

Another type of DNA polymerase used in genetic engineering is *Taq* DNA polymerase which is used in PCR (Polymerase Chain Reaction).

Reverse transcriptase is also an important type of DNA polymerase enzyme for genetic engineering. It uses RNA as a template for synthesizing a new DNA strand called as cDNA or complementary DNA). Its main use is in the formation of cDNA libraries. Apart from all these above mentioned enzymes, a few other enzymes also

mark their importance in genetic engineering.

e) Terminal deoxynucleotidyl transferase enzyme

It adds single stranded sequences to 3′-terminus of the DNA molecule. One or more deoxynbonucleotides (dATP, dGTP, dTTP, dCTP) are added onto the 3′-end of the blunt-ended fragments.

f) Alkaline Phosphatase Enzyme

It functions to remove the phosphate group from the 5′-end of a DNA molecule.

g) Polynucleotide Kinase Enzyme

It has an effect reverse to that of Alkaline Phosphatase, i.e. it functions to add phosphate group to the 5′-terminus of a DNA molecule.

2. Cloning Vectors:

It is another important natural tool which geneticists use in rec DNA technology. The cloning vector is the DNA molecule capable of replication in a host organism, into which the target DNA is introduced producing the rec DNA molecule.

A cloning vector may also be termed as a cloning vehicle or earner DNA or simply as a vector or a vehicle a great variety of cloning vectors are present for use with *E. coli* is the host organism. Different types of DNA molecules may be used as cloning vehicles such as they may be plasmids, bacteriophages, cosmids, phasmids or artificial chromosomes.

3. Host Organism:

A good host organism is an essential tool tor genetic engineering. Most widely used host for rec DNA technology is the bacterium *E. coli*. Because cloning and isolation of DNA inserts is very easy in this host. A good host organism is the one winch easy to transform and in which the replication of rec DNA is easier. There should not be any interfering element against the replication of rec DNA in the host cells.

4. DNA Insert or Foreign DNA:

The desired DNA segment which is to be cloned is called as DNA insert or foreign DNA or target DNA. The selection of a suitable target DNA is the very first step of rec DNA technology.

The target DNA (gene) may be of viral, plant, animal or bacterial origin.

Following points must be kept in mind while selecting the foreign DNA:

- ✓ It can be easily extracted from source.
- ✓ It can be easily introduced into the vector.
- ✓ The genes should be beneficial for commercial or research point of view.

5. Linker and Adaptor Sequences:

Linkers and adaptors are the DNA molecules which help in the modifications of cut ends of DNA fragments. These can be joined to the cut ends and hence produce modifications as desired.

Both are short, chemically synthesized, double stranded DNA sequences. Linkers have (within them) one or more restriction endonuclease sites and adaptors have one or both sticky ends. Different types of linkers and adaptors are used for different purposes.

Steps in Recombinant DNA Technology:

The basic 7 steps involved in gene cloning are as follows –

(i) Isolation of DNA (gene of interest) fragments to be cloned.

(ii) Insertion of isolated DNA into a suitable vector to form recombinant DNA.

(iii) Introduction of recombinant DNA into a suitable organism known as host.

(iv) Selection of transformed host cells and identification of the clone containing the gene of interest.

(v) Multiplication/Expression of the introduced Gene in the host.

(vi) Isolation of multiple gene copies/Protein expressed by the gene.

(vii) Purification of the isolated gene copy/protein.

Isolation of DNA fragment or gene:

- ❖ The target DNA or gene to be cloned must be first isolated. A gene of interest is a fragment ofgene whose product (a protein, enzyme or a hormone) interests us. For example, gene encoding for the hormone insulin.
- ❖ The desired gene may be isolated by using restriction endonuclease (RE)

enzyme, which cut DNA at specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease) producing blunt or sticky ends.

- Sometimes, reverse transcriptase enzyme may also be used which synthesizes complementaryDNA strand of the desired gene using its mRNA.

Selection of suitable cloning vector:

- The vector is a carrier molecule which can carry the gene of interest (GI) into a host, replicate there along with the GI making its multiple copies.
- The cloning vectors are limited to the size of insert that they can carry. Depending on the size and the application of the insert the suitable vector is selected.
- The different types of vectors available for cloning are plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs) and mammalian artificial chromosomes (MACs).
- However, the most commonly used cloning vectors include plasmids and bacteriophages (phage λ) beside all the other available vectors.

Formation of RNA Technology:

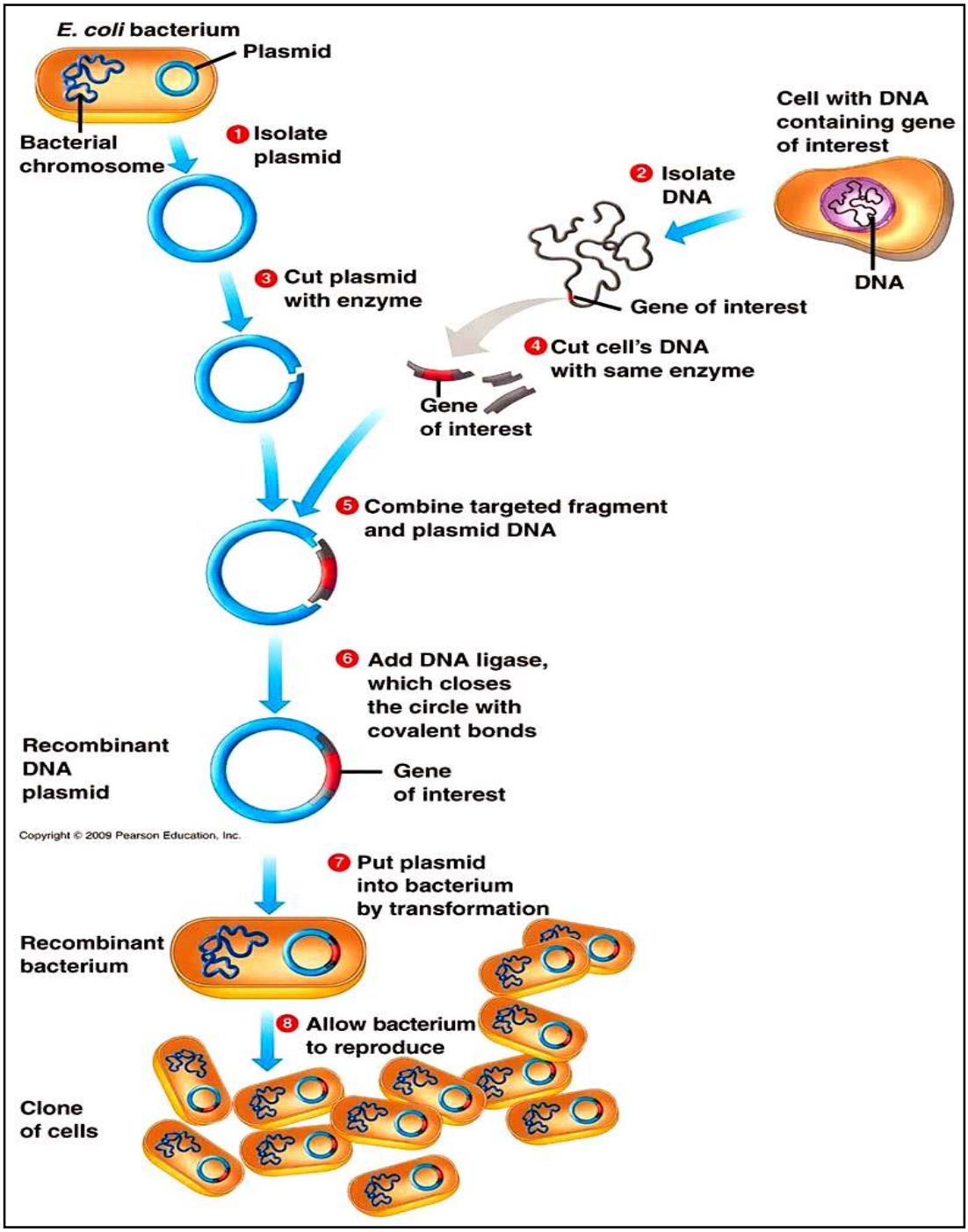
- The plasmid vector is cut open by the same RE enzyme used for isolation of donor DNA fragment.
- The mixture of donor DNA fragment and plasmid vector are mixed together.
- In the presence of DNA ligase, base pairing of donor DNA fragment and plasmid vector occurs.
- The resulting DNA molecule is a hybrid of two DNA molecules – the GI and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination.
- Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the recombinant DNA technology.

Transformation of recombinant vector into suitable host:

- The recombinant vector is transformed into suitable host cell mostly, a bacterial cell.
- This is done either for one or both of the following reasons:
- To replicate the recombinant DNA molecule in order to get the multiple copies of the GI.
- To allow the expression of the GI such that it produces its needed protein product.
- Some bacteria are naturally transformable; they take up the recombinant vector automatically.
- For example: *Bacillus*, *Haemophillus*, *Helicobacter pylori* which are naturally competent.
- Some other bacteria, on the other hand require the incorporation by artificial methods such asCa^{++} ion treatment, electroporation, etc.

Isolation of Recombinant cells:

- The transformation process generates a mixed population of transformed and non-transformed host cells.
- The selection process involves filtering the transformed host cells only.
- For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector isemployed.
- For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knocks out Ampicillin resistant gene from the plasmid, so that the recombinant cell becomes sensitive to Ampicillin.


E. coli bacterium
Plasmid
Bacterial chromosome
1 Isolate plasmid
Cell with DNA containing gene of interest
2 Isolate DNA
DNA
Gene of interest
3 Cut plasmid with enzyme
4 Cut cell's DNA with same enzyme
Gene of interest
5 Combine targeted fragment and plasmid DNA
6 Add DNA ligase, which closes the circle with covalent bonds
Recombinant DNA plasmid
Gene of interest
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7 Put plasmid into bacterium by transformation
Recombinant bacterium
8 Allow bacterium to reproduce
Clone of cells

Multiplication of Selected Host Cells:

- Once transformed host cells are separated by the screening process; becomes necessary to provide them optimum parameters to grow and multiply.
- In this step the transformed host cells are introduced into fresh culture media.
- At this stage the host cells divide and re-divide along with the replication of the recombinant DNA carried by them.
- If the aim is obtaining numerous copies of GI, then simply replication of the host cell is allowed. But for obtaining the product of interest, favourable conditions must be provided such that the GI in the vector expresses the product of interest.

Isolation and Purification of the Product:

The step involves isolation of the multiplied GI attached with the vector or of the protein encoded by it. This is followed by purification of the isolated gene copy/protein.

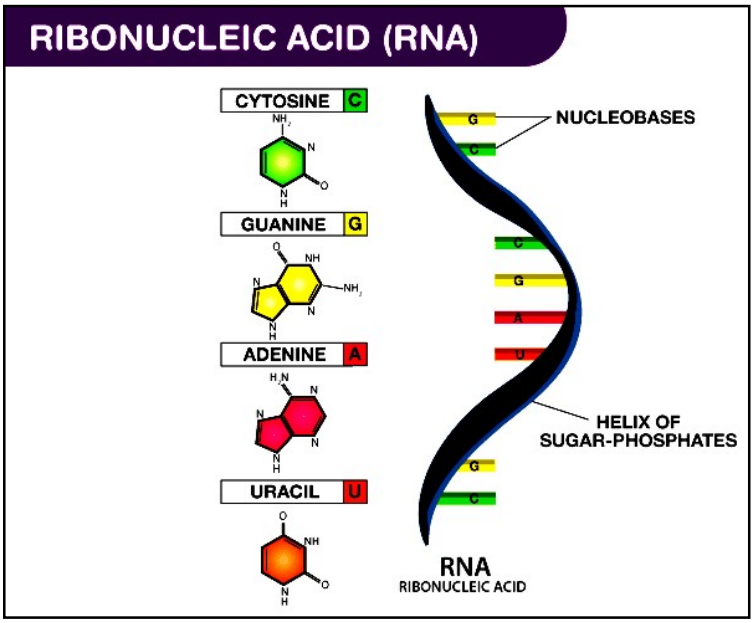
4. Biology of RNA types: Ribosomal RNA, transfer RNA and messenger RNA; mRNA modifications: capping, splicing and tailing, Regulatory RNAs and their role in gene silencing, antisense RNA, Ribozyme, Riboswitch.

Ribonucleic acid (RNA) is a polymeric molecule essential in various biological roles in coding, decoding, regulation and expression of genes. RNA and DNA are nucleic acids, and, along with lipids, proteins and carbohydrates constitute the four major macromolecules essential for all known forms of life.

Structure of RNA:

- Ribonucleic acid, or RNA is one of the major biological macromolecules that are essential forall known forms of life (along with DNA and proteins)

- Each nucleotide in RNA contains a ribose sugar, with carbons numbered 1' through 5'. A base is attached to the 1' position, in general, adenine (A), cytosine (C), guanine(G), and uracil (U).
- Back bone is sugar and phosphate group.
- Nitrogenous bases linked to sugarmoiety project from the backbone.
- Nitrogenous bases are linked to pentose sugar through N-glycosidic linkage to form a nucleoside.
- Phosphate group is linked with 3'OH of nucleoside through phosphoester linkage.
- Two nucleotides are linked through 3'- 5'-phosphodiester linkage to form a dinucleotide.
- More and more such groups will be linked to form a polynucleotide chain.



- Such a polymer has a free phosphate moiety at 5' end of ribosesugar and it is called as 5'-end of polynucleotide chain.
- At other end, ribose has free 3'-OH group which is called as the 3'-end of polynucleotide chain.
- In RNA, every nucleotide has an additional-OH present at 2'-position of ribose.

Types of RNA:

In all prokaryotic and eukaryotic organisms, three main classes of RNA molecules exist-

1. Messenger RNA(mRNA)
2. Transfer RNA (tRNA)
3. Ribosomal RNA (rRNA)

The others are –

- small nuclear RNA (SnRNA),
- micro RNA(mi RNA),
- small interfering RNA(Si RNA) and
- heterogeneous nuclear RNA (hn RNA).

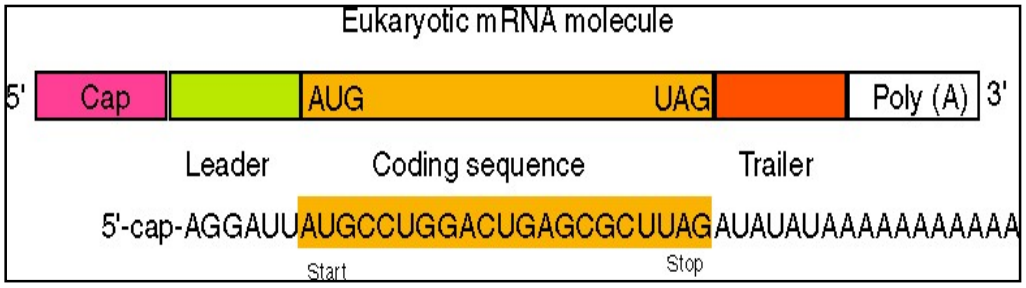
1. Messenger RNA (mRNA)

- Comprises only 5% of the RNA in the cell.
- Most heterogeneous in size and base sequence.
- All members of the class function as messengers carrying the information in a gene to theprotein synthesizing machinery.

Structural Characteristics of mRNA:

- The 5' terminal end is capped by 7- methyl guanosine triphosphate cap.
- The cap is involved in the recognition of mRNA by the translating machinery.
- It stabilizes mRNA by protecting it from 5' exonuclease.
- The 3'end of most mRNAs has a polymer of Adenylate residues (20-250).
- The tail prevents the attack by 3' exonucleases.
- Histones and interferons do not contain polyA tails.
- On both 5' and 3' end there are non coding sequences which are not translated (NCS).
- The intervening region between non coding sequences present between 5'and 3' end is calledcoding region. This region encodes for the synthesis of a protein.
- The mRNA molecules are formed with the help of DNA template during the process oftranscription.
- The sequence of nucleotides in mRNA is complementary to the sequence of nucleotides ontemplate DNA.
- The sequence carried on mRNA is read in the form of codons.
- A codon is made up of 3 nucleotides.

❖ The mRNA is formed after processing of heterogeneous nuclear RNA.



2. Transfer RNA (tRNA)

- Transfer RNA is the smallest of three major species of RNA molecules.
- They have 74-95 nucleotide residues.
- They are synthesized by the nuclear processing of a precursor molecule.
- They transfer the amino acids from cytoplasm to the protein synthesizing machinery, hencethe name tRNA.
- They are easily soluble, hence called "Soluble RNA or s-RNA.
- They are also called Adapter molecules, since they act as adapters for the translation of the sequence of nucleotides of the mRNA in to specific amino acids.
- There are at least 20 species of tRNA one corresponding to each of the 20 amino acids required for protein synthesis.

Structural Characteristics of tRNA:

I. **Primary structure** – The nucleotide sequence of all the tRNA molecules allows extensive intrastand complimentarity that generates a secondary structure.

II. **Secondary structure** – Each single tRNA shows extensive internal base pairing and acquires a clover leaf like structure. The structure is stabilized by hydrogen bonding between the bases and is a consistent feature. Secondary structure (***Clover leaf structure***) of all tRNA contain 5 main arms or loops which are as follows –

a) Acceptor arm
b) Anticodon arm
c) DHU arm
d) T Ψ C arm
e) Extra arm

a) Acceptor arm

The acceptor arm is at 3' end.

- It has 7 base pairs.
- The end sequence is unpaired Cytosine, Cytosine-Adenine at the 3' end.
- The 3' OH group terminal of Adenine binds with carboxyl group of amino acids.
- The t RNA bound with amino acid is called Amino acyl tRNA.

CCA attachment is done post transcriptionally.

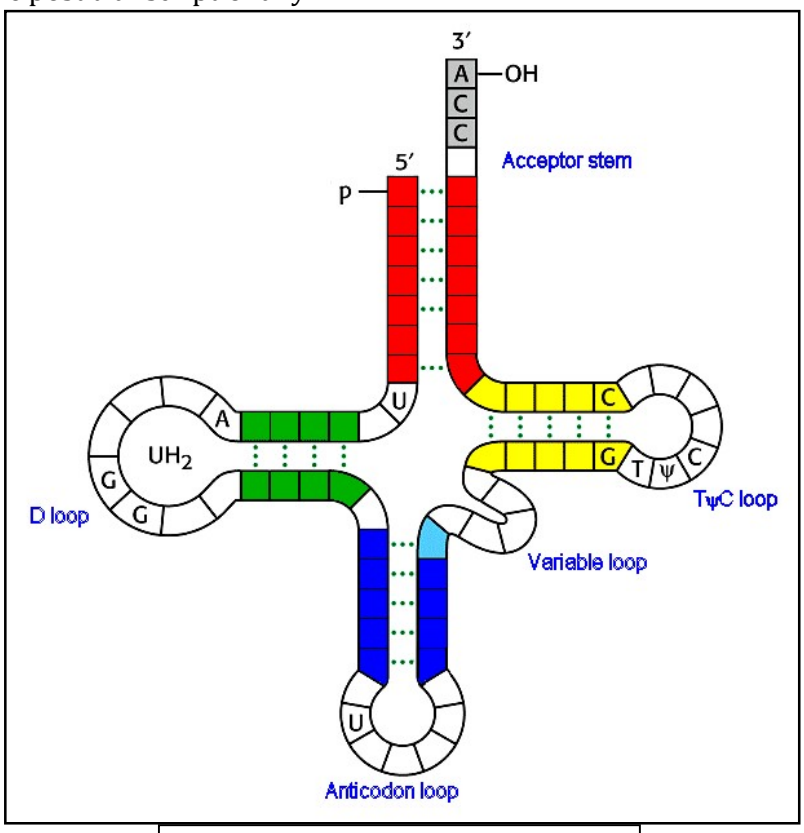


Fig.: Structure of tRNA

b) Anticodon arm

- Lies at the opposite end of acceptor arm.
- 5 base pairs long.
- Recognizes the triplet codon present in the mRNA.
- Base sequence of anticodon arm is complementary tothe base sequence of mRNA codon.
- Due to complimentarity it can bind specifically withmRNA by hydrogen bonds.

c) DHU arm

- It has 3-4 base pairs
- Serves as the recognition site for the enzyme (amino acyl tRNA synthetase) that adds the amino acid to the acceptor arm.

d) T Ψ C arm

- This arm is opposite to DHU arm.
- Since it contains pseudo uridine that is why it is sonamed.
- It is involved in the binding of tRNA to theribosomes.

e) Extra arm

- About 75 % of tRNA molecules possess a short extra arm
- If about 3-5 base pairs are present the tRNA is said to be belonging to class 1. Majority t-RNA belongs to class 1.
- The tRNA belonging to class 2 have long extra arm, 13-21 base pairs in length.

III. **Tertiary structure** – The L shaped tertiary structure is formed by further folding of the clover leaf due to hydrogen bonds between T and D arms. The base paired double helical stems get arranged in to two double helical columns, continuous and perpendicular to one another.

3. Ribosomal RNA (rRNA)

The mammalian ribosome contains two major nucleoprotein subunits—a larger one with a molecular weight of 2.8 x 106 (60S) and a smaller subunit with a molecular weight of 1.4 x 106 (40S).

- The 60S subunit contains a 5S ribosomal RNA (rRNA), a 5.8S rRNA, and a 28S rRNA; there are also probably more than 50 specific polypeptides.
- The 40S subunit is smaller and contains a single 18S rRNA and approximately 30 distinct polypeptide chains.
- All of the ribosomal RNAmolecules except the 5S rRNAare processed from a single 45S precursor RNA molecule in the nucleolus.

5S rRNA is independently transcribed.

- The functions of the ribosomal RNA molecules in the ribosomal particle are not fully understood, but they are necessary for ribosomal assembly and seem to play key roles in the binding of mRNA to ribosomes and its translation.
- Recent studies suggest that an rRNA component performs the peptidyl transferase activity and thus is an enzyme (a ribozyme).

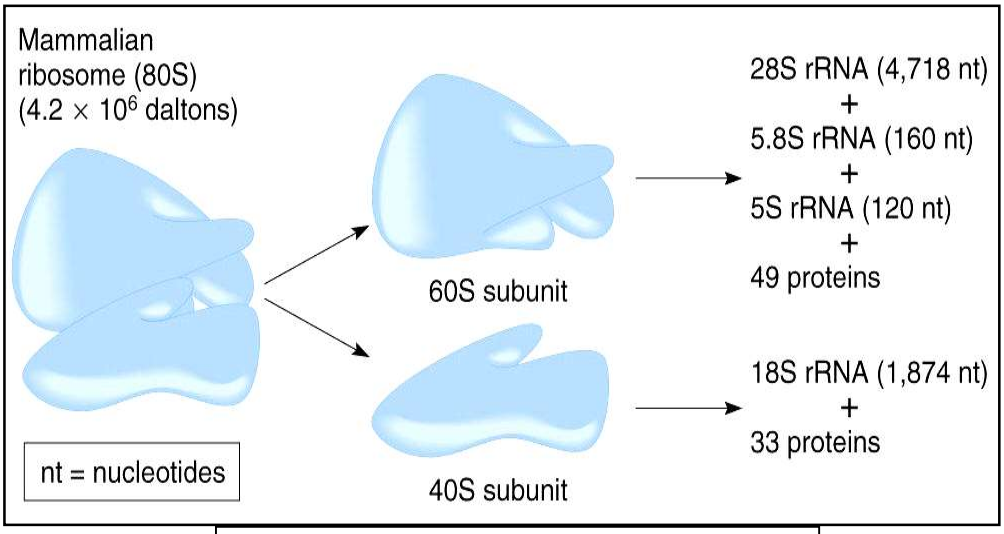
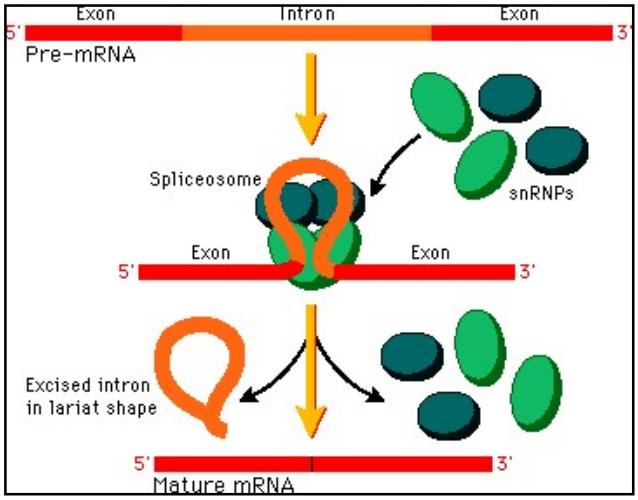


Fig.: Structure of Eukaryotic rRNA

Small RNA:

Most of these molecules are complexed with proteins to form ribonucleoproteins and are distributed in the nucleus, in the cytoplasm, or in both. They range in size from 20 to 300 nucleotides and are present in 100,000–1,000,000 copies per cell.

Small nuclear RNAs (snRNA): Many RNAs are involved in modifying other RNAs. Introns are spliced out of pre-mRNA by spliceosomes, which contain several small nuclear RNAs (snRNA), or the introns can be ribozymes that are spliced by them. Of the several



snRNAs, U1, U2, U4, U5, and U6 are involved in intron removal and the processing of hnRNA into mRNA. The U7 snRNA is involved in production of the correct 3' ends of histone mRNA—which lacks a poly(A) tail.

Small nucleolar RNAs (snoRNA): RNA can also be altered by having its nucleotides modified to nucleotides other than A, C, G and U. In eukaryotes, modifications of RNA nucleotides are in general directed by small nucleolar RNAs (snoRNA; 60–300 nt), found in the nucleolus and cajal bodies. The snoRNAs associate with enzymes and guide them to a spot on an RNA by base pairing to that RNA. These enzymes then perform the nucleotide modifications. The rRNAs and tRNAs are extensively modified, but snRNAs and mRNAs can also be the target of base modification. RNA can also be methylated.

Small Interfering RNAs (siRNAs):

Small interfering RNAs and miRNAs were discovered in different works, but their biogenesis and assembly into RNA-protein complexes and their function in down regulating gene

expression are closely related. Short interfering RNAs and mi RNAs share common RNAse III processing enzyme, the dicer enzymes and closely related effector complexes for post-transcriptional repression of protein synthesis. On the other hand, siRNAs and miRNAs differ in their molecular origins. In the cytoplasm the dicer enzymes split the dsRNA primer molecules. The finished siRNAs in animals are usually 21-22 nitrogen bases long, similar to miRNAs.

Micro-RNAs (miRNAs):

Micro-RNAs are a class of small, non-coding RNAs that regulate gene expression in a sequence specific manner as required in embryonic development. Micro-RNAs have been found throughout diverse eukaryotes genomes including plants. They can inhibit protein expression by shutting off translation or by targeting mRNA for degradation. Micro-RNAs were first discovered in 2001 in the widely studied worm *Caenorhabdtis elegans*.

Micro-RNAs genes produce short (~22 nitrogen bases) ss segments that fold over on themselves forming a short section of dsRNA in hairpin like structure. Humans express over 460 genetically encoded miRNAs. These miRNAs make up more than 1% of human genome and may regulate over 30% of all protein coding genes. Micro-RNAs can pair exactly with a mRNA and cause its cleavage and destruction, or it can pair partially with mRNA and produce translational inhibition (block the ribosome). It is presumed that they are involved in regulating development by controlling as transcriptional factor.

mRNA modifications:

1. Capping: Early in the elongation process, the 5' ends ofeukaryotic pre-mRNAs are modified by the addition of 7-methyl guanosine (7-MG) caps. These 7-MG caps are added when the growing RNA chains are only about 30 nucleotides long. The 7-MG cap contains an unusual 5-5' triphosphate linkage and two or more methyl groups. These 5' caps are added co-transcriptionally by the biosynthetic pathway. The 7-MG caps are recognized by protein factors involved in the initiation of translation and also help protect the growing RNA chains from degradation by nucleases.

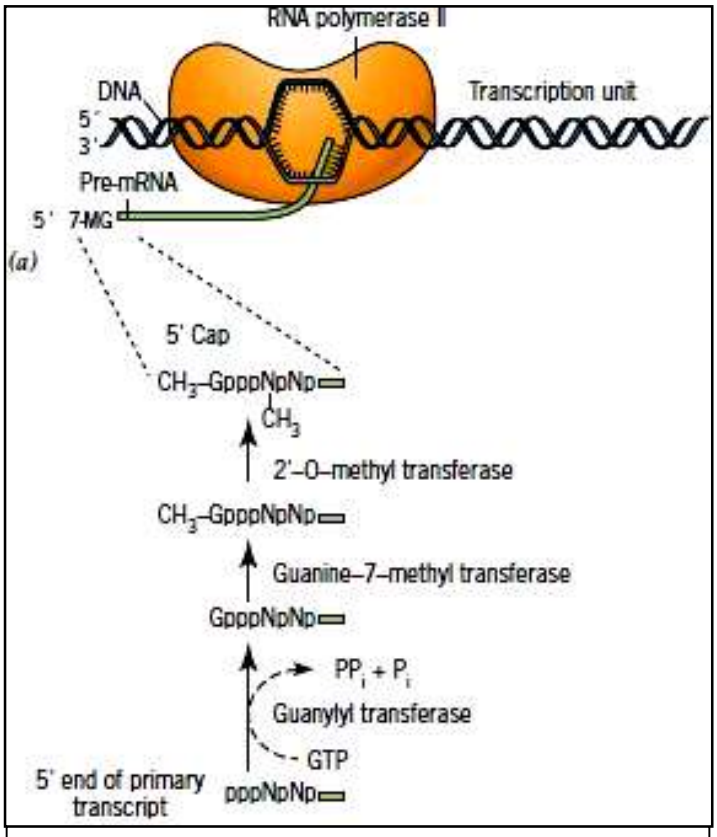
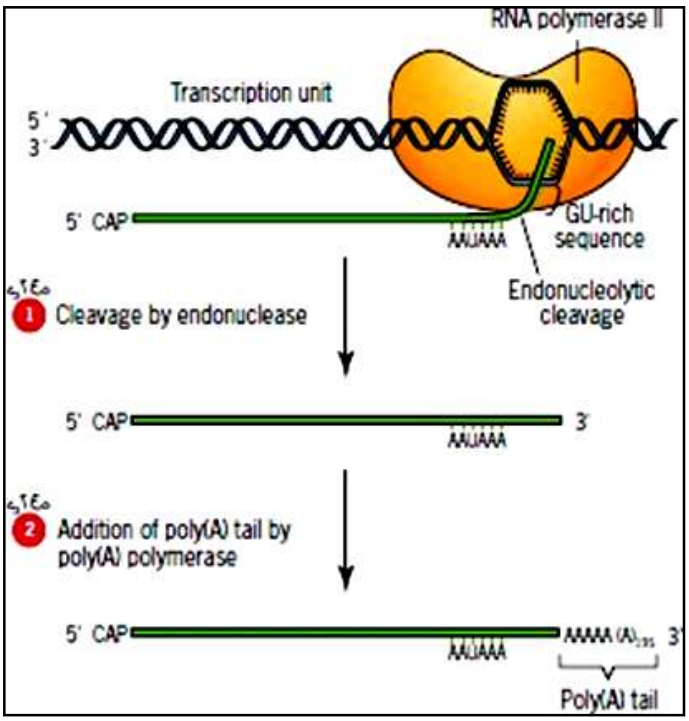


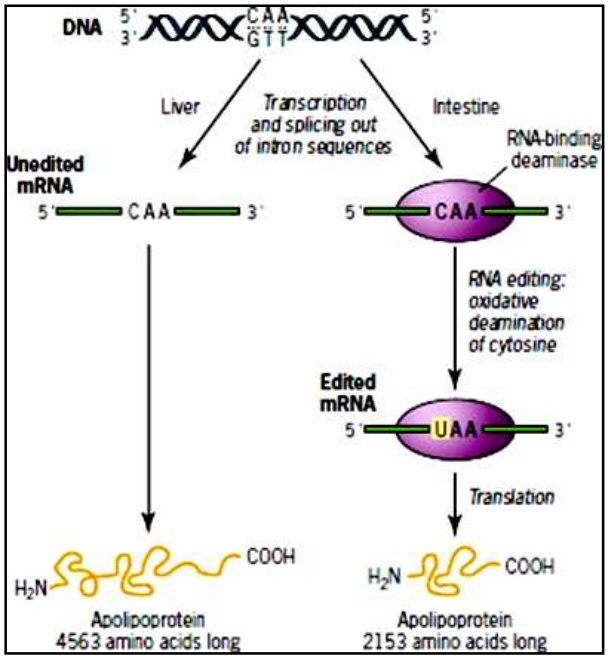
Fig: Pathway of biosynthesis of the 7-MG cap

2. Poly A tail: The cleavage event that produces the 3' end of a transcript usually occurs at a site 11 to 30 nucleotides downstream from a conserved polyadenylation signal, consensus AAUAAA, and upstream from a GU-rich sequence located near the end of the transcript. After cleavage, the enzyme poly(A) polymerase adds poly(A) tails, tracts of adenosine monophosphate residues about 200 nucleotides long, to the 3' ends of the transcripts. The addition of poly(A) tails to eukaryotic mRNAs is called polyadenylation. To examine the polyadenylation signal of the human HBB (globin) gene, check out Solve It: Formation of the 3'-Terminus of an RNA Polymerase II Transcript. The formation of poly tails on transcripts requires a specificity component that recognizes and binds to the AAUAAA sequence, a stimulatory factor that binds to the GU-rich sequence, an endonuclease, and the poly(A) polymerase. These proteins form amultimeric complex that carries out both thecleavage and

the polyadenylation in tightly coupled reactions. The poly(A) tails of eukaryotic mRNAs enhance their stability and play an important role in their transport from the nucleus to the cytoplasm.



3. RNA editing: Normally, the genetic information is not altered in the mRNA intermediary. However, the discovery of RNA editing has shown that exceptions do occur.



RNA editing processes alter the information content of gene transcripts in two ways: (1) by changing the structures of individual bases and (2) by inserting or deleting uridine monophosphate residues.

The first type of RNA editing, which results in the substitution of one base for another base, is rare. This type of editing was discovered in studies of the apolipoprotein-B (apo-B) genes and

mRNAs in rabbits and humans. Apolipoproteins are blood proteins that transport certain types of fat molecules inthe circulatory system. In the liver, the apo-B mRNA encodes a large protein 4563 amino acids long. In the intestine, the apo-B mRNA directs the synthesis of a protein only 2153 amino acids long. Here, a C residue in the pre-mRNA is converted to a U, generating an internal UAA translation– termination codon, which results in the truncated apolipoprotein.

4. Splicing: This process takes place in the nucleus and involves the removal of noncoding intron sequences from pre-mRNAs to produce mature mRNAs in which the coding sequences, corresponding to the exons, are continuous. The mature spliced mRNA, an accurate template for protein synthesis, is then exported to the cytoplasm where it acts as a templatefor protein synthesis. Splicing depends on the presence of signal sequences in the pre-mRNA. In almost all genes, the first two nucleotides at the 5′ end of an intron are GT and the last two at the 3′ end are AG. These are part of larger signal sequences present at the 5′ and 3′ ends of the introns. The complete 5′ signal sequence is 5′ AGGTAAGT 3′ and the 3′ sequence is 5′ YYYYYYNCAG 3′ (Y = pyrimidine, N = any nucleotide).

A branch point sequence is present in vertebrates, in the introns 10-40 bases upstream of the 3′ signal sequence. A morespecific sequence 5′ UACUAAC 3′, occursin introns of yeast. Splicing occurs in two steps (Fig. 16.8A). In the first step, the 2′ hydroxyl group of the adenine of the branch point sequence attacks the phosphodiester bond 5′ to the G of the GT (5′ splice site).

Splicing a pre-mRNA in Eukaryotes:

The bond is broken releasing the 5′ end of the intron and attach•ing it to the branch point sequence. The intron now forms a tailed loop structure called a lariat. In the second step, the 3′ end of the Intron iscleaved after G of the AG (3′ splice site), the intron is released and the two exon sequences are joined together.

Splicing is catalyzed by a group of molecules called small nuclear ribonucleoproteins (snRNPs) – U1, U2, U4, U5 and U6. These are composed of small RNA molecules rich in uracil, called U RNAs or small nuclear RNAs (snRNAs) that exist complexed with proteins. The U1 snRNP binds to the 5′ splice site and the U2 snRNP binds to the branch point sequence.

The remaining snRNPs, U5 and U4/U6, then form a complex with U1 and U2 causing the

intron to loop out and the exons to be brought together. The combination of the pre-mRNA and the snRNPs is called the spliceosome and this is responsible for folding the pre-mRNA into the correct conformationfor splicing (Fig.).

The spliceosome also catalyzes the cutting and joining reactions that excise the intron and ligate the exons. Once splicing is completed the spliceosome dissociates.

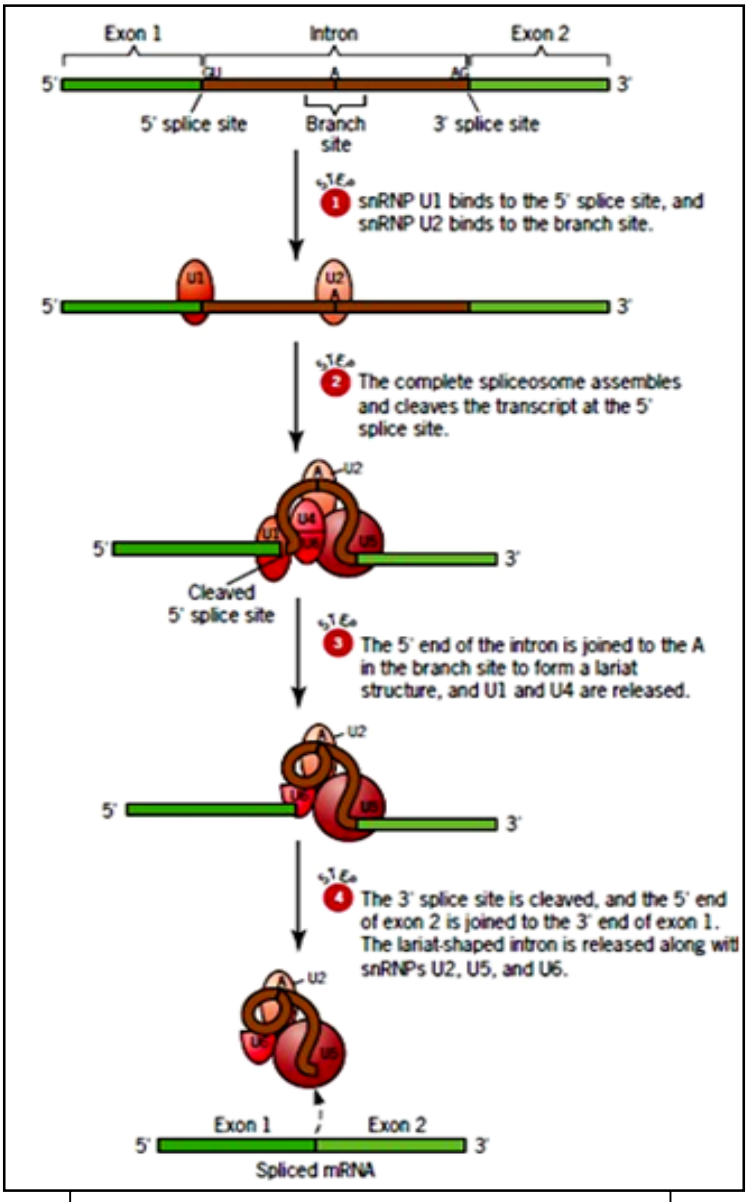
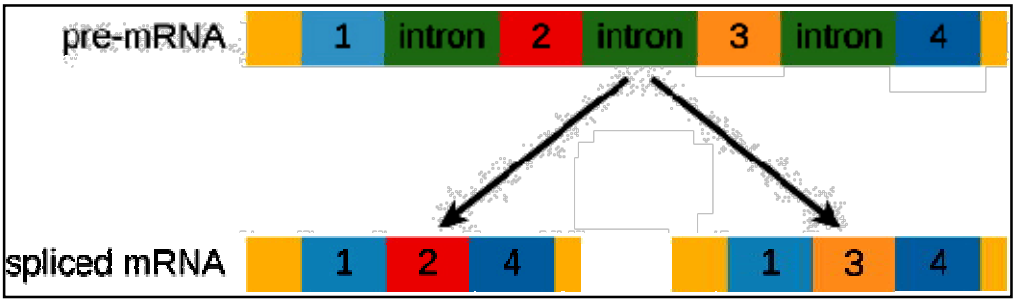


Fig.: Splicing a pre-mRNA in Eukaryotes

Alternative splicing:

Most pre-mRNA molecules have sections that are removed from the molecule, called introns, and sections that are linked or together to make the final mRNA, called exons. This process is called splicing. In the process of alternative splicing, different portions of an mRNA can be selected for use as exons. This allows either of two (or more) mRNA molecules to be made from one pre-mRNA.



Alternative splicing is not a random process. Instead, it is typically controlled by regulatory proteins. The proteins bind to specific sites on the pre-mRNA and "tell" the splicing factors which exons should be used. Different cell types may express different regulatory proteins, so different exon combinations can be used in each cell type, leading to the production of different proteins.

Small Regulatory RNAs and their role in gene silencing:

Small Regulatory RNAs in eukaryotes fall into two main groups-

1. miRNAs (microRNAs)
2. siRNAs (short interfering RNAs)

Both of these RNAs are non-coding, i.e., they are untranslated and therefore do not specify a polypeptide product.

1. miRNAs:

- MicroRNAs are ssRNA regulatory molecules about 21-23 nucleotide (nt) long that derivefrom RNA transcripts.
- These miRNAs are coded by genes in the genome of all multicellular eukaryotes, as well as some unicellular ones (budding yeast- *Saccharomyces cerevisiae*).
- In humans, several hundred miRNA genes are scattered throughout all the

chromosomesexcept of the Y chromosome.

- ~30% of mammalian miRNA genes are located in intergenic regions i.e. between protein-coding genes of the genome.
- They are transcribed by RNA polymerase II resulting in capped, polyadenylated transcripts.
- Some miRNA genes are located in transposons.
- Rests are situated within other genes- many are in introns of protein-coding genes, whilesome are in introns and exons of non-protein coding genes.
- In all cases, the miRNA sequence is transcribed by an RNA polymerase as part of thetranscript of the host gene.
- In few cases, an intron-located miRNA gene is transcribed independently by RNA polymerase II.

2. MicroRNAs (miRNAs) mediated gene silencing:

- The transcript containing a miRNA is called the primary miRNA transcript or pri-miRNA.
- The pri-miRNA molecule contains a hairpin structure about 70 nt long, within which is theeventual miRNA.
- The hairpin is cut out of the pri-miRNA in the nucleus by the dsRNA-specific endonucleaseDrosha complexed to an accessory protein (*Pasha* in *Drosophila*).
- Drosha makes staggered cuts resulting in a ~2 nt 3' single-stranded overhang.
- The excised hairpin- pre-miRNA is exported rapidly to the cytoplasm.
- In the cytoplasm, another dsRNA-specific endonuclease, Dicer, complexed to an accessory protein (*Loq* in *Drosophila*), makes staggered cuts in the pre-miRNA, releasing a short miRNA:miRNA* dsRNA consisting of some the former paired sides of the hairpin.
- The two RNA strands are imperfectly paired: "miRNA" is the mature miRNA strand that subsequently functions in the cell for RNA silencing, while miRNA* is its partial complement and does not function in RNA silencing.
- Because the miRNA directs RNA silencing, it is termed the guide strand, while the miRNA* is termed the passenger strand.

- Next the dsRNA, Dicer and accessory protein bind to Ago1, a member of the Argonaute family of protein and other proteins to form the pre-microRNA-induced silencing complex, orpre-miRISC.
- Ago1 is another RNA endonuclease; more generally called Slicer. It makes a single cut withinthe miRNA* passenger strand.
- A helicase that is part of the pre-miRISC then unwinds to two pieces from the miRNA guidestrand, and they dissociate from the complex.
- The result is the mature miRISC, the ribonucleoprotein complex that can silence geneexpression.

How does a miRISC function in Post-transcriptional gene silencing?

(i) The miRNA in the miRISC is a trans-acting RNA regulatory molecule, meaning that it targetsmRNAs that are not the same as the RNA molecules from which the miRNA is derived. This is one distinguishing feature of miRNAs compared with siRNAs.

(ii) A miRISC binds to a target mRNA through complementary base pairing involving the miRNA.

(iii) Usually, the sequence to which the miRNA binds are short sequence in the 3' UTR of themRNA.

(iv) An mRNA molecule may have one or more sequences in its 3' UTR to which the same miRNA can bind and/or it may have several sequences in its 3' UTR to which several different miRNAs can bind. The latter raises the possibility of regulating the expression of the same gene (through its mRNA) by various combinations of miRNA regulator molecules.

(v) Here, one miRISC is shown binding to a 3' UTR sequence for simplicity.

(vi) Binding of most of the miRISC to their target mRNAs involves imperfect pairing between themiRNA and the 3' UTR region of the mRNA.

(vii) Such pairing triggers translational repression – translation of that mRNA becomes inhibited.

(viii) The translationally repressed mRNA with its associated miRISC(s) is then sequestered from the translation machinery by becoming or moving into a P body.

(ix) P body is a cytoplasmically located aggregate of translationally repressed mRNAs complexed with proteins, and proteins for mRNA decapping and mRNA degradation.

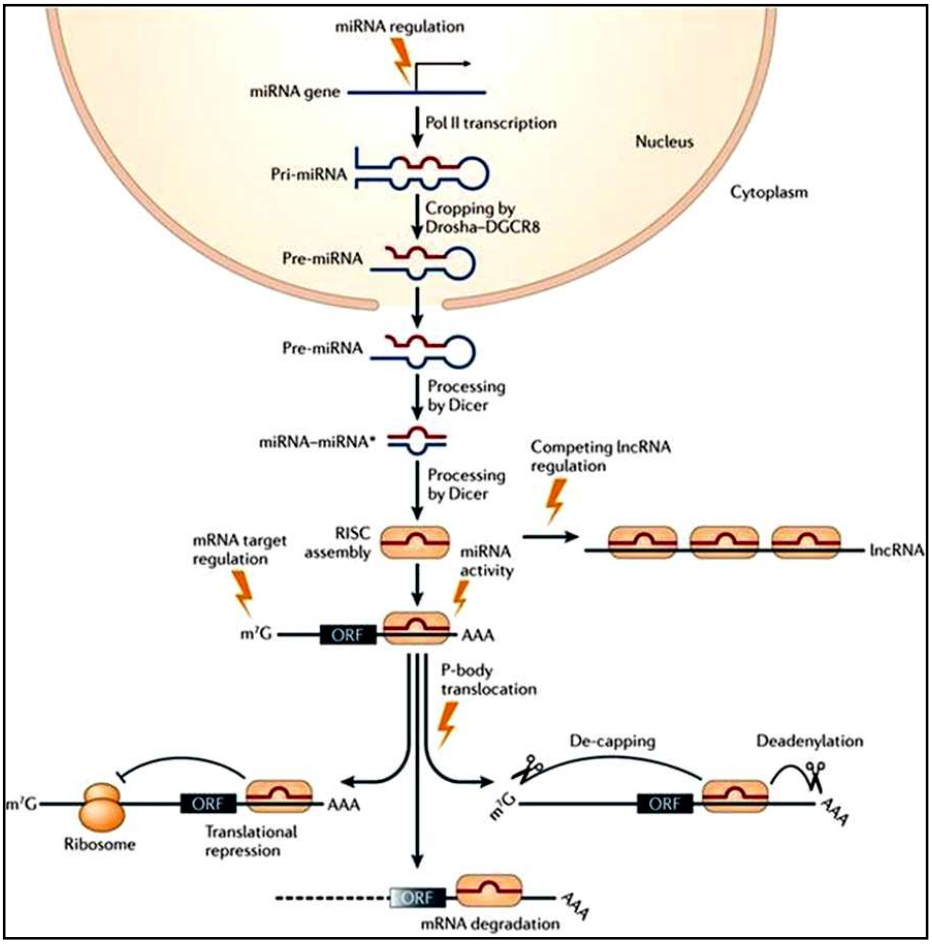
(x) The mRNAs is P bodies may be degraded using the contained mRNA degradation machinery or stored in ribonucleoprotein complexes.

(xi) Stored mRNAs can be returned to translation at a later time. Whether degraded or stored the effect of miRNA action is to reduce the expression of the gene encoding the targeted mRNAat the translational level.

(xii) In plants, binding of most miRISCs to their target mRNAs involves perfect or near-perfect pairing between much of the miRNA and the 3' UTR region of the mRNA.

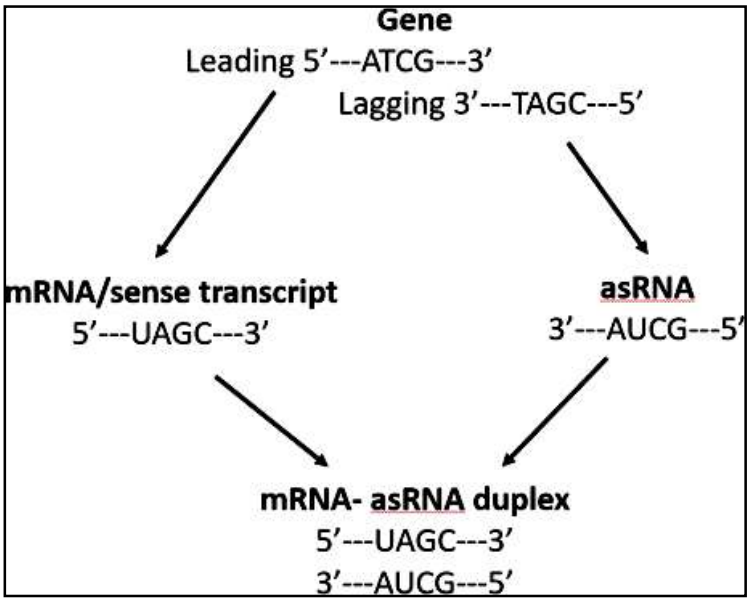
(xiii) Perfect pairing triggers mRNA degradation rather than translational repression.

(xiv) Here, the Ago1 Slicer protein cuts the target mRNA into two and the mRNA-miRISC complex forms, or it is moved to a P body where degradation of the mRNA is completed.



Antisense RNA:

Antisense RNA (asRNA), also referred to as antisense transcript, natural antisense transcript (NAT) or antisense oligonucleotide, is a single stranded RNA that is complementary to a protein coding messenger RNA (mRNA) with which it hybridizes, and thereby blocks its translation into protein. as RNAs (which occur naturally) have been found in both prokaryotes and eukaryotes, antisense transcripts can be classified into short (<200 nucleotides) and long (>200 nucleotides) non-coding RNAs (ncRNAs). The primary function of asRNA is regulating gene expression. asRNAs may also be produced synthetically and have found wide spread use as research tools for gene knockdown. They may also have therapeutic applications.



Ribozymes:

Ribozymes are catalytic RNA enzymes that act to alter covalent structure in other classes of RNAs and certain molecules. They occur in ribosomes, nucleus and chloroplasts of eukaryotic organisms. Some viruses including several bacteriophages also have ribozymes. An optimum concentration of metal ions such as Mg^{+2} and K^{+2} is associated with their effective functioning. Ribozymes generally act as molecular scissors cutting precursor RNA molecules at specific sites. Surprisingly, they also serve as molecular staplers, which ligate or join two RNA

molecules together. Ribozymes are involved in the transformation of large precursor molecules of tRNA, rRNA and mRNA into smaller final products. In their active form, ribozymes are complexed with protein molecules, e.g., the enzyme ribonuclease-P (RNAse-P) is found in all living cells.

Riboswitch:

RNA domains at the front of messenger RNA, referred to as riboswitches, can directly interact with small molecules and can control gene expression. The vast majority of riboswitches have been found in bacteria and so far it is only in bacteria that experimental evidence for riboswitch operation exists. Three distinct tricks for switching gene expression have been revealed:

- The RNA element can cause premature termination of transcription of the mRNA, it can block ribosomes from translating the mRNA, or it can even cleave the mRNA and thereby promote its destruction. This involves an RNA unit directly binding a small-molecule metabolite, which switches the RNA into a conformation that activates its intrinsic self-cleavage activity. This "ribozyme riboswitch" represents a new type of biological activity for a catalytic RNA.
- Many antibiotics bind to ribosomal RNAs and selectively inhibit bacterial growth.
- Riboswitches might also be targeted by new classes of antibiotics.

Given the significant role that riboswitches play in bacterial genetic control and the fact that they haveevolved to bind metabolites, drug compounds could be created that disrupt bacterial genetic control.

Engineered riboswitches might function as designer genetic control elements.

TPP riboswitch: this riboswitch binds TPP (thiamin pyrophosphate inorder to regulate the transport and synthesis of thiamin as well asother metabolites with similar properties.

Lysine riboswitch: binds to lysine and regulates its biosynthesis, catabolism, and transport.

Glycine riboswitch: this riboswitch regulates glycine metabolism. This is the only riboswitch knowncurrently to be able to perform cooperative binding.

FMN riboswitch: this riboswitch binds FMN (flavin mononucleotide) in order to regulate the

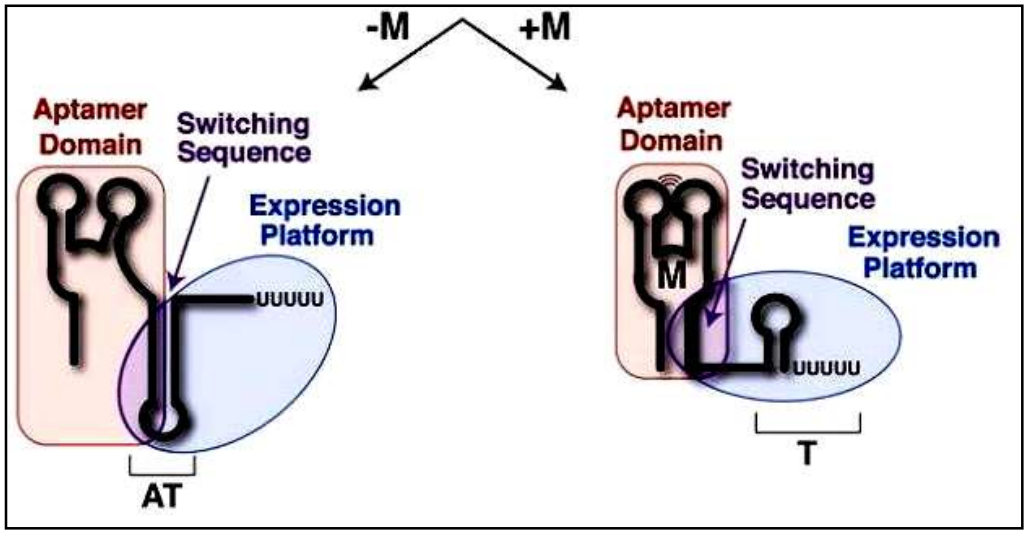
transport and synthesis of riboflavin.

Purine riboswitch: binds purines to regulate its transport and metabolism. Different forms of this riboswitch are able to bind either guanine or adenine depending on the pyrimidine in the riboswitch.

Cobalamin riboswitch: this riboswitch binds adenosylcobalamin, the coenzyme form of B12 vitamin, in order to moderate the synthesis and transport of cobalamin and other similar metabolites. as well as many others such as SAM riboswitch, PreQ1 riboswitch, SAH riboswitch, glmS riboswitch,and cyclic di-GMP riboswitch.

Structure:

- Two domains
- Aptamer-(Ligand binding domain)
- Ligand recognation and binding.
- Highly conserved.
- Expression platform.
    - Less conserved
    - Adopts two mutually exclusive conformations.
    - Shine-dalgano sequence locates in this domain.



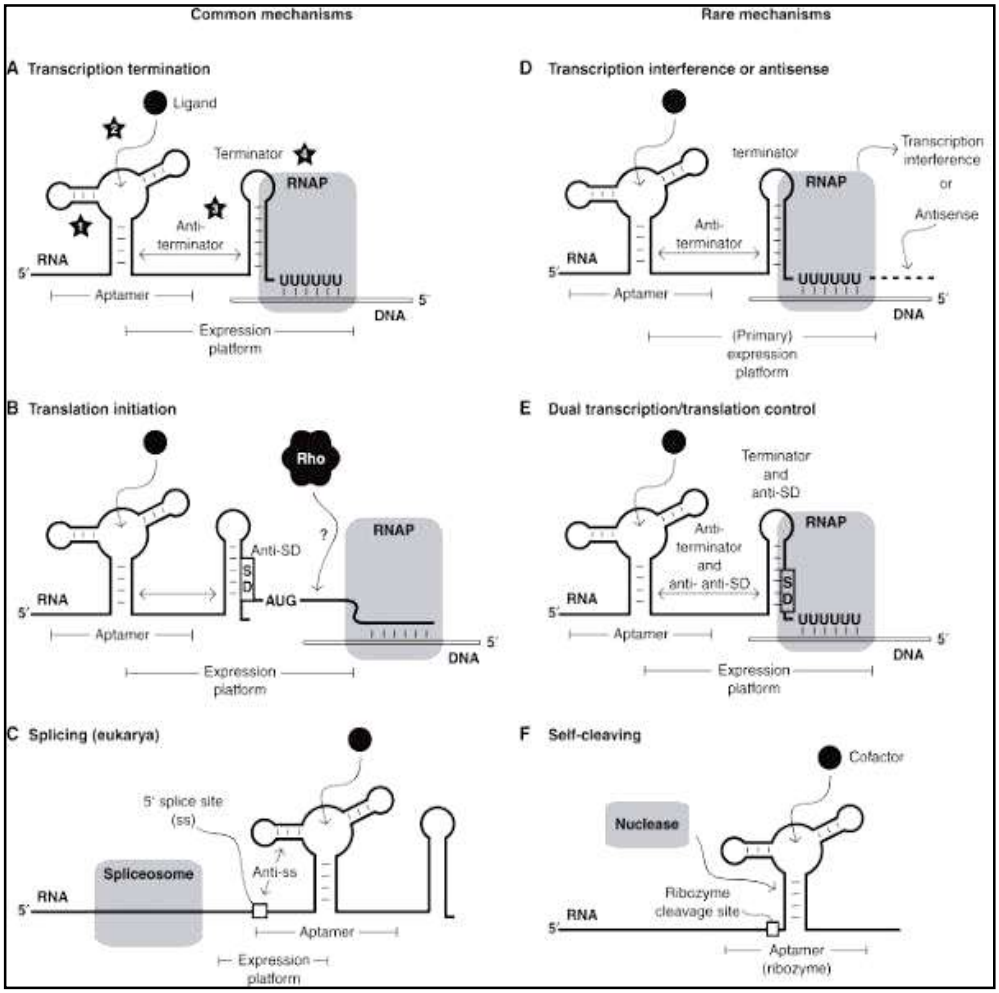
Mechanism:

Three mechanisms are as follows –

a) The terminator loop reduces the stability of either the mRNA:RNA polymerase interaction and/or of the DNA:RNA hybrid causing the RNA polymerase to dissociate, terminating transcription prematurely

b) When no metabolite is bound, the Shine-Dalgarno (SD) site is exposed- ribosome can bind and initiate translation. Binding of the metabolite to the 5' leader region of the mRNA induces the formation of an SD: anti-SD stem-loop structure that masks the ribosome binding site such that initial step of translation, is not achieved.

c) The conformational change induced by the binding of the ligand to the riboswitch brings adjacent nucleotides in line with each other in an orientation that favours cleavage-ribozyme action eg. Glms box.



Application:

- Synthetic analogs of riboswitch ligands could be engineered to shut off central metabolic pathways, arresting the growth of the bacteria-less toxic as RNA is targeted instead of protein.
- Can be used in the synthetic aptamers that led to their discovery: as molecular chemosensors for measuring chemical composition or biochemical secretions
- Using riboswitch fusions to trans-genes as a means to regulate gene inserts through small molecule inducers. This could have widespread applications in genetic research, and even in medicine and gene therapy.
- Use of riboswitches in taxonomic studies: Though regions of riboswitches are well-conserved, there are distinct variable regions that have been indicated as being dependant on taxonomy.

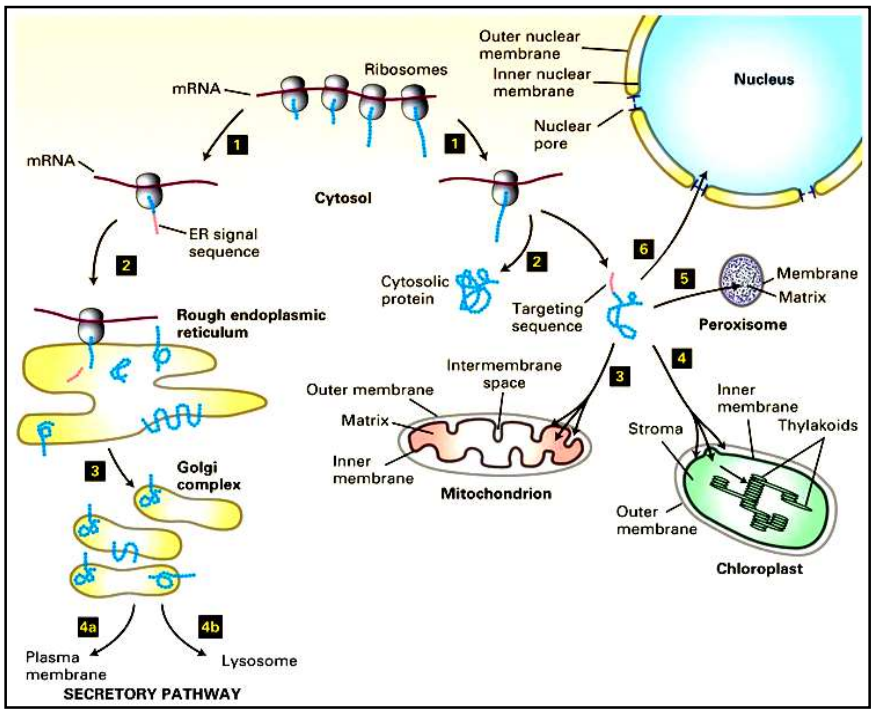
5. Protein sorting: Protein targeting in organelles.

Introduction:

Both in prokaryotes and eukaryotes, newly synthesized proteins must be delivered to a specific sub cellular location or exported from the cell for correct activity. Protein targeting is necessary for proteins that are destined to work outside the cytoplasm. This delivery process is carried out based on information contained in the protein itself. Correct sorting is crucial for the cell; errors can lead to diseases.

The endomembrane system and secretory pathway:

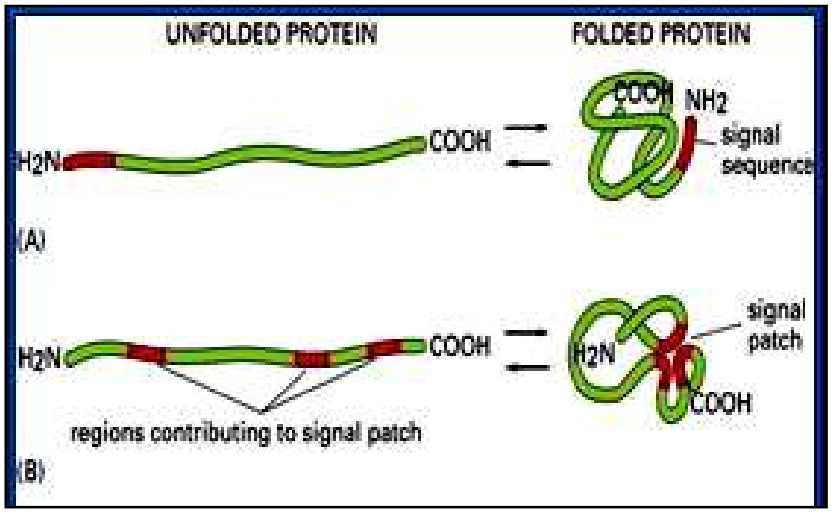
Proteins destined for any part of the endomembrane system (or the outside of the cell) are brought to the ER during translation and fed in as they are made.



Types of Sorting Signals in Proteins

1. **Signal Sequence**
   - *continuous sequence of 15-60 aminoacids (aa's)*
   - *sometimes removed from finishedprotein*
   - *sometimes a part of finished protein*

2. **Signal Patch**
   - *specific 3D arrangement of atoms onprotein surface; aa's distant*
   - *persist in finished protein*



Signal peptide:

The signal peptide that sends a protein into the endoplasmic reticulum during translation is a series of hydrophobic ("water-fearing") amino acids, usually found near the beginning (N-terminus) of the protein. When this sequence sticks out of the ribosome, it's recognized by a protein complex called the signal-recognition particle (SRP), which takes the ribosome, to the ER. There, the ribosome feeds its amino acid chain into the ER lumen (interior) as it's made.

(i) Signal recognition particle (SRP) binds to the signal peptide as it emerges from the ribosome.

(ii) SRP brings the ribosome to the ER by binding to a receptor on the ER surface. The receptor isassociated with other proteins that make a pore.

(iii) The ribosome resumes translating, feeding the polypeptide through the pore and into the ERlumen (interior).

(iv) An enzyme associated with the pore snips off the signal peptide.

(v) Translation continues, and the growing amino acid chain slides into the ER lumen.

(vi) The completed polypeptide is released into the ER lumen, where it floats freely.

(vii) In some cases, the signal peptide is snipped off during translation and the finished protein is released into the interior of the ER (as shown above). In other cases, the signal peptide or another stretch of hydrophobic amino acids

gets embedded in the ER membrane. This creates a transmembrane (membrane-crossing) segment that anchors the protein to the membrane.

Table: Some Typical Signal Sequences

FUNCTION OF SIGNAL SEQUENCE	EXAMPLE OF SIGNAL SEQUENCE
Import into nucleus	-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-
Export from nucleus	-Leu-Ala-Leu-Lys-Leu-Ala-Gly-Leu-Asp-Ile-
Import into mitochondria	$^{+}H_3N$-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-
Import into plastid	$^{+}H_3N$-Met-Val-Ala-Met-Ala-Met-Ala-Ser-Leu-Gln-Ser-Ser-Met-Ser-Ser-Leu-Ser-Leu-Ser-Ser-Asn-Ser-Phe-Leu-Gly-Gln-Pro-Leu-Ser-Pro-Ile-Thr-Leu-Ser-Pro-Phe-Leu-Gln-Gly-
Import into peroxisomes	-Ser-Lys-Leu-COO^-
Import into ER	$^{+}H_3N$-Met-Met-Ser-Phe-Val-Ser-Leu-Leu-Leu-Val-Gly-Ile-Leu-Phe-Trp-Ala-Thr-Glu-Ala-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln-
Return to ER	-Lys-Asp-Glu-Leu-COO^-

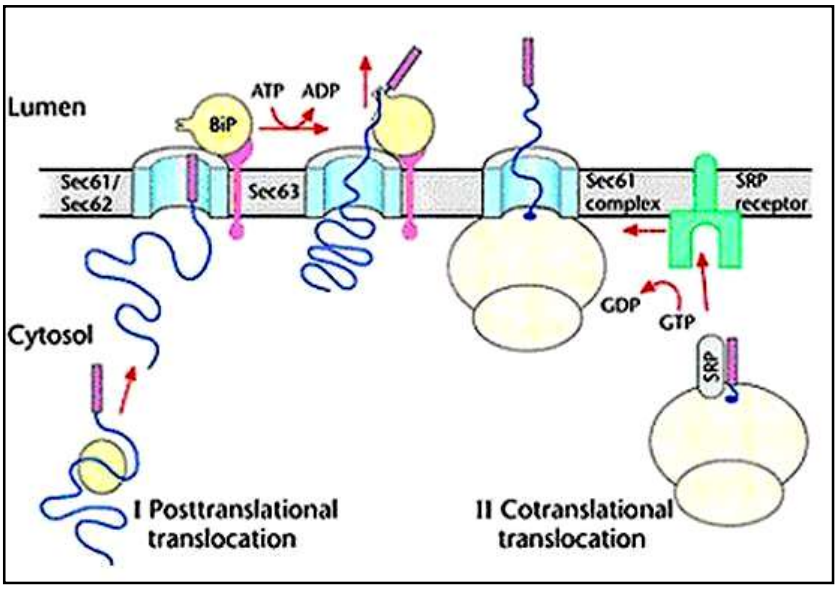
Some characteristic features of the different classes of signal sequences are highlighted in color. Where they are known to be important for the function of the signal sequence, positively charged amino acids are shown in *red* and negatively charged amino acids are shown in green. Similarly, important hydrophobic amino acids are shown in *yellow* and hydroxylated amino acids are shown in *blue*. $^{+}H_3N$ indicates the N-terminus of a protein; COO^- indicates the C-terminus.

Protein translocation:

In 1970, **Günter Blobel** conducted experiments on the translocation of proteins across membranes. He was awarded the Nobel Prize (1999) for his findings. He discovered that many proteins have a signal sequence, that is, a short amino acid sequence at one end that functions like a postal code for the target organelle.

Sorting or translocation of protein can occur in two ways:

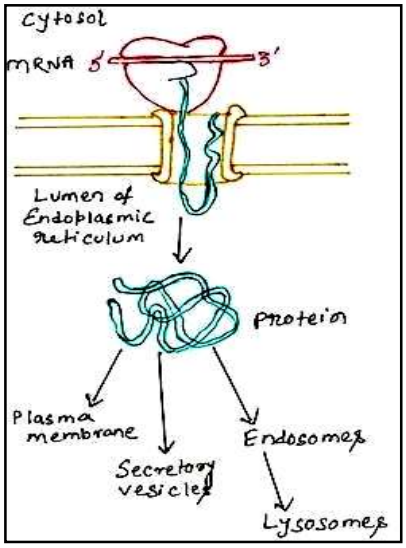
1. **Co-translational:** Synthesised protein is transferred to an SRP receptor on the endoplasmic reticulum (ER), a membrane-enclosed organelle. There, the nascent protein is inserted into the translocation complex
2. **Post-translational:** Even though most proteins are co translationally translocated, some are translated in the cytosol and later transported to their destination. This occurs for proteins that go to a mitochondrion, a chloroplast, or a peroxisome.

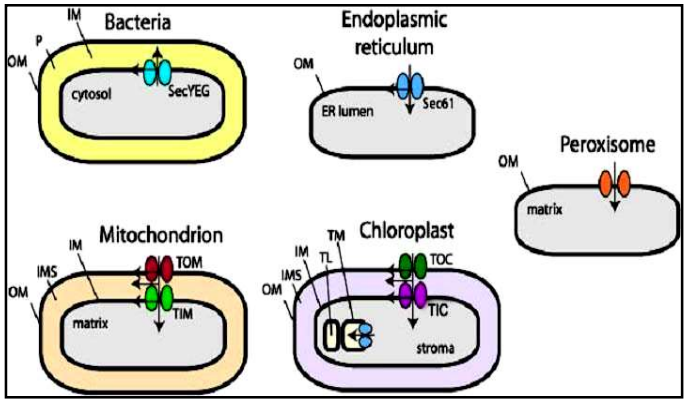


Transport through the endomembrane system:

Eukaryotic cells posses distinct membrane bound organelles which are absent in prokaryotic cells. The membrane bound organelles have different functions and these organelles provide discrete compartments in which specific cellular activities take place. The complex internal organization of eukaryotic cells generates hardship for transport of proteins to their destinations.

Many proteins destined for the endoplasmic reticulum, the Golgi apparatus, lysosomes, the plasma membrane and secretion from the cell are synthesized on ribosomes that are bound to the membrane of endoplasmic reticulum.





IM-inner membrane, IMS- inner membrane space, P- Periplasm, OM- outer membrane, TL-thylakoidlumen, TM- thylakoid membrane and Sec YEG, Sec61, TOM, TIM, TOC are the protein subunits of the translocation systems.

Protein targeting to the endoplasmic reticulum:

The signal sequences cover about twenty amino acids, including a stretch of hydrophobic residues, usually at the amino terminus of the polypeptide chain. As soon as the signal sequences of the growing polypeptide chain emerge from the ribosome, they are recognised and bound by a signal recognition particle (SRP) consisting of six polypeptides and a small cytoplasmic RNA (srp RNA). Then the complex containing the growing polypeptide chain, ribosome, and SRP is specifically targeted to the endoplasmic reticulum membrane by an interaction with a membrane-bounrd receptor, the SRP receptor or docking protein. In the next step, the SRP is released from both the ribosome and the signal sequence, where GTP (guanosine triphosphate) plays a key role. The ribosome then binds toa protein translocation complex in the membrane of the endoplasmic reticulum, and the signal sequence is inserted into a membrane channel or translocon. The translocons are complexes of three transmembrane proteins, known as Sec61 proteins. Transfer of the ribosome from the SRP to the translocon allows translation to resume, and the growing polypeptide chain is transferred directly into the translocon channel and across the membrane of the endoplasmic reticulum as translation proceeds. As translocation proceeds, the signal sequence is cleaved by the signal peptidase and the polypeptideis released into the lumen of the endoplasmic reticulum. Finally, GTP hydrolysis leads to the dissociation of the SRP from its receptor, and a new targeting cycle can begin. The actual transfer of the polypeptide through the membrane does not require the SRP or its receptor and commences only after their disengagement. Two basic functions are done by the SRP, where first it targets the polypeptide chain to the Endoplasmic reticulum membrane by interacting both with the signal sequence and with the translocation apparatus and secondly it keeps the bound signal sequence segregated from the rest of the polypeptide chain and thereby prevents aberrant, premature folding.

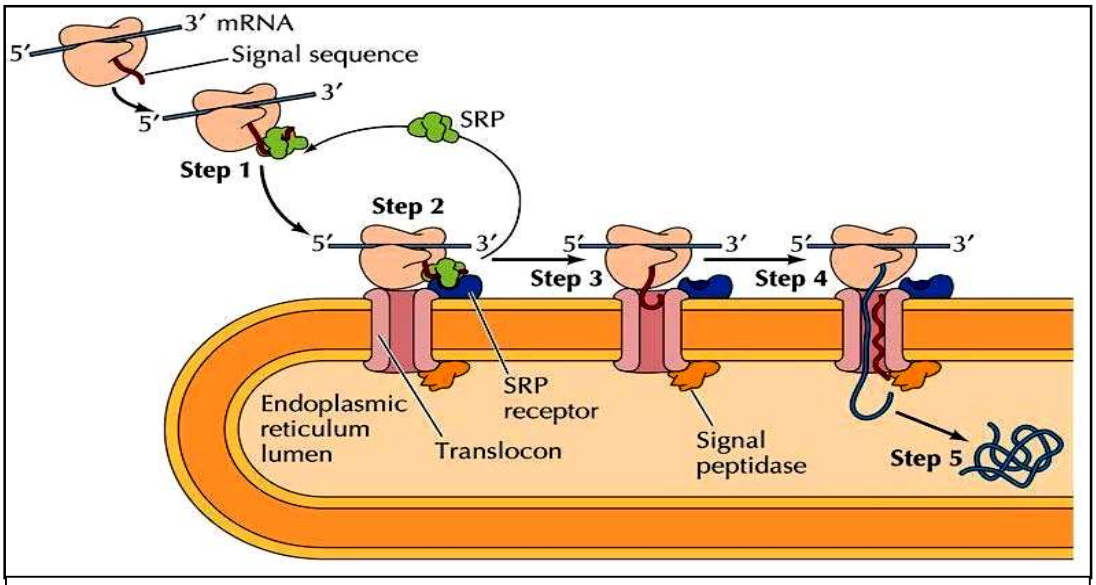


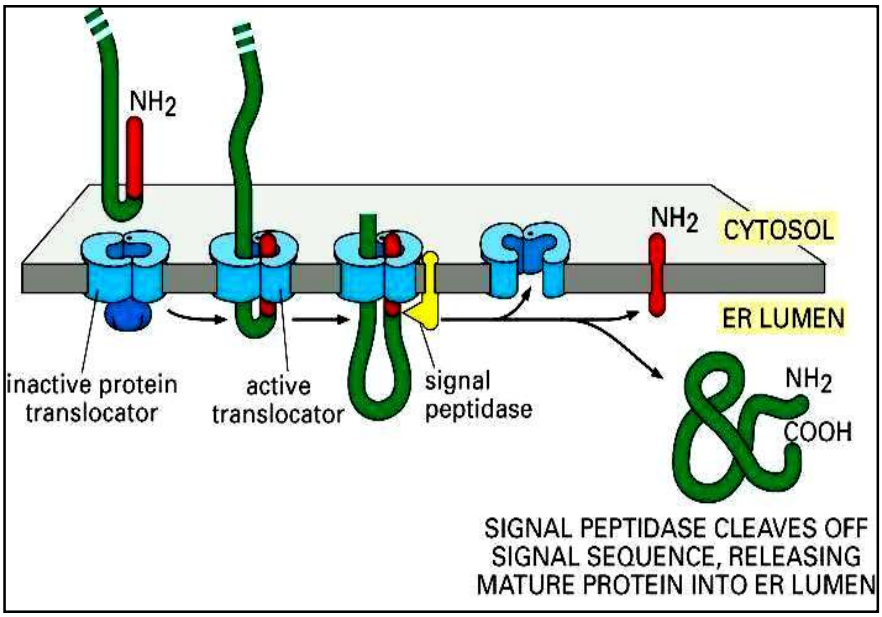
Fig.: The co-translational pathway of transport of secretory proteins to the endoplasmic reticulum.

Some proteins in mammals and many proteins in yeast are transported through post-translational pathway. These proteins are synthesized on free cytosolic ribosomes and these proteins do not require a signal recognition particle (SRP) for their transport. Their signal sequences are recognised by distinct receptor proteins associated with the translocon in the endoplasmic reticulum membrane. The polypeptide chains are remained in an unfolded conformation by the cytosolic Hsp70 chaperones.

ER and protein Trafficking:

Signal sequence is removed from soluble proteinstwo signaling functions:

I. Directs protein to ER membrane
II. Serves as “start transfer signal” to open poreSignal peptidase removes terminal ER signal sequence upon release of protein into the lumen



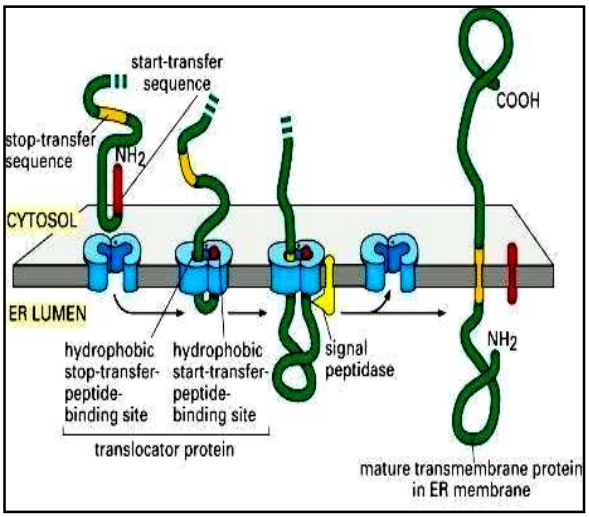
Signal Pass Transmembrane Proteins:

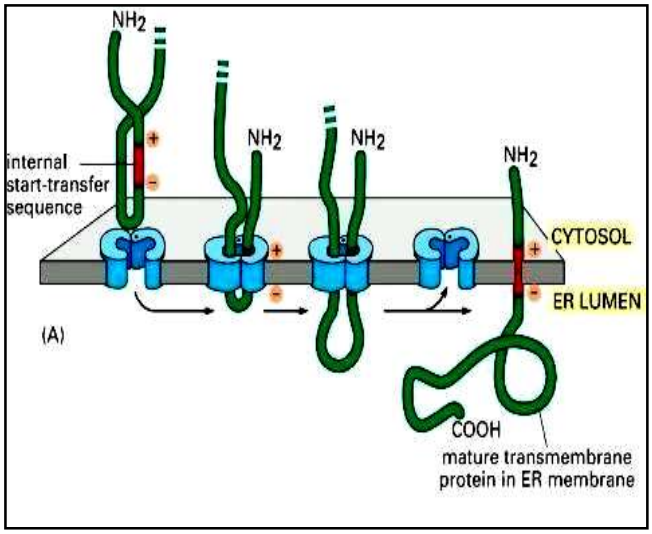
(i) N-terminal signal sequence initiates trans-location and additional hydrophobic "stop sequenceanchors protein in membrane

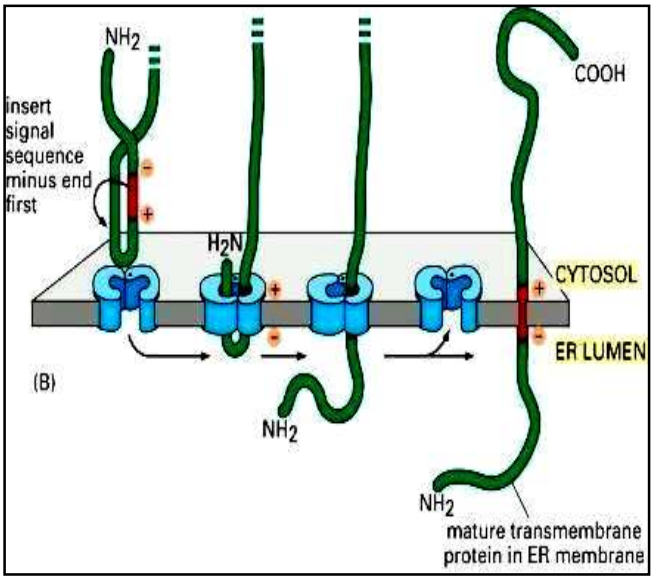
(ii) Signal sequence is internal and remains in lipid bilayer after release from translocator

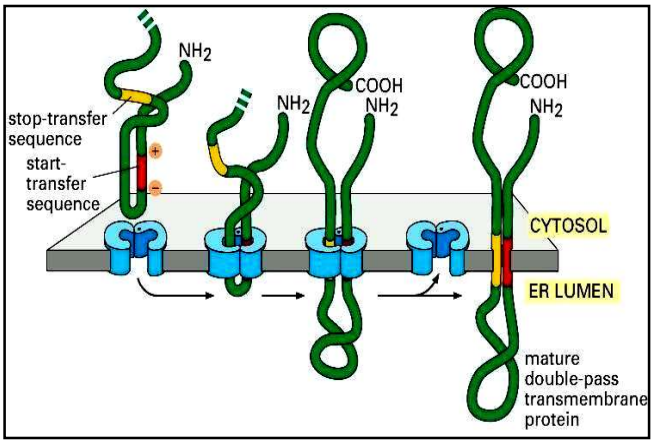
(iii) Internal signal sequence in opposite orientation

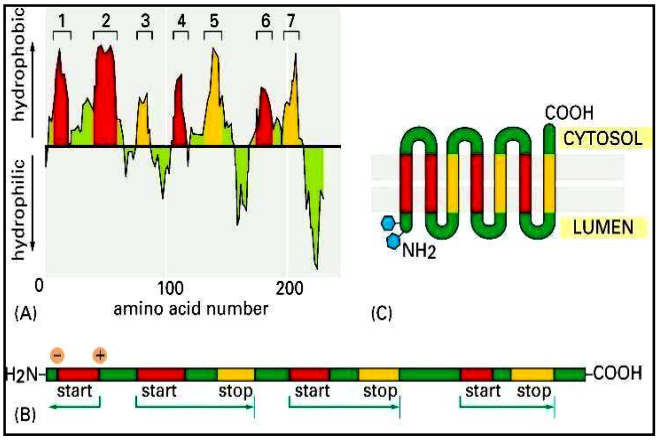
(iv) Orientation of start-transfer sequence governed by distribution of nearby charged amino acid




NH2
NH2
NH2
NH2
internal
start-transfer
sequence
CYTOSOL
ER LUMEN
(A)
COOH
mature transmembrane
protein in ER membrane


NH2
COOH
insert
signal
sequence
minus end
first
H2N
CYTOSOL
ER LUMEN
(B)
NH2
NH2
mature transmembrane
protein in ER membrane


NH2
COOH
NH2
COOH
NH2
stop-transfer
sequence
start-
transfer
sequence
CYTOSOL
ER LUMEN
mature
double-pass
transmembrane
protein



Multipass transmembrane Proteins:

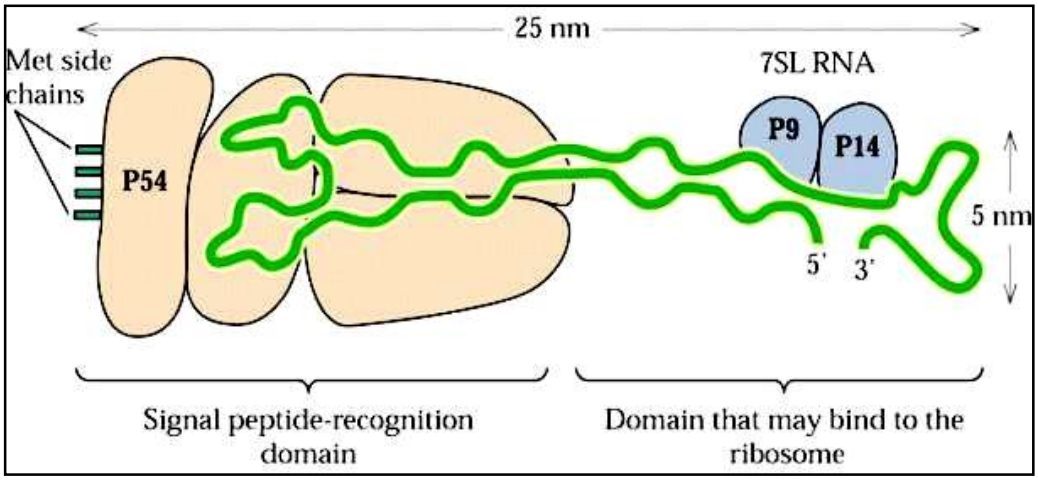
- Combinations of start- and stop-transfer signals determine topology
- Whether hydrophobic signal sequence is a start- or stop-transfer sequence depends upon itslocation in polypeptide chain
- All copies of same polypeptide have same orientation

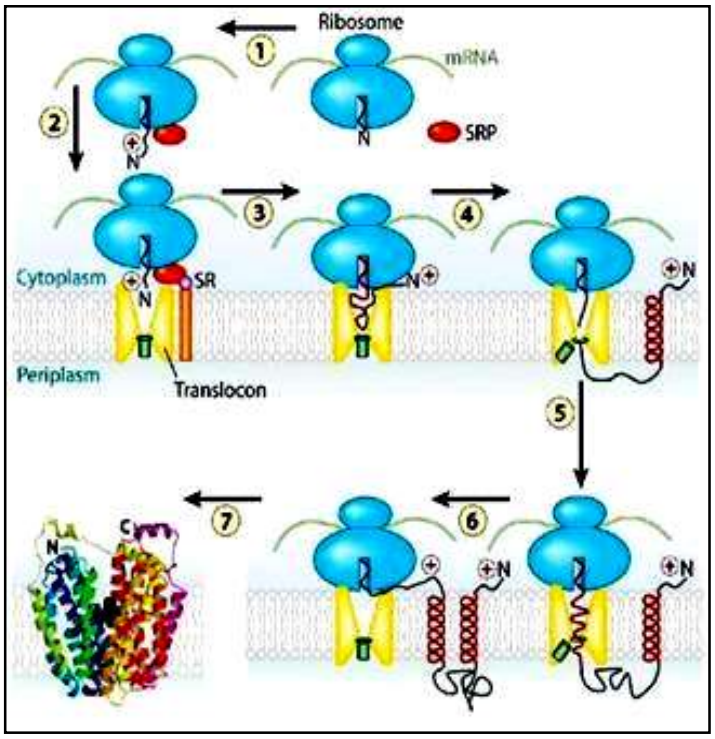
The signal and receptor machine:

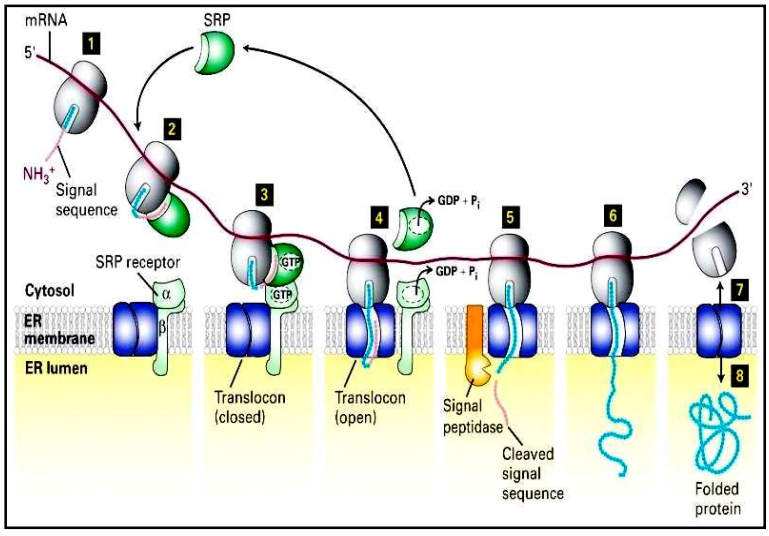
a) The signal peptide is often a hydrophobic stretch of 20-40 AA at the N-terminus of the protein.Cleaved after translocation into ER.

b) The SRP is a protein-RNA complex (one RNA of 300 base long and 5 proteins). SRP54 is the majorcomponent that recognizes and binds the signal peptide. It is a GTPase.

c) SRP is normally located in the cytosol but can associate to the membrane by interacting with itsreceptor in the ER membrane.

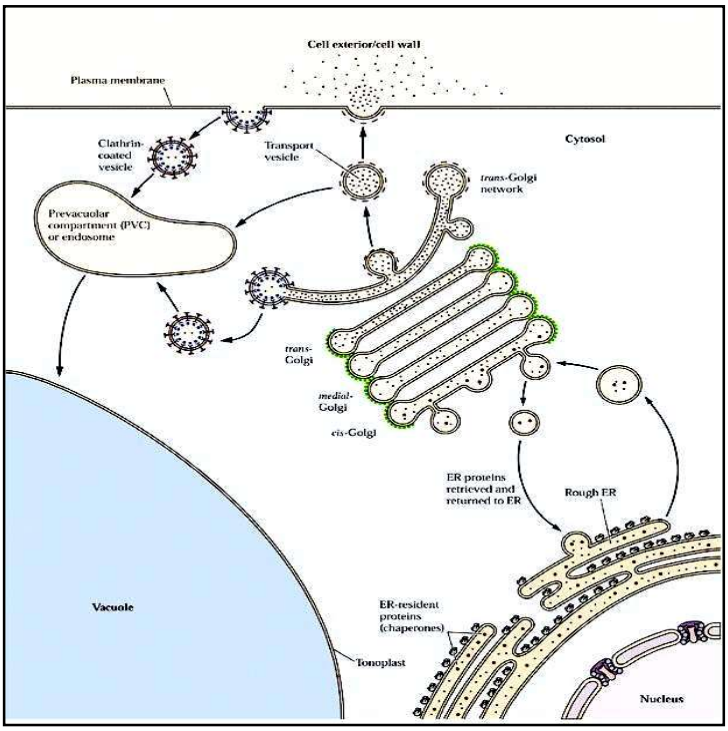






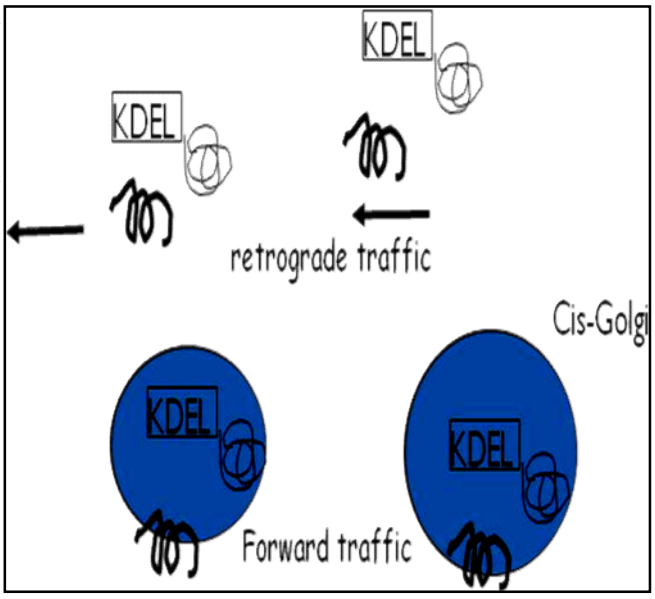
From ER to Golgi and beyond vesicular transport:

Cargo proteins are transported in a sealed container that is made of membrane vesicle.



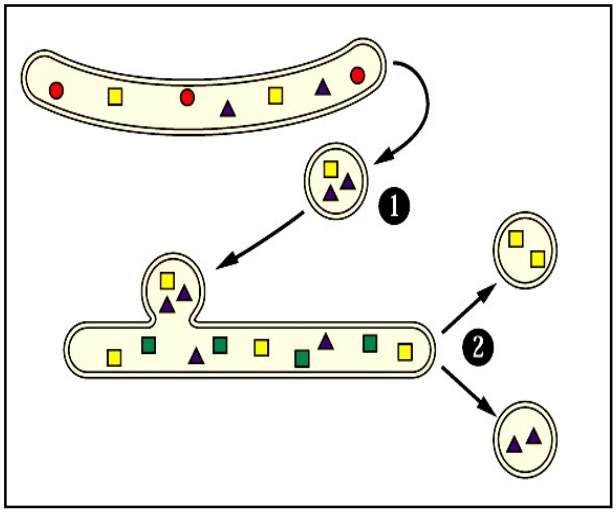
From Er to cis-Golgi:

- The Golgi complex: three separated compartments: (Close to ER) cis/medial/trans (farther fromER)
- Which proteins move forward from ER—cis Golgi? Forward traffic
- How does ER residents find ER destination? The retrograde traffic and ER retention signal KDELat the C-terminus of the ER lumen proteins.



From cis to medial and trans Golgi network (TGN):

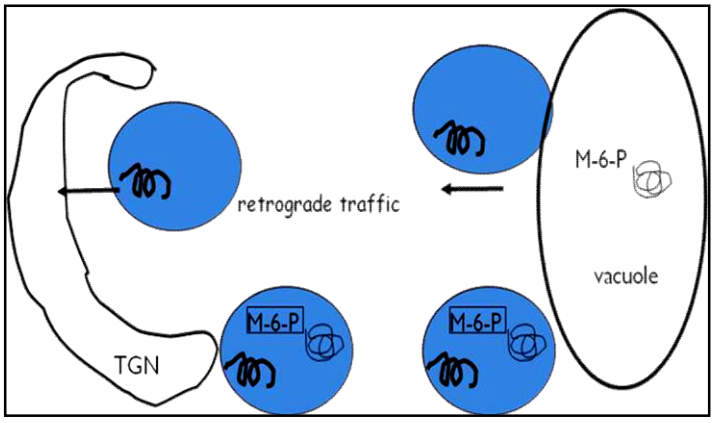
- Resident proteins stayed behind and other proteins move forward to the TGN.
- An important modification in Golgi is O-linked glycosylation following N-linked sugar addition in ER.
- Different compartments of Golgi (cis/medial/trans) have different enzymes that do different things to the protein passengers.



From TGN to vacuole or Plasma membrane/cell wall:

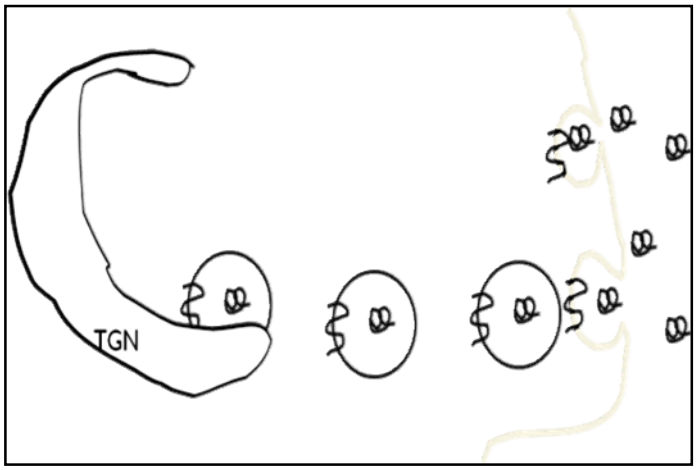
1) ***From TGN to lysosome/vacuole: a receptor-mediated pathway***

In animals and yeast: The receptors recognize a specific sugar label called mannose-6-phosphate added to the protein in cis-Golgi. The enzyme recognizes the specific protein substrates and phosphorylates the mannose-OH group. Such M-6-P serves as ligand for the receptors in the vesicular membrane.



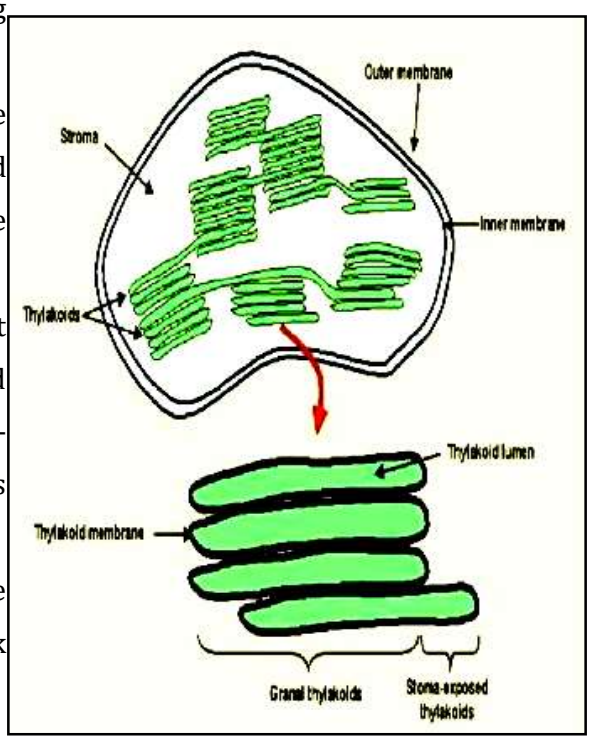
2) *From TGN to PM/CW: a default pathway*

All passengers without the M-6-P tag will be deposited to the vesicles that will fuse with plasma membrane and soluble proteins secreted out of the cell.



Sorting of proteins to chloroplast:

- The preprotein for **chloroplast** may contain a stromal import sequence or a stromal and thylakoid targeting sequence.
- The majority of preproteins are translocated through the Toc and Tic complexes located within the chloroplast envelope.
- In the stroma the stromal import sequence is cleaved off and folding as well as intra-chloroplast sorting to thylakoids continues.
- Proteins targeted to the envelope of chloroplasts usually lack cleavable sorting sequence.



3 pathways for protein targeting into and across thylakoid membranes (to lumen):

(i) secA-dependent

(ii) pH gradient-dependent (or Tat pathway)

(iii) SRP-dependent

secA-dependent pathway for protein targeting:

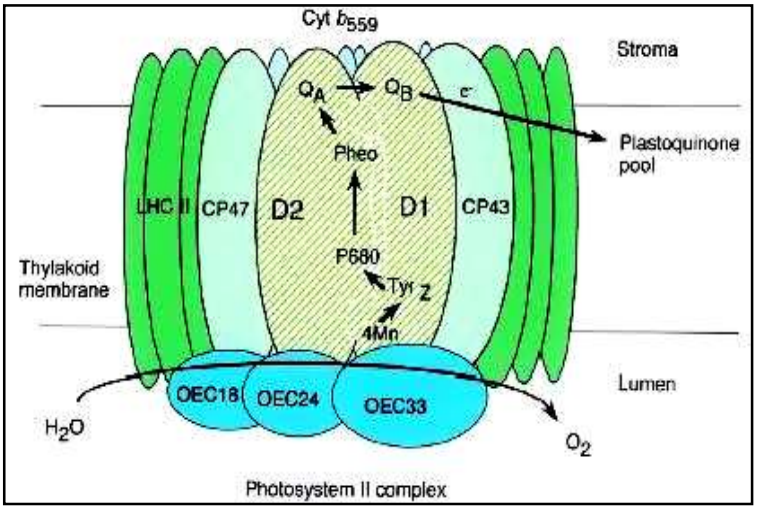
(i) Involves a soluble, secA (bacterial gene) homologue

(ii) requires ATP

(iii) pH gradient stimulates

(iv) Examples of proteins transported this way:

- Plastocyanin
- OE33 : 33 kDa protein of the oxygen evolving component of PSII (OEC)
- OEC (or OE) proteins of PSII mediate water splitting: Found in thylakoid lumen



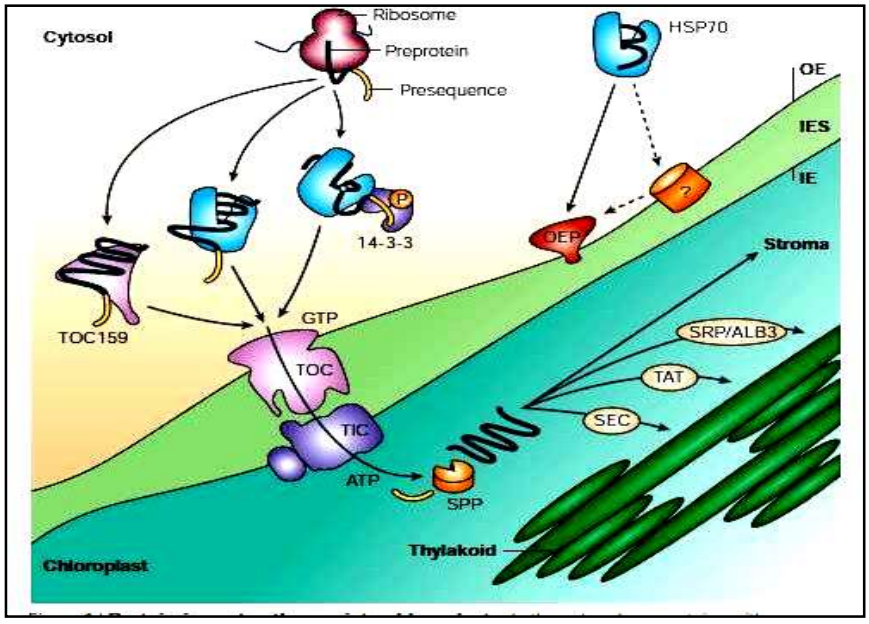
pH gradient-dependent (or Tat pathway):

- Requires the pH gradient across thylakoid membrane (generated by photosynthesis)
- Examples of proteins transported by this pathway:
  - OE24 and OE17 subunits of the OEC

- Transit peptides of these proteins have twin-arginine (Tat) motif that is essential for transportacross thylakoids
    - also occurs in bacteria

SRP dependent pathway for protein targeting:

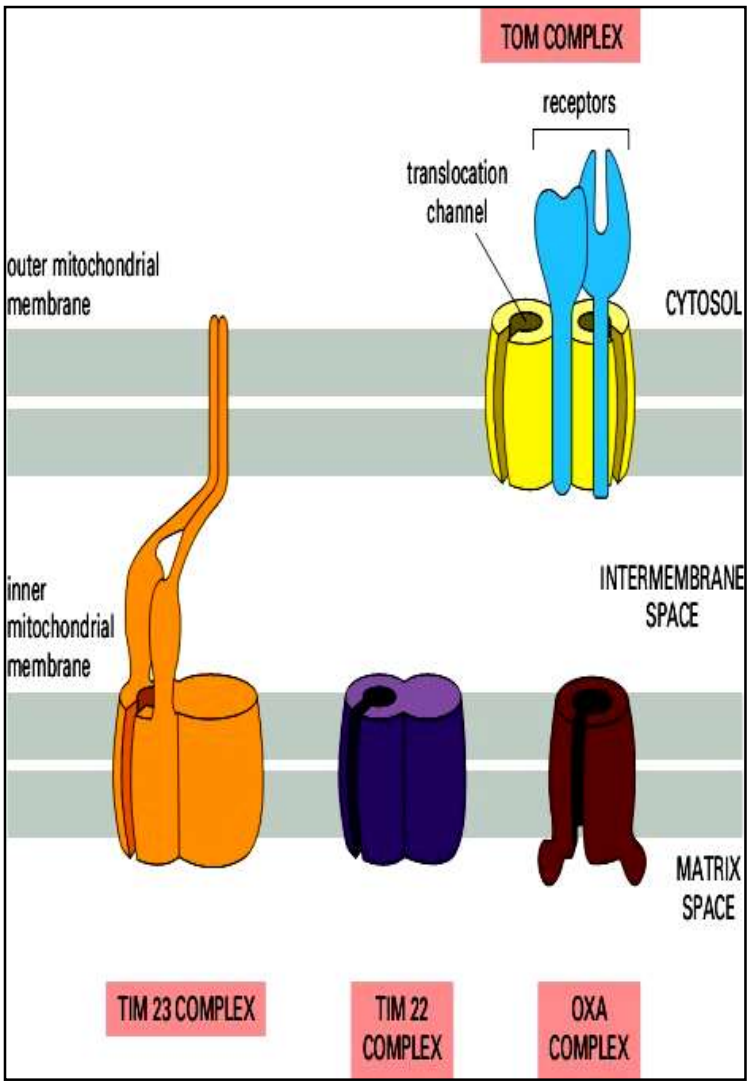
- Involves a signal recognition particle (SRP)-like protein (cSRP54)
    - SRP occurs in prokaryotes and eukaryotes
    - Green plant chloroplast SRP does not have an RNA subunit
- requires GTP
- pH gradient stimulates
- Examples of proteins transported by this pathway-
    - LHCPs: light-harvesting chlorophyll proteins (cab genes)



Sorting of proteins in mitochondria:

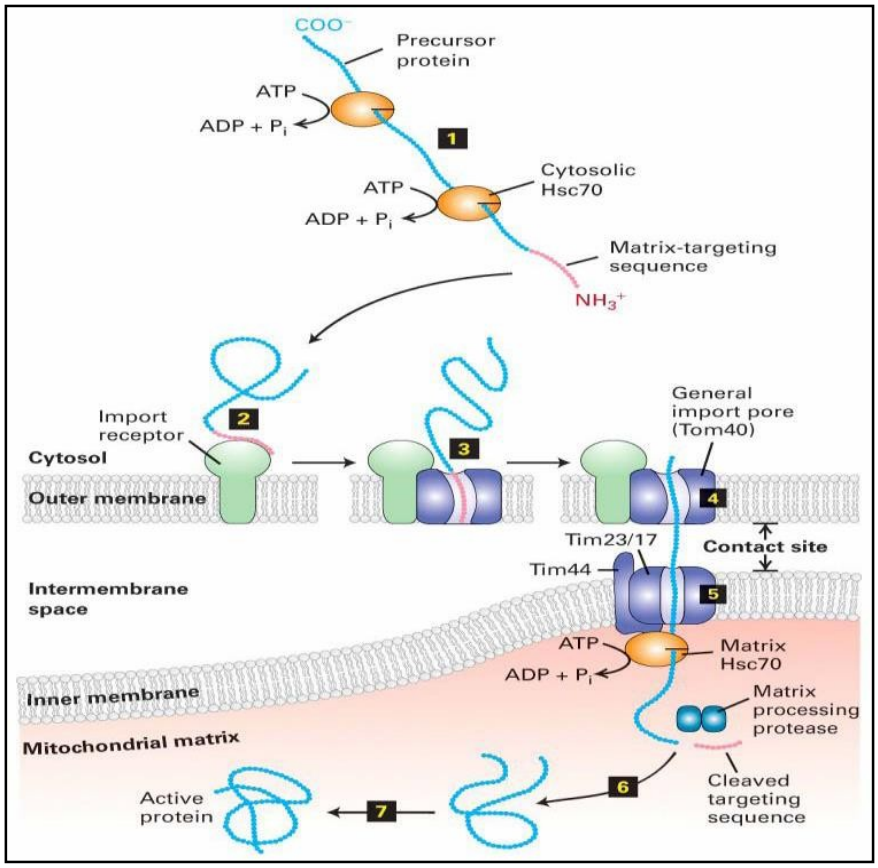
- Most mitochondrial proteins are encoded bynuclear DNA.
- Only very few are encoded by mitochondrial DNA and synthesized on mitochondrial ribosomes.
- Usually located at N-terminus of precursorpolypeptide.

- Usually removed in mitochondrial matrix.
- Protein translocation across mitochondrial membranes is mediated by multi-subunit protein complexes that function as **proteintranslocators**.
- **TOM ,TIM 23,TIM22 ,OXA.**
- TOM transports-**mitochondrial precursor proteins**, nucleus-encoded mitochondrial proteins.
- TIM23-proteins **into the matrix space**.
- TIM22-**mediates the insertion of a subclass of inner membrane proteins**, including the carrier protein that transports ADP, ATP, andphosphate.
- OXA-mediates the **insertion of innermembrane proteins**.



Process:

1) Mitochondrial proteins are synthesized in cytosol as precursors.
2) Bind to cytosolic **chaperones (Hsp 70)** to keep them unfolded until they ready to betranslocated.
3) Energy from ATP.
4) Some outer membrane proteins insert themselves in the membrane while in transit.
5) Intermembrane space proteins remain there and fold.
6) Protein destined to matrix passes through Tom 40 and then Tim (inner membrane translocon).



7) The vast majority of chloroplast proteins are synthesized as precursor proteins (preproteins) in the cytosol and are imported post-translationally into the organelle.

8) Most proteins that are destined for the **thylakoid membrane**.
9) Preproteins that contain a cleavable transit peptide are recognized in a GTP-regulated manner12 by receptorsof the outer-envelope translocon, which is called **theTOC complex**.
10) The preproteins cross the outer envelope through an aqueous pore and are then transferred to the translocon in the inner envelope,which is called **the TIC complex.**
11) The TOC and TIC translocons function together during the translocation process Completion of import requires energy, which probably comes from the ATP-dependent functioning of molecular chaperones in the stroma.
12) The stromal processing **peptidase** then cleaves the transit sequence to produce the mature form of the protein, which can fold into its native form.

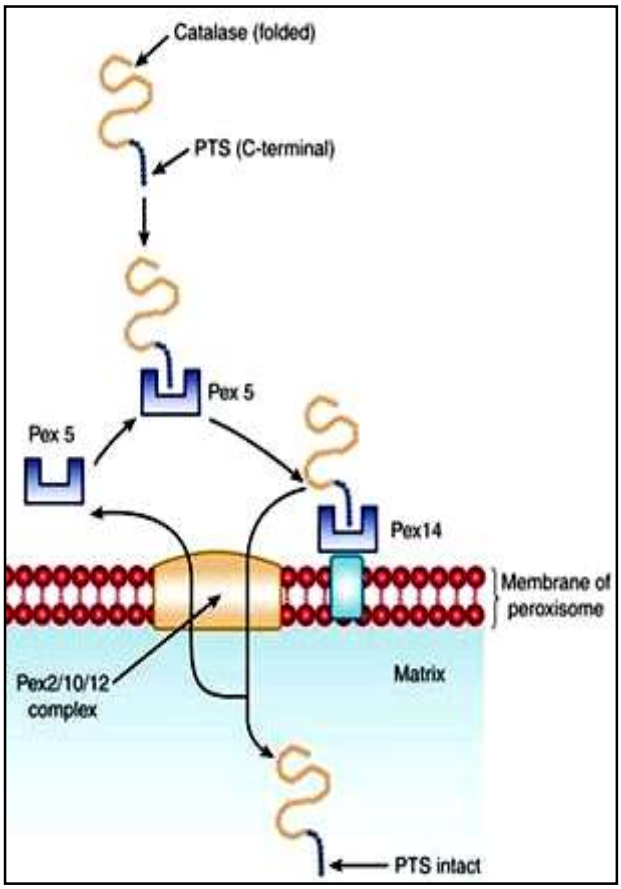
Sorting of proteins to both chloroplasts and mitochondria:

- Mitochondria and chloroplasts are **double-membrane-enclosed organelles**.
- They specialize in the synthesis of ATP, using energy derived from **electron transport** and oxidative phosphorylation in mitochondria **and** from photosynthesis in chloroplasts**.**
- Both organelles contain their **own DNA, ribosomes**, and other components required forprotein synthesis.
- Their **growth depends** mainly on the import of **proteins from the cytosol**.

Sorting of proteins to peroxisomes:

- Single membrane organelle
- Matrix contains oxidative enzymes
    - Lipid oxidation without ATP production
- Proteins encoded by nuclear DNA (all have to be imported)
- Peroxins - peroxisome transport receptors
- Bind to proteins with PTS1 and dock to the translocation channel
- The complex is transported through the membrane

- Protein is released and recycled
- **Peroxisome targeting signal 1 (PTS1):** a C- terminal tripeptide with a consensus sequence. The most common PTS1 is serine-lysine- leucine (SKL). Most peroxisomal matrix proteins possess a PTS1 type signal.
- **Peroxisome targeting signal 2 (PTS2):** a nonapeptide located near the N-terminus witha consensus sequence



Transport into the nucleus:

- All proteins found in the nucleus aresynthesized in the cytoplasm
- Examples:
    - Histones
    - Ribosomal proteins
    - DNA and RNA polymerases

- Transcription factors

➢ Transport requires nuclear localizationsequences (NLS)

➢ Transport occurs through the nuclear pores

- Nuclear import receptor (Importin α and β)
- Energy from GTP
- GTPase Ran

➢ Fully folded proteins are transported

➢ Importin α and β bind to the protein to be transported

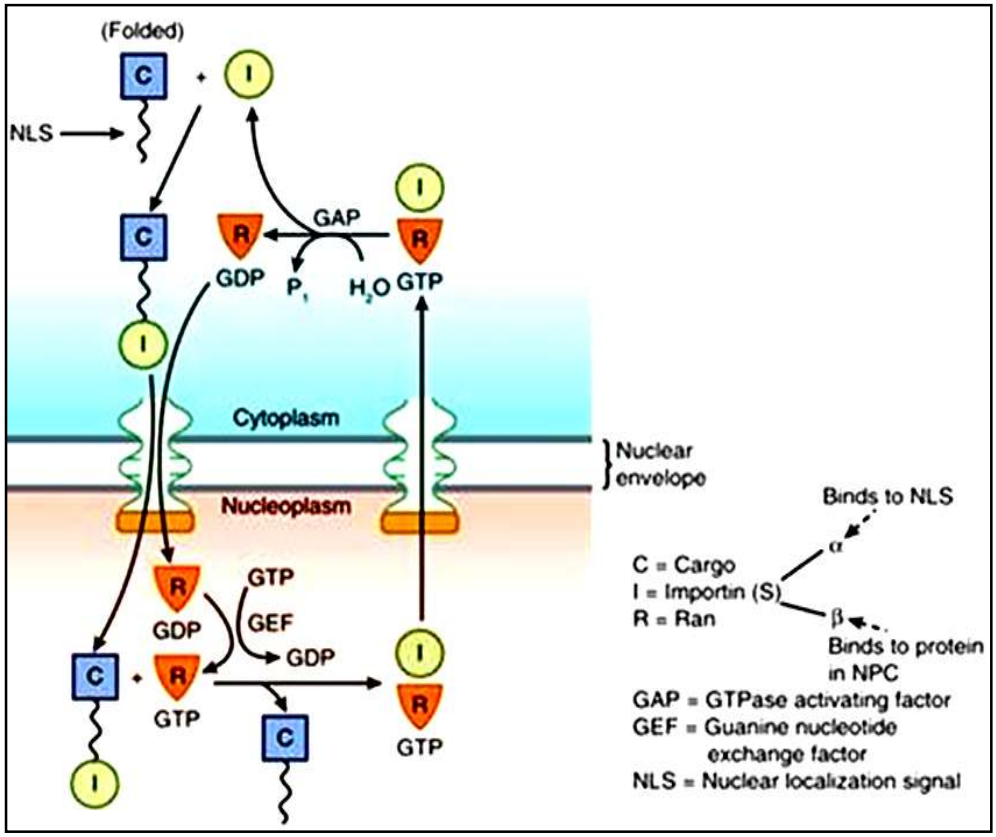
- Nuclear localization signal binds to importin α

➢ The complex is translocated through the nuclear membrane

➢ Activated Ran (GTP) causes the complex to dissociate

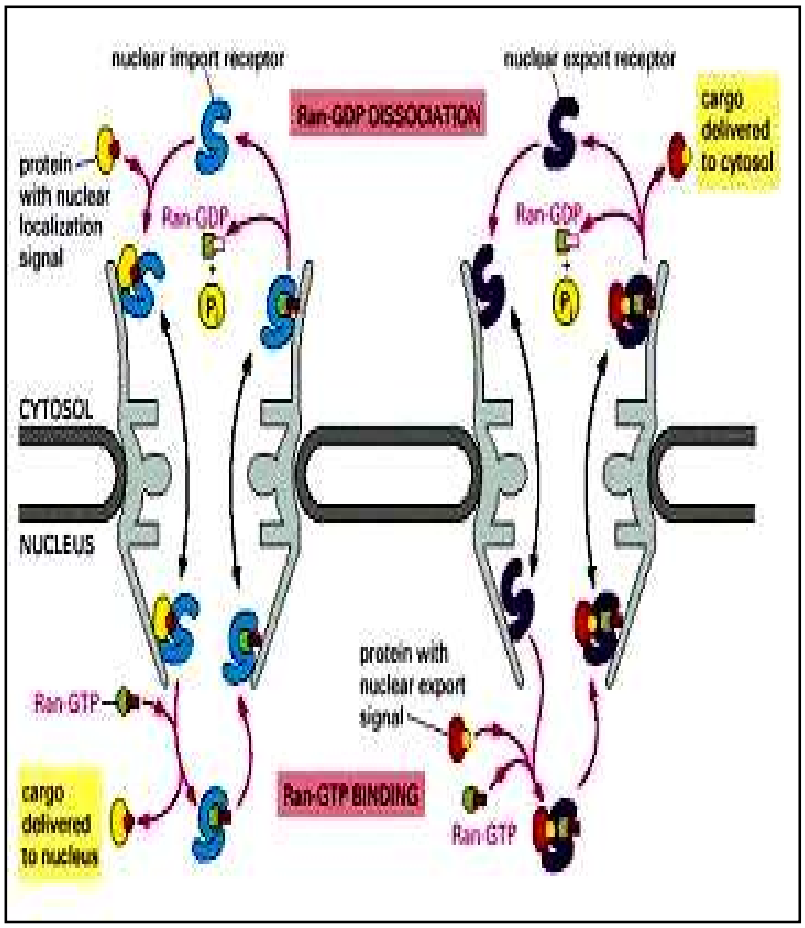
➢ Ran transports importin β back to cytosol

➢ Importin α becomes a part of export receptor



Import and export protein to nucleus:

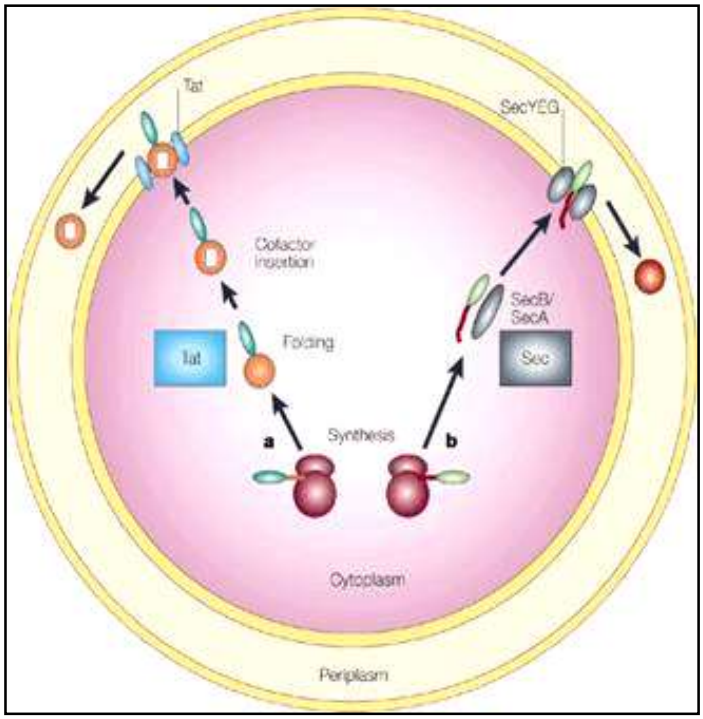
- Protein encodes a receptor protein that is specialized for the transport of a group of nuclear proteins sharing structurally similar nuclear localization signals.
- Nuclear import receptors do not always bind to nuclear proteins directly. Additional adaptor proteins are sometimes used that bridge between the import receptors and the nuclear localization signals on the proteins to be transported.
- **Export -ribosomal subunits and RNA molecules.**
- **For import and export requires energy**

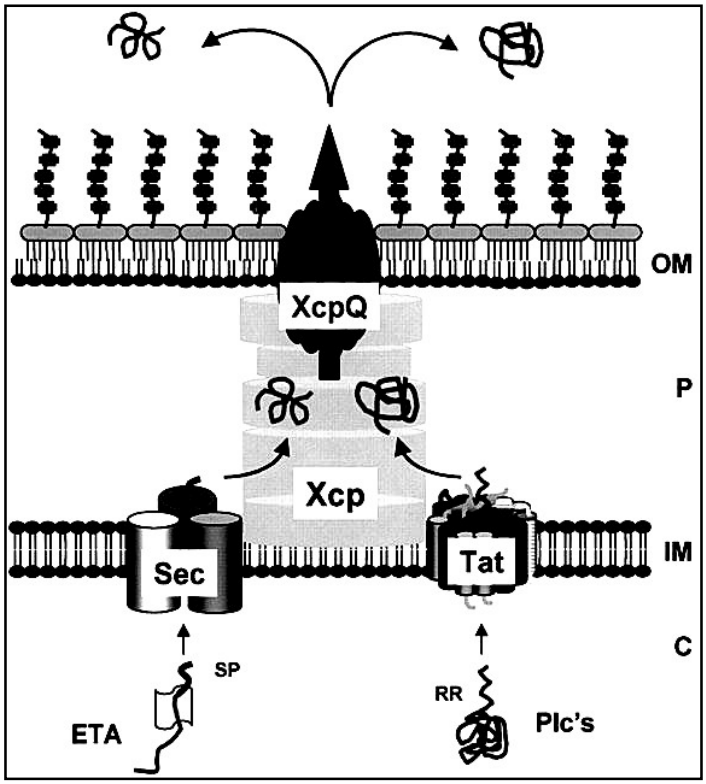


Protein targeting in *E. coli*:

- Tat: for periplasmic redox proteins & thylakoid lumen
- Sec pathway

- Periplasmic proteins with the correct signals (exposed after cleaving signal peptide) are exported by XcpQ system





6. Genetic transformation: *Agrobacterium* mediated (co- culture, in planta, agroinfection); Direct method (PEG, electroporation, particle gun method); Reporter genes- screenable and selectable markers.

In molecular biology, **genetic transformation** is a process by which the genetic material carried byan individual cell is altered by the incorporation of foreign (exogenous) DNA into its genome. Genetic transformation discovered by **British bacteriologist Frederick Griffith in 1928** has revolutionized molecular biology, but it was not until the recombinant DNA was produced from Escherichia coli with the use of biochemical scissor called restriction enzymes that genetic transformation of cells started.

In plant cells, gene transfer to plant cells achieved using two different methods:

I. Indirect methods (Vector-mediated methods)
II. Direct methods (Vector less methods)

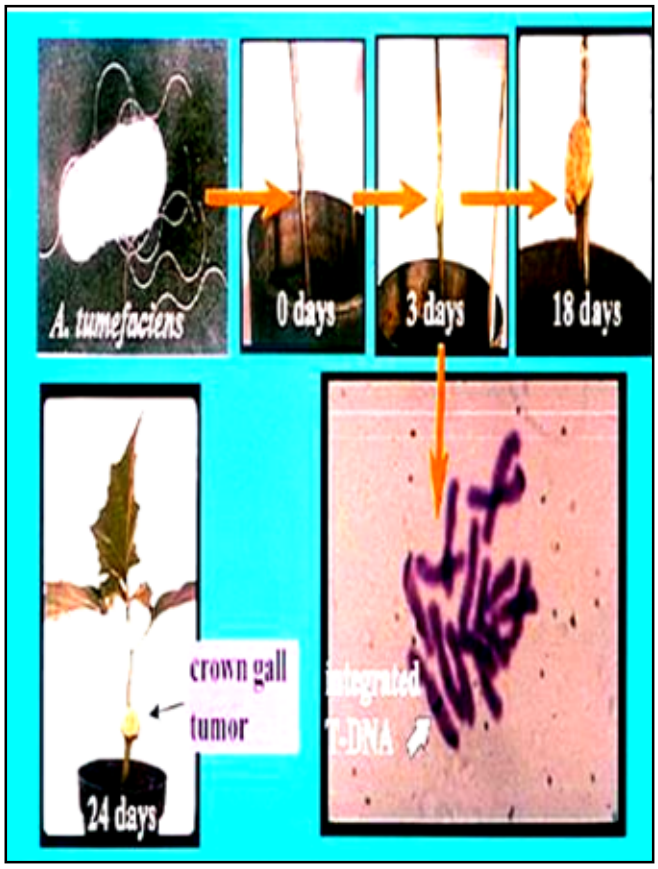
I. Indirect methods (Vector mediated methods):

The vector mediated methods exploit the natural ability of certain bacteria (*Agrobacterium* species) and viruses to naturally transfer DNA to the genomes of infected plant cells.

Agrobacterium mediated gene transfer:

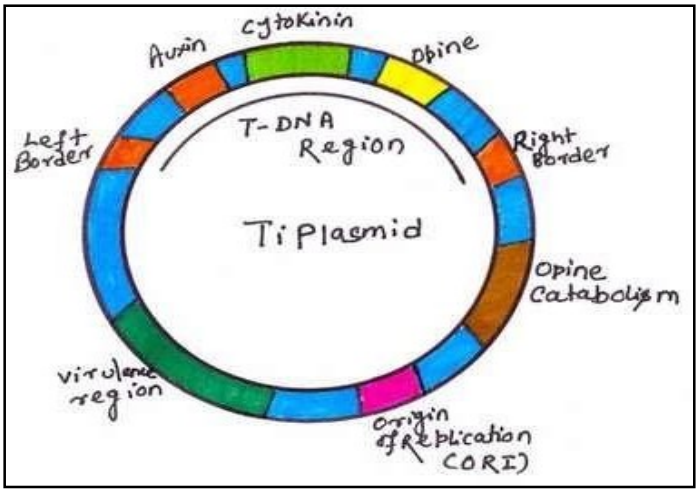
Agrobacteria are natural plant parasites. Their natural ability to transfer genes provides another engineering method. To create a suitable environment for them, these *Agrobacteria* insert their genes into plant hosts, resulting in a proliferation of modified plant cells near the soil level (**crown gall**). The genetic information for tumor growth is encoded on a mobile, circular DNA fragment (plasmid). When *Agrobacterium* infects a plant, it transfers this T-DNA to a random site in the plant genome. When used in genetic engineering the bacterial T-DNA is removed from the bacterial plasmid and replaced with the desired foreign gene. The bacterium is a vector, enabling transportation of foreign genes into plants. This method works especially well for dicotyledonous plants like potatoes, tomatoes, and tobacco. *Agrobacteria* infection is less successful in crops like wheat and maize. *A.tumifaciens* cause crown gall

(tumor) and *A rhizogenes* hairy root disease in dicot plants by infecting through wounds on roots or stem at the soil surface. The bacterium contains Ti (Tumor inducing) and Ri (Root inducing) plasmids . Both these plasmids can transfer part of their DNA (T-DNA) into plant cell chromosome by which Plant cells become transformed by expression of T-DNA gene which induce disease.



Description of *Agrobacterium* as Natural Genetic Engineer:

Agrobacterium is a soil plant pathogenic bacterium. This bacterial cell possesses a Ti plasmid in addition to bacterial chromosome. The bacteria can infect plants specifically dicots through wounds near soil surface. During infection a segment (T-DNA) of Tiplasmid gets transferred and become integrated in the plant cell genome. This T-DNA carries genes for expression of Disease.



Components of Ti/Ri Plasmid:

- T- (Transferable) DNA region
- Vir (Virulence) region
- Host Specificity Region
- Ori (origin of Replication) region

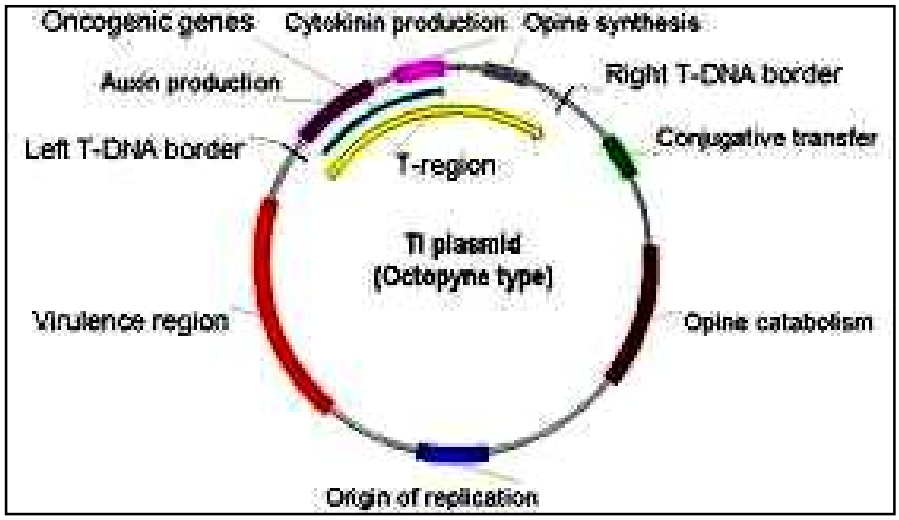
The Ti plasmids are classified into different (about 14) types depending upon the specific opine being synthesized.(octapine/nopaline/Agropine).

The most important is T DNA region which carries genes encoding plant hormones and opines. The Vir region contains the virulence gene which helps

in transfer of T DNA Crown gall disease results from transformation of the plant genome with this part of the plasmid in a process analogous to bacterial conjugation Third region of plasmidcontains conjugation genes concerned with whole plasmid transfer between bacteria. Fourth regionencodes genes concerned with opine utilization.

- It is ~200 kb megaplasmid
- T-DNA (15 – 40 kb) region contains genes for synthesis of Auxins, Cytokinins and Opines.
- Auxins and cytokinin genes are expressed in plant tissue inducing disease.
- Opines (unusual amino acids) produced by infected cells are used as nutrients by *Agrobacterium.*

- T-DNA region is bordered on both sides by 25bp repeat which helps in its transfer to plantgenome.
- Virulence Region contains about 8 operons having about 24- 25 genes
- These genes help in transfer of T-DNA
- Host specificity region has gene for conjugative transfer and opine catabolism
- Ti plasmid also has origin of replication



Organization of T-DNA:

- T-DNA is ~ 23kb (15-40kb) segment bordered on both sides by 25bp direct repeat sequences.
- T-DNA contains genes for tumor induction (IAAM, IAAH & IPT) by forming auxins,cytokinins and Opines.
- All the genes in T-DNA region contain eukaryotic regulatory sequences, so they are expressedonly in plant cells

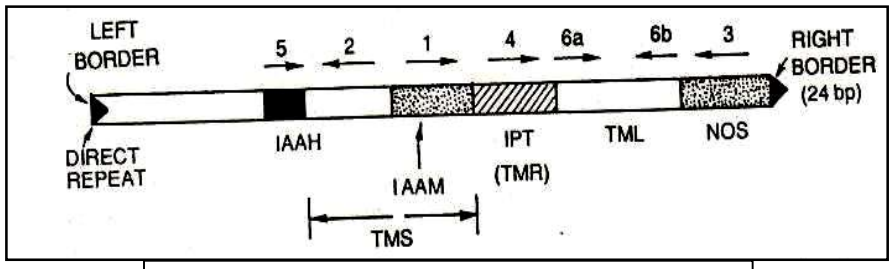


Fig.: Organization of T-DNA of Ti Plasmid

Organisation of Vir region:

- Vir region contains 8 operons (VirA, B, C, D, E, F , G & H) which together have 25 genes.
- Vir region mediates transfer of T-DNA into plant genome. It is itself not transferred.
- VirA and Vir G are constitutive operons encoding Vir A and VirG Proteins.
- Other Vir operons encode various proteins involved in T-DNA transfer.

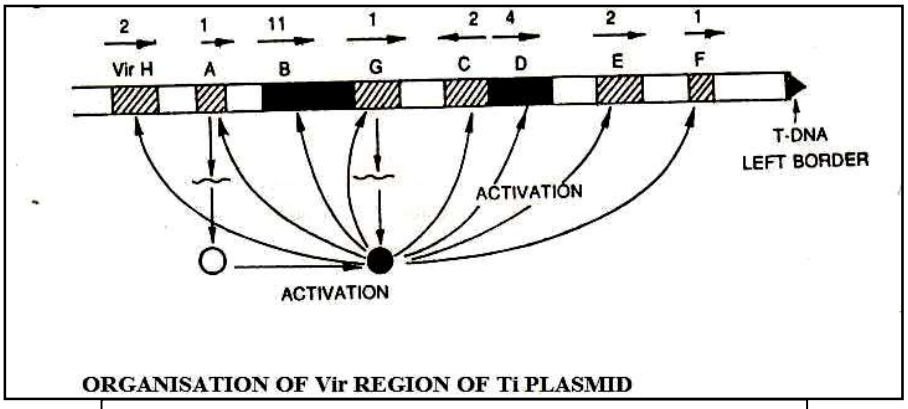
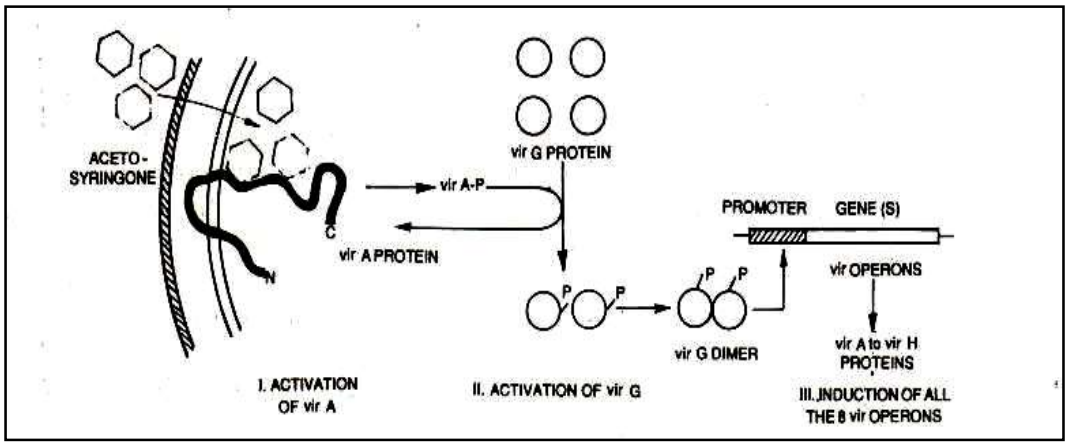


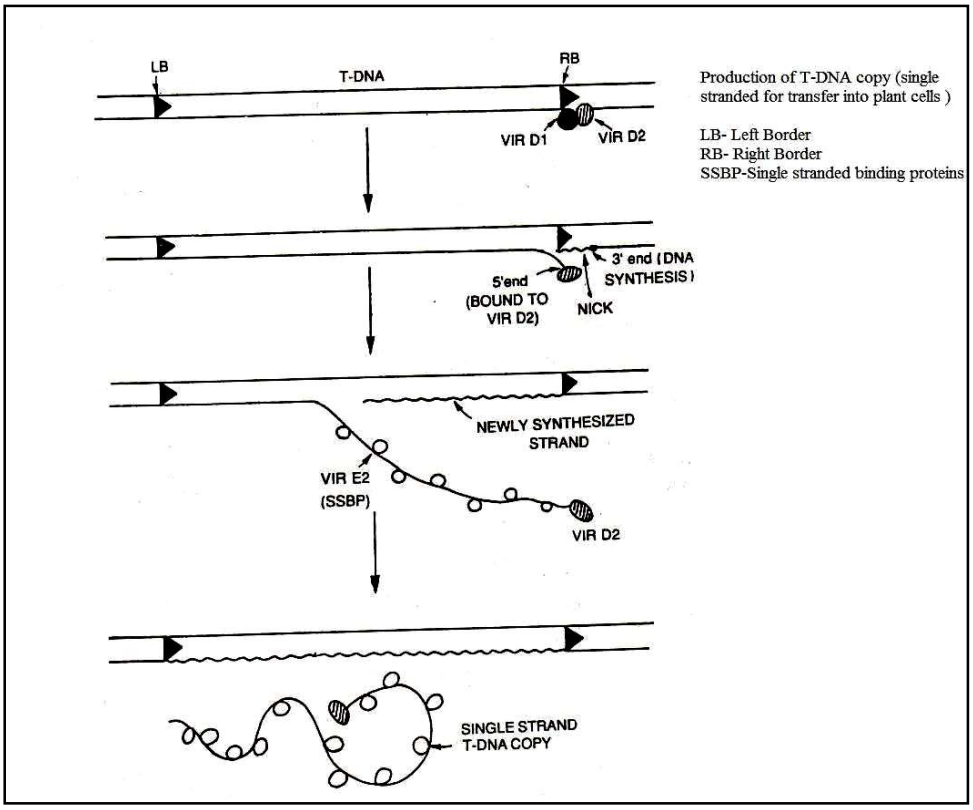
Fig.: Organization Vir region of Ti Plasmid

Mechanism of transfer of T-DNA:

- Transfer of T-DNA is a step wise process.
- Vir region of Ti plasmid becomes activated by the phenolic signal molecules.
- Acetosyringone and α-hydroxyacetosyringone released by wounded tissue of dicot plantswhich constitute wound response as follows –



- Acetosyringone and α-hydroxyacetosyringone bind with Vir A protein (located in the inner membrane) and activates it. It start functioning as autokinase to phosphorylate itself by ATP. Phosphorylated Vir A protein then phosphorylates Vir G protein which then dimerises.
- Phosphorylated Vir G protein has DNA binding function. It induces expression of rest of Vir operons
- Vir D1 protein has topoisomerase and endonuclease activity.
- It binds to right border sequence of T-DNA and facilitate the action of Vir D2 protein which is also endonuclease and nicks at the right border and remains bound to 5' end so generated.



- The 3'end produced at the site of nick serves as a primer for DNA synthesis in 5' to 3' direction as a result of which one strand of T-DNA is displaced from the DNA duplex.
- The T-DNA strand is again nicked at the left border to generate a single strand

copy of T-DNA.

- To this single strand copy Vir E 2 protein (single strand DNA binding proteins) bind for its protection against exonucleases.
- Vir B operon consisting of 11 genes encode membrane bound Vir B proteins. These along with Vir D4 proteins participate in conjugal tube formation between bacterial and plant cells for transfer of T-DNA.
- Vir D2 which remains bound to 5'end of T DNA has a signal sequence which drives it intothe nucleus of plant cell.

Integration of T-DNA into plant genome:

- T-DNA enters plant cell as a single stranded structure which is immediately converted intodouble stranded form.
- Vir E2 also has nuclear localization sequence and is responsible for transfer of T-DNA intoplant cell nucleus
- Double stranded T-DNA integrates at random sites in the host plant genome.

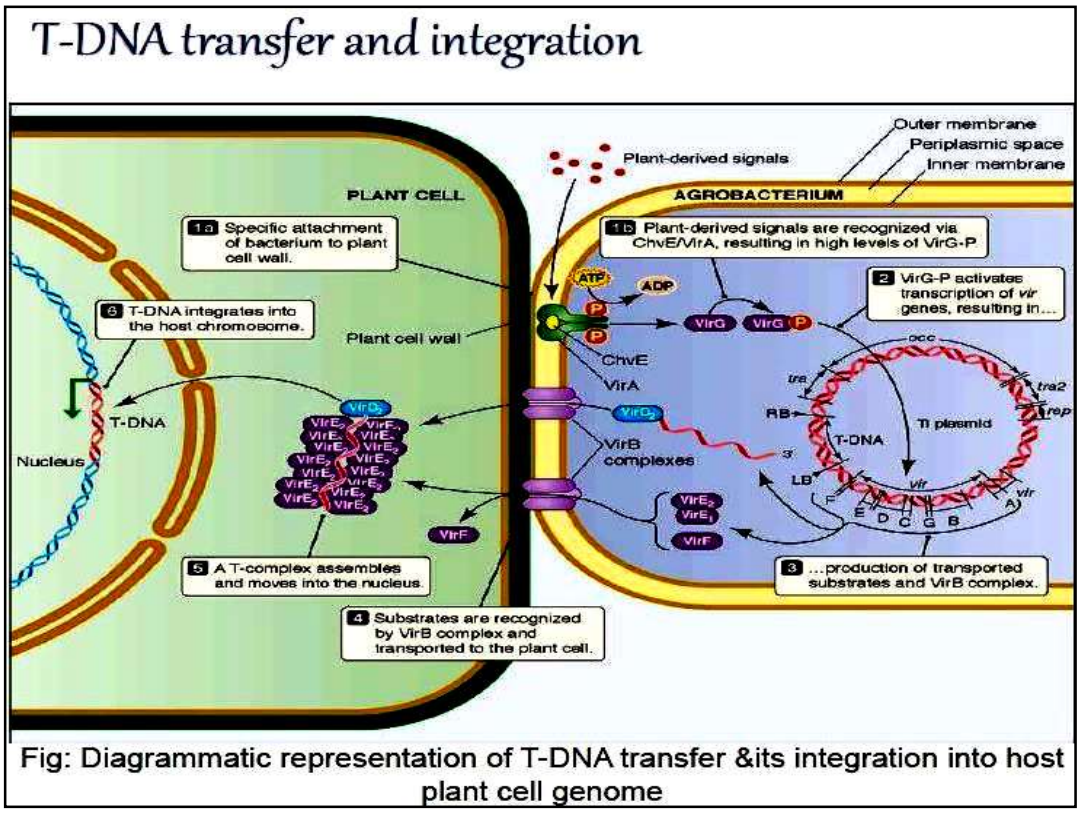


Fig: Diagrammatic representation of T-DNA transfer &its integration into host plant cell genome

- For integration 23-79 base pair deletion takes place at the integration or target site
- After integration of T-DNA into plant genome ,the genes for auxins, cytokinins and opinesexpress themselves which result in uncontrolled growth in the form of tumor.

Harnessing *Agrobacterium* in transferring foreign gene into plant:

1. It is cleared that *Agrobacterium* has the potentiality to transfer prokaryotic DNA to eukaryotic Genome as a Natural Genetic Engineer
2. It will be made on strategies involved for harnessing

Agrobacterium for introducing new (desired) gene into plant cells

*Agrobacterium'*s ability to introduce its DNA into plant genome with efficiency makes Ti plasmid an attractive vector for gene transfer into plants After knowing about how T-DNA is transferred and what modifications are needed , Genetic engineers employed different strategies and before actual transfer of desired gene Genetically engineered Agrobacterium were produced with disarmed T-DNA region in which oncogenes and opine biosynthesis genes were replaced by desired (Foreign) gene anda selectable marker gene. Along with this some other sequences as unique restriction sites were added. The natural Ti plasmids are unsuitable to be used directly as vectors for invitro manipulation due to following reasons

- ✓ Large size
- ✓ Tumor induction (Oncogenic) property
- ✓ Absence of unique restriction enzyme site

Now it is also well known that disarmed TDNA, left and right borders along with genes of Vir regionare essential elements for designing of transformation vectors.

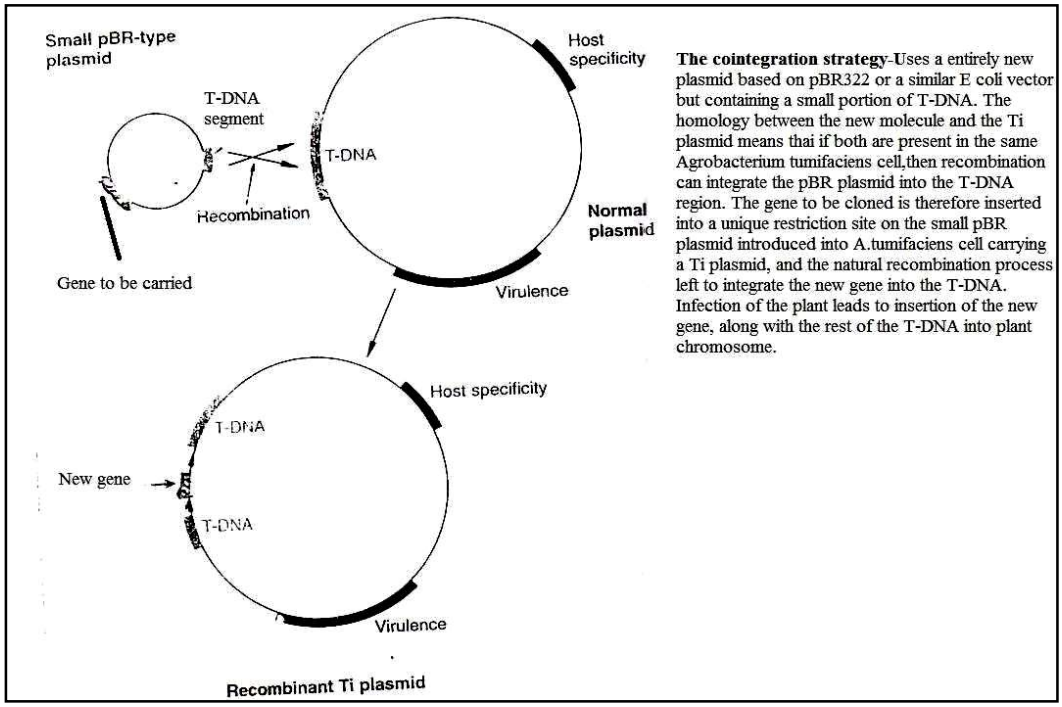
Now the strategies involved for harnessing *Agrobacterium* for introduction of new/desired/foreign genes into plants are:

- ✓ Only useful attributes of Mega Ti plasmid (~200kb) have been exploited in designing planttransformation vector.
- ✓ For using *Agrobacterium* for in vitro genetic engineering purposes following manipulation are to be made.

- ✓ Ti plasmids are disarmed. Genes from T DNA responsible for growth hormones (Oncogenes) and Opine formation are deleted so that no disease will be produced and as opines will not be synthesized further growth of *Agrobacterium* inside the host tissue stops.
- ✓ The space between lB and RB is used to insert desired gene which is to be integrated and expressed in host tissue for transformation. The foreign gene is inserted by RDT.
- ✓ A selectable marker gene providing resistance against antibiotic like kanamycin is also inserted in the T-DNA region.
- ✓ The oncogenic problem can be solved by use of disarmed T-DNA from which these genes are removed.

The large size problem of Ti plasmid can be tackled by any of following two ways using manipulated *Agrobacterium* vectors.

1. **The Co-integrated vector** For this purpose a suitably modified *E coli* plasmid or vector is integrated into disarmed Ti plasmid (pTi), this gives rise to co-integrate vector.



During disarming oncogenes of T-DNA are replaced by gene insert and other sequences of *E coli* plasmid. For this both plasmids are introduced into same *Agrobacterium* cell. Because of homology through recombination pBR plasmid is integrated into the T-DNA region. The gene to be cloned is therefore inserted into unique restriction site on the small pBR plasmid, introduced into *A tumifaciens* cell carrying a Ti plasmid and natural recombination process left to integrate the new gene into T-DNA.

2. **Binary (Two) Vector strategy** (*Agrobacterium* containing two different recombinant plasmids) First is *Agrobacterium E coli* shuttle vector containing disarmed. T DNA with 25 bp repeats flankingthe gene insert to be introduced and a selective marker (often a Neo gene which provide cells resistence to antibiotic Kanamycin) This Mini plasmid (pBIN19) is designed to replicate in both Ecoli and Agrobacterium and is capable of conjugal transfer between two bacterial species also has a copy of lacZ gene containing multiple cloning sites. Second is helper Ti plasmid (p AL4404) from which T DNA segment has been removed. It has a functional Vir region. The Vir genes of the helper Ti induce the transfer of T DNA present on first plasmid in the same bacterial cell. As a result a gene insert within T-region is also transferred into plant cell. Many binary vectors have been developed which differ in size and source of 25bpnrepeat sequence, plant selection marker and cloning sites. In both cases (co-integrate or binary vector) ,the manipulated T-DNA is transferred to the plant genome efficiently.

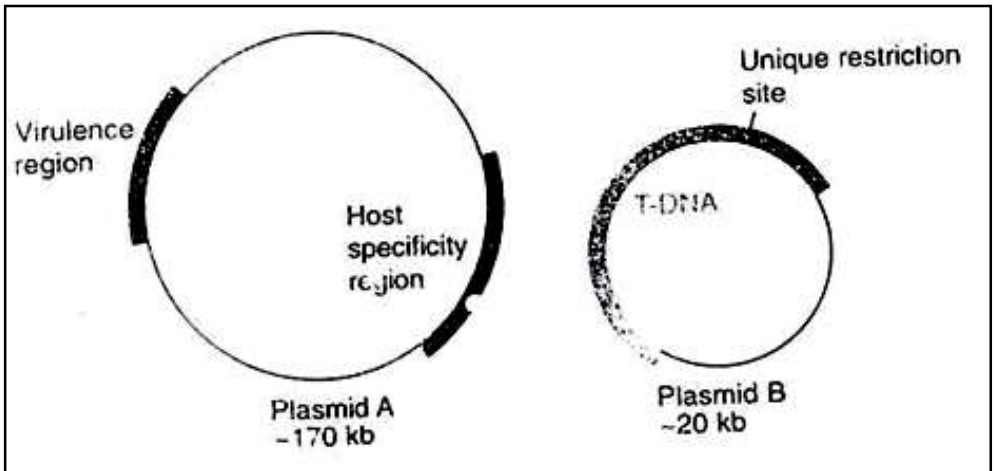


Fig.: The Binary Vector Strategy – Plasmid A and B complement each other when present in the same *Agrobacterium tumifaciens* cell.

Transformation technique using *Agrobacterium*

Some prerequisities for integration of foreign gene and production of transgenic plants are as follows:

- The plant explants must produce acetosyringone to induce Vir genes for virulence
- Induced Agrobacterium should have access to cells that are competent for transformation or to take up DNA
- Cells must be meristematic, often transformed tissue or explants do not regenerate and are nottotipotent, therefore transformation and regeneration competent cells should be taken as explants.
- The transformed explants cells should allow expression of transgene
- Disease causing genes should not be transferred and expressed, which is helped by disarmed T DNA

***Agrobacterium* gene transfer can be achieved in the following two ways:**

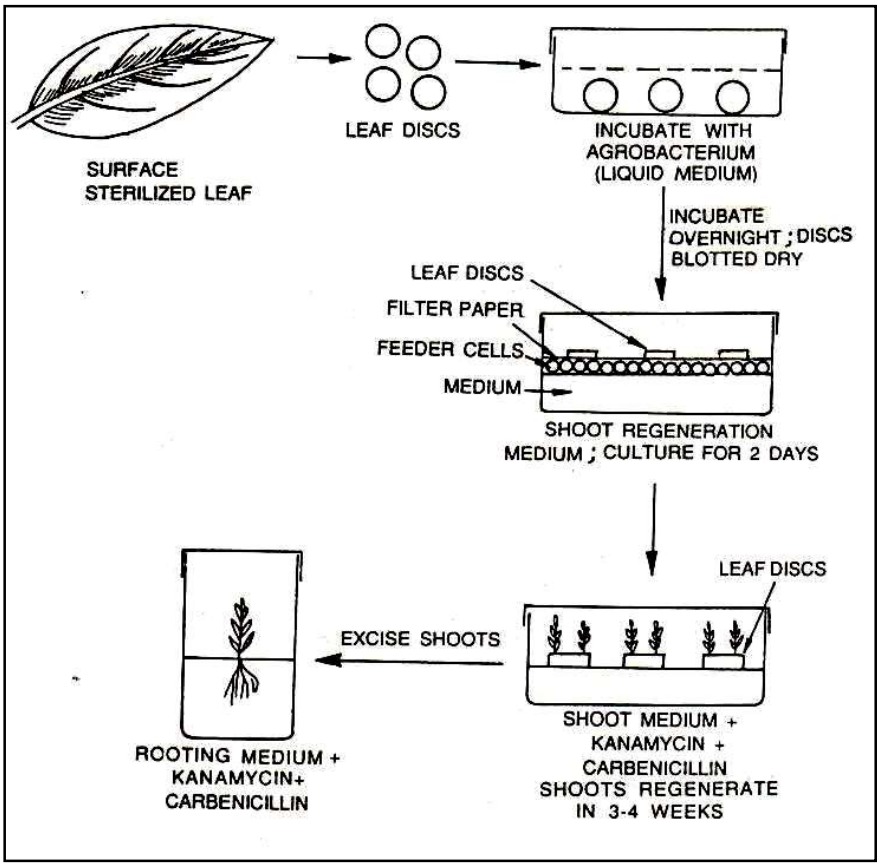
- Co culture with tissue explants
- In planta transfusion

Co culture with tissue explants

For transformation explants like protoplast, callus, tissue slices, leaf disc, stem or floral tissue etc. canbe co-cultured with genetically engineered. *Agrobacterium* with recombinant vectors for about 2 days.During co-culture acetosyringone(phenolic signal molecule) released from wounds (cut leaf disc) induce the Vir genes which bring about the transfer of recombinant T-DNA into many of the plant cells.

The explants can then be transferred to shoot inducing (regeneration medium) containing kanamycin and carbenicillin. Kanamycin allows only transformed plant cells to divide and regenerate shoots in about 3-4 weeks, while carbenicillin kills *Agrobacterium* cells.

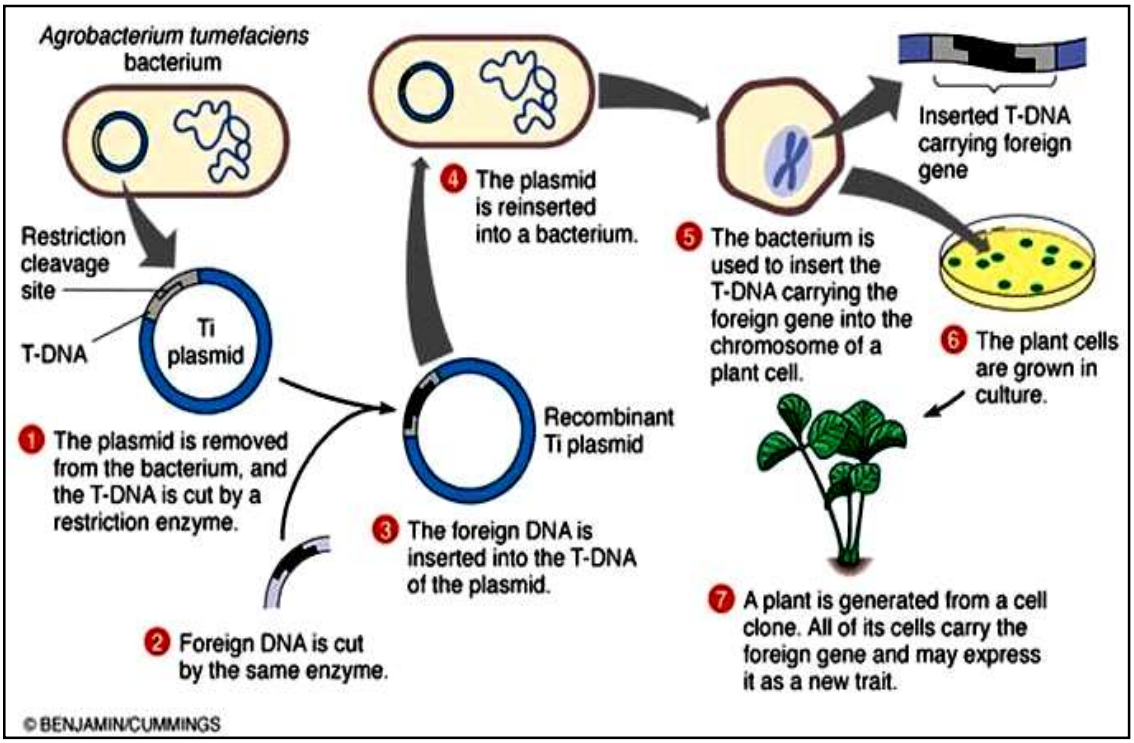
The shoots are separated and transferred to root inducing medium and finally after few weeks are transferred to soil.



By this co-culture method only dicot plants can be transformed. *Agrobacterium* mediated transformation of monocot plant (cereals) cells can be achieved by adding acetosyringone in the medium during co-culture.

In planta transfusion

Transformation can also be achieved by imbibition of seeds in fresh culturs of *Agrobacterium*. T- DNA becomes integrated into plant genome. It appears that *Agrobacterium* cells enter the seedling during germination, are retained within the plants, when flowers develop, the zygote or cells become transformed.

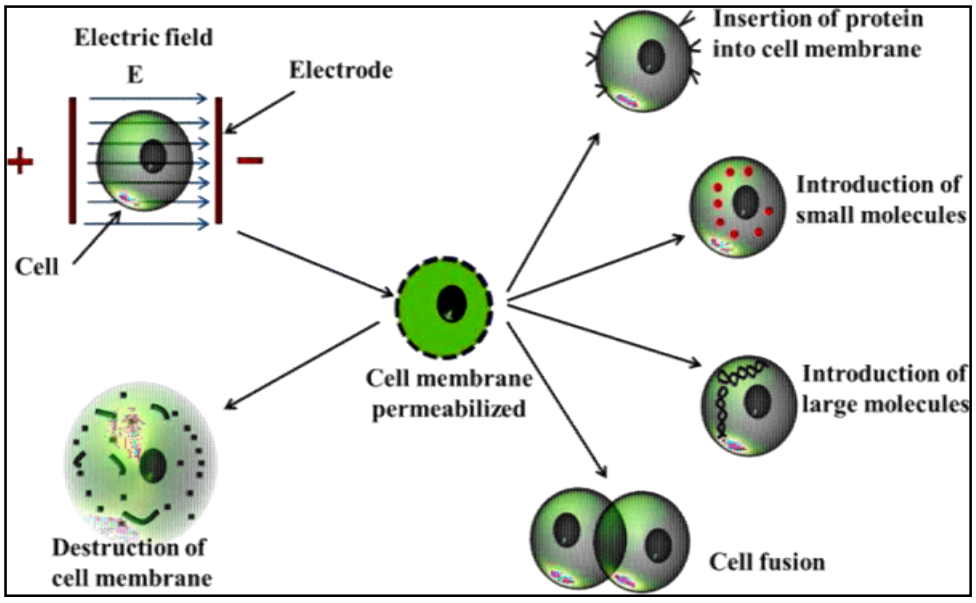


II. Direct methods (Vector less methods):

Vectorless methods use chemical or physical means to introduce DNA into plant cell. These methodsare species and genotype independent in terms of DNA delivery.

Physical Methods:

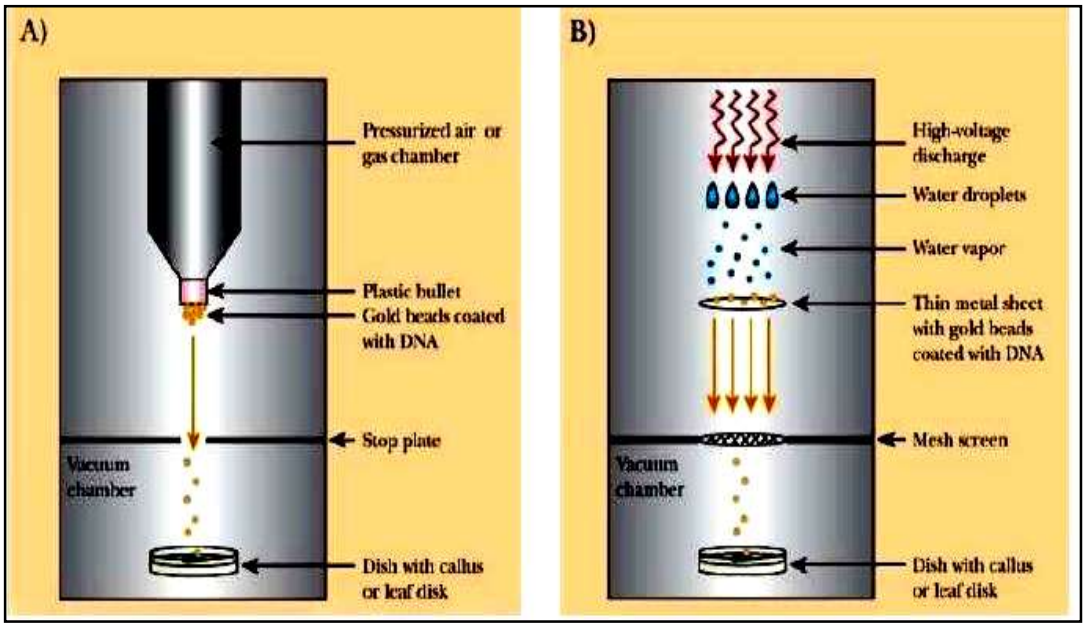
1. ***Electroporation:*** Electroporation involves the creation of pores in the cell membrane using electrical pulses of high field strength. If DNA is present in the buffer solution at a sufficient concentration, it will be taken up through these pores. Plant cell electroporation generally utilizes the protoplast because thick plant cell walls restrict macromolecule movement. Electrical pulses are applied to a suspension of protoplasts with DNA placed between electrodes in an electroporation cuvette. Short high voltage electrical pulses induces the formation of transient micropores in cell membranes allowing DNA to enter the cell andthen the nucleus.



Advantage: Simple, fast, low cost.

Drawbacks: Low efficiency, requires laborious protocols, and transforms mainly protoplasts.

2. ***Particle bombardment:*** It is also known as microprojectile bombardment, biolistics, gene gun, etc. Foreign DNA coated with high velocity gold or tungsten particles to deliver DNA into cells. This method is widely being used because of its ability to transfer foreign DNA into themammalian cells and microorganisms. It was developed by Prof. Sanford and coworkers of Cornell University (USA) in 1987. As the term denotes, it shoots foreign DNA into plant cells or tissue at a very high speed. This technique is most suitable for those plants which hardly regenerate and do not show sufficient response to gene transfer through Agrobacterium for example, rice, wheat corn, sorghum, chickpea and pigeon-pea.



Advantages:

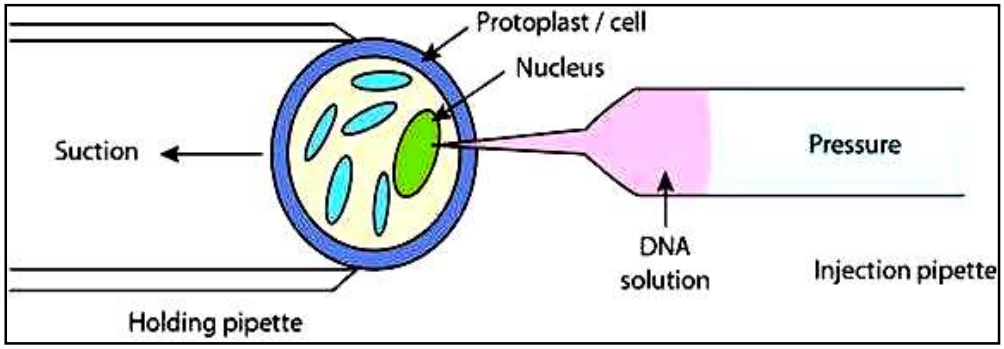
- ❖ It is clean and safe.
- ❖ The ability to engineer organized and potentiality regenerable tissue permits introduction offoreign genes into elite germplasm.
- ❖ Transient gene expression has been demonstrated in numerous tissues representing manydifferent species.
- ❖ By this process transformation of recalcitrant species such as rice, maize, wheat etc can bedone.
- ❖ Transformation frequency is very high.
- ❖ Gene transfer occurs without protoplast isolation.
- ❖ Suitable transformation of monocot plant.

Disadvantages:

- ❖ Limited by depth of penetration.
- ❖ Lack of control over the velocity of bombardment, which often lead to substantial damage tothe target cells.
- ❖ The emergence of chimeral plants.

- ❖ Less ability to regenerate.
- ❖ Very expensive method.

3. ***Microinjection:*** Microinjection is a direct physical method involving the mechanical insertion of the desirableDNA into a target cell. The technique of microinjection involves the transfer of the gene through a micropipette intothe cytoplasm or nucleus of a plant cell or protoplast. The most significant use of this is the introduction of DNA into the oocyte and the eggs ofanimals, either the transient expression analysis or to generate transgenic animals. The major limitations of microinjection are that it is slow, expensive, and has to be performed by trained and skilled personnel. Examples – e.g. Tobacco, Brassica napus has been successfully transformed by this approach.



Advantages:

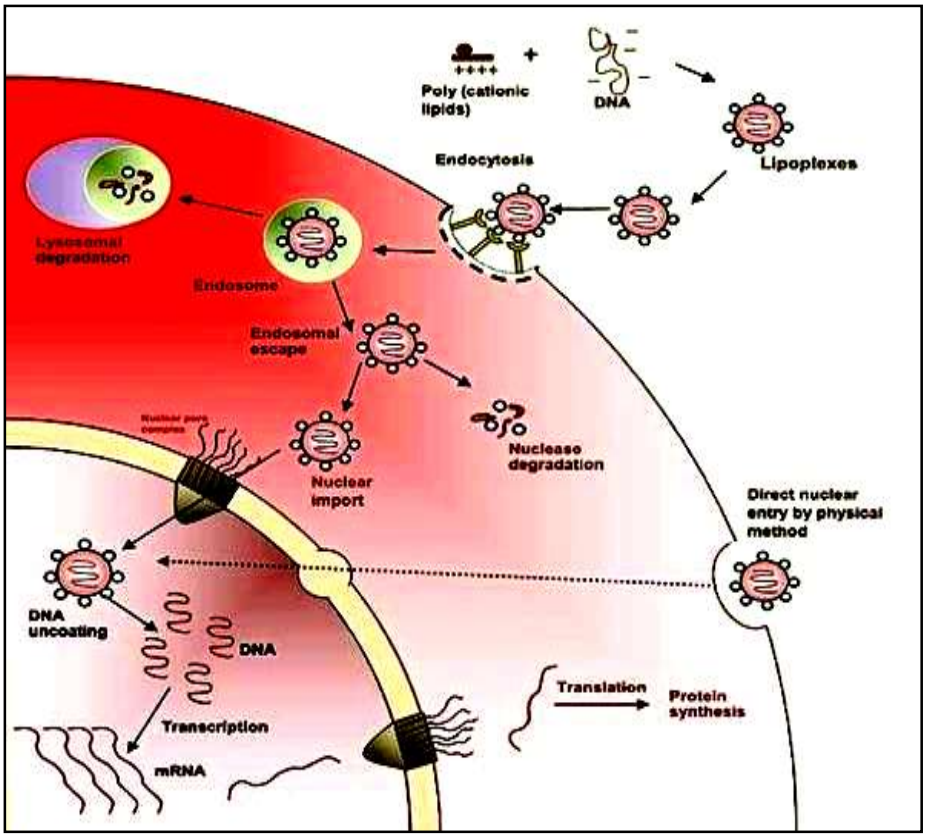
- ❖ The amount of DNA delivered per cell is not limited by the technique and can be optimized.This improves the chance for integrative transformation.
- ❖ The delivery is precise, again increasing the chance of integrative transformation.
- ❖ The small structures can be injected containing only a few cells and with high regenerationpotential.
- ❖ Since it is a direct physical approach, it is host-range independent.

Disadvantages:

- ❖ Injection can cause damage that affects embryonic survival and can result in quite highmortalities.
- ❖ Only one cell is targeted per injection.

- ❖ The handling requires specialized skill and instrumentation.
- ❖ Has low transformation rate.

4. ***Liposome mediated transformation:*** Liposome mediated transformation involves adhesion of liposomes to the protoplast surface, its fusionat the site of attachment and release of plasmids inside the cell.



<u>Chemical gene mediated transfer method:</u>

1. ***Polyethylene glycol- mediated transformation:*** Polyethylene glycol (PEG), in the presence of divalent cations, destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. A large number of protoplasts can be simultaneously transformed. This technique can be successfully used for a wide variety of plant species. It has certain limitations:

- ❖ 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.

2. ***DEAE-Dextran mediated transfer:*** 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 transformants.

Selectable marker genes:

The selection is based on the survival of transformed cells when grown on a medium containing a toxic substance (antibiotic, herbicide, antimetabolite). This is due to the fact that the selectable marker gene confers resistance to toxicity in the transformed cells, while the non-transformed cells will get killed. Some of them are given below:

Selective agent	**Marker gene/ Reporter gene**	**Source of gene**
Kanamycin (antibiotic)	NPT (neomycin phosphotransferase)	*E. coli*
Streptomycin(antibiotic)	SPT(streptomycin phosphotransferase)	streptomyces
Hygromycin(antibiotic)	HPT(hygromycin phosphotransferase)	*E. coli*
Phosphinothricin(herbicide)	BAR(phosphinothricin acetyltransferase)	streptomyces
B-glucuronidase	gus	*E.coli*
Glyphosate(herbicide)	EPSP synthase	Plant/microorganism
Bleomycin	Ble	*E. coli*

Reporter genes:

An assay for the reporter gene is carried out by estimating the quantity of the protein it produces or the final products formed. Reporter genes are often used as an indication of whether a certain gene has been taken up by or expressed in the cell or organism population. Some of the important ones are given below:

Opine synthase (ocs), β-Glucuronidase (gus), Bacterial luciferase (luxA), Firefly luciferase (luc).

Screenable marker gene:

The screenable markers presently used are mostly derived from bacterial genes coding for an enzymesthat is readily detected by the use of chromogenic or other substances. A screenable marker gene is functional only if an enzyme with comparable activity is not present in non-transformed/non-transfected cells. Several reporter genes are used and the most commonly used examples of reporter genes are:

- **Green fluorescent protein** makes cells glow green under UV light. A specialized microscope is required to see individual cells. Yellow and red versions are also available, so scientists can look at multiple genes at once. It is commonly used to measure gene expression.
- **GUS assay (using β-glucuronidase)** is an excellent method for detecting a single cell by staining it blue without using any complicated equipment. The drawback is that the cells are killed in the process. It is particularly common in plant science.
- **Blue white screen** is used in both bacteria and eukaryotic cells. The bacterial lacZ gene encodes a beta-galactosidase enzyme. When media containing certain galactosides (e.g. X-gal) is added, cells expressing the gene convert the X-gal to a blue product and can be seen with the naked eye.
- **Chloramphenical acetyl transferase (CAT)** encoding gene of E.coli: The CAT gene encodes the enzyme chloramphenicol acetyltransferase, which transfer acetyl groups from acetyl CoAonto the antibiotic chloramphenicol.
- **Luciferase** encoding gene of firefly, Photinus pyralis, catalyses the oxidation of luciferin withthe emission of yellow-green light which can be detected easily even at low levels.

7. Blotting techniques: Southern, Northern and Western Blot; DNA fingerprinting, DNA foot printing, basic idea of proteomics and genomics, c-DNA and genomic library.

Biochemistry studies molecules such as DNA, RNA and proteins. Blotting techniques are what scientists use to separate these types of molecules. In cells, they exist as a mixture. Blotting allows researchers to find one protein among many, like a needle in a haystack. Blotting is generally done by letting a mixture of DNA, RNA or protein flow through a slab of gel. This gel allows small molecules to move faster than bigger ones. The separated molecules are then pressed against a membrane, which helps move the molecules from the gel onto the membrane. The molecules stick to the membrane, but stay in the same location, apart from each other, as if they were still in the gel.

Southern blot:

Southern blotting is the original blotting technique, which started the naming system. It was invented by Edwin Southern. The Southern blot is used to detect the amount of DNA in a mixture. Just as with protein and RNA, the DNA of a cell can be released when that cell is broken open. Southern blotting separates DNA from different cell types by size. The DNA from each sample is spread into neat, parallel lanes. Individual pieces of DNA can be detected using a radioactive or fluorescent probe, which is designed to bind only to that piece of DNA. The energy signal from a radioactive probe, or the flashes of light from a fluorescent signal, tell researchers how much of that piece of DNA is in each sample.

Principle:

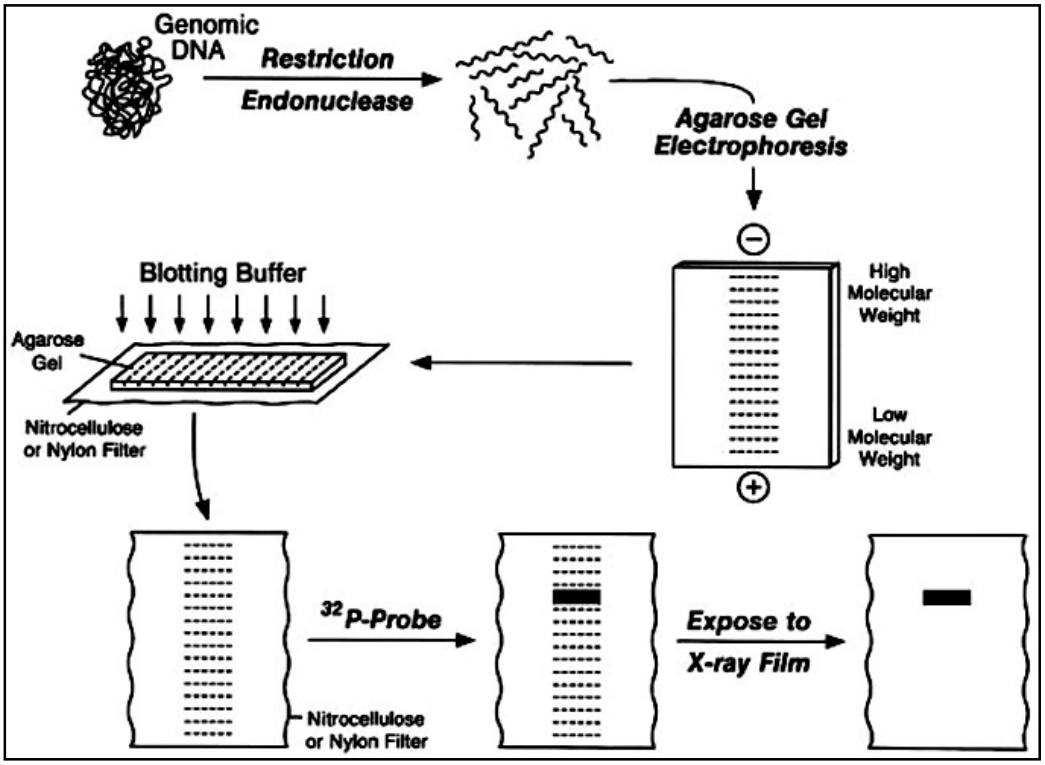
- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of DNA from the mixture of other similar molecules. This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by

labelled probe hybridization.

- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/Steps:

I. Restriction digest: by RE enzyme and amplification by PCR
II. Gel electrophoresis: SDS gel electrophoresis
III. Denaturation: Treating with HCl and NaOH
IV. Blotting
V. Baking and Blocking with casein in BSA
VI. Hybridization using labelled probes
VII. Visualization by autoradiogram



Step I: Restriction digest

- ✓ The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specificsite generating fragments
- ✓ The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- ✓ The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

- ✓ The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denaturethe double stranded DNA fragments.
- ✓ DNA strands get separated

Step IV: Blotting

- ✓ The separated strands of DNA are then transferred to positively charged membrane nylonmembrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- ✓ After the DNA of interest bound on the membrane, it is baked on autoclave to fix in themembrane.
- ✓ The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates allthe binding site of membrane

Step VI: Hybridization with labelled probes

- ✓ The DNA bound to membrane is then treated with labelled probe
- ✓ The labelled probe contains the complementary sequences to the gene of interest
- ✓ The probe bind with complementary DNA on the membrane since all other non-specificbinding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- ✓ The membrane bound DNA labelled with probe can be visualized under autoradiogram whichgive pattern of bands.

Application of Southern blotting:

- ∗ Southern blotting technique is used to detect DNA in given sample.
- ∗ DNA finger printing is an example of southern blotting.

* Used for paternity testing, criminal identification, victim identification.
* To isolate and identify desire gene of interest.
* Used in restriction fragment length polymorphism.
* To identify mutation or gene rearrangement in the sequence of DNA.
* Used in diagnosis of disease caused by genetic defects.
* Used to identify infectious agents

Northern blot:

Northern blotting is used to detect RNA. Cells can be broken open to release their RNA. The RNA from different cell types can be run on separate lanes on a gel. The gel spreads the different RNA by size. These neat, parallel rows of RNA allow a researcher to compare which cell type has how much of which RNA. This method allows a researcher to determine if cells from a certain disease have more of this RNA or less of that RNA. Northern blotting may reveal how a disease is working at the level of RNA production.

Principle:

As all normal blotting technique, northern blotting starts with the electrophoresis to separate RNA samples by size. Electrophoresis separates the RNA molecules based on the charge of the nucleic acids. The charge in the nucleic acids is proportional to the size of the nucleic acid sequence. Thus the electrophoresis membrane separates the Nucleic acid sequence according to the size of the RNA sequence. In cases where our target sequence is an mRNA, the sample can be isolated through oligo cellulose chromatographic techniques, as mRNA are characterized by the polyA tail. Since gel molecules are fragile in nature, the separated sequences are transferred to the nylon membranes. The selection of nylon membrane is contributed to the factor that nucleic acids are negatively charged in nature. Once the RNA molecules are transferred it is immobilized by covalent linkage. The probe is then added, the probe can be complementary an ss DNA sequence. Formamide is generally used as a blotting buffer as it reduces the annealing temperature.

Procedure:

I. The tissue or culture sample collected is first homogenized. The samples may be representative of different types of culture for comparison or it can be for the study of different stages of growth inside the culture.

II. The RNA sequence is separated in the electrophoresis unit an agarose gel is used for the purpose of the nucleic acid separation.

III. Now the separated RNA sequence is transferred to the nylon membrane. This is done by two mechanisms capillary action and the ionic interaction.

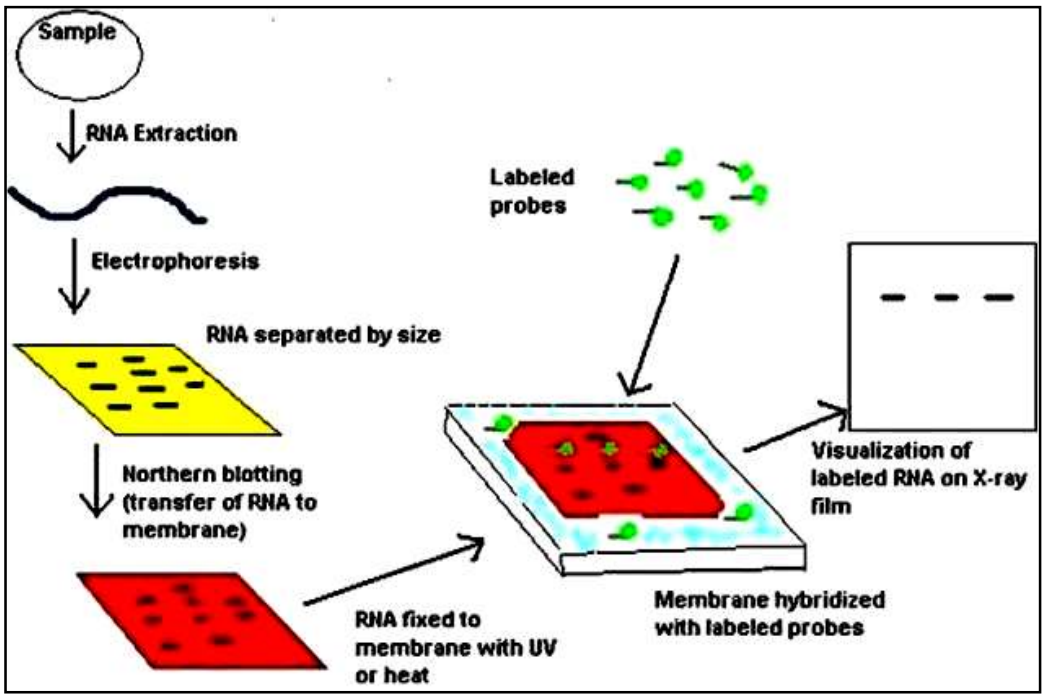
IV. The transfer operation is done by keeping the gel in the following order. First, the agarose gel is placed on the bottom of the stack, followed by the blotting membrane. On top of these paper towels a mild weight (glass plate) is placed. The entire setup is kept in a beaker containing transfer buffer.

V. RNA transferred to the nylon membrane is then fixed using UV radiation.

VI. The fixed nylon membrane is then mixed with probes. The probes are specifically designed for the gene of interest, so that they will hybridize with RNA sequences on the blot corresponding to the sequence of interest.

VII. The blot membrane is washed to remove unwanted probe

VIII. Labeled probe is detected by chemiluminescence or autoradiography. The result will be dark bands in x-ray film.



Application of Northern blotting:

Northern blotting allows one to observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment. The technique has been used to show overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue, as well as the gene expression in the rejection of transplanted organs. If an upregulated gene is observed by an abundanceof mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding.

The expression patterns obtained under given conditions can provide insight into the function of that gene. Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight into the size of the product, suggesting alternative splice products of the same gene or repetitive sequence motifs.The variance in size of a gene product can also indicate deletions or errors in transcript processing. By altering the probe target used along the known sequence it is possible to determine which region of the RNA is missing.

BlotBase is an online database publishing northern blots. BlotBase has over 700 published

northern blots of human and mouse samples, in over 650 genes across more than 25 different tissue types.Northern blots can be searched by a blot ID, paper reference, gene identifier, or by tissue. The results of a search provide the blot ID, species, tissue, gene, expression level, blot image (if available),and links to the publication that the work originated from. This new database provides sharing of information between members of the science community that was not previously seen in northern blotting as it was in sequence analysis, genome determination, protein structure, etc.

Advantages:

Analysis of gene expression can be done by several different methods including RT-PCR, RNase protection assays, microarrays, RNA-Seq, serial analysis of gene expression (SAGE), as well as northern blotting. Microarrays are quite commonly used and are usually consistent with data obtained from northern blots; however, at times northern blotting is able to detect small changes in gene expression that microarrays cannot. The advantage that microarrays have over northern blots is that thousands of genes can be visualized at a time, while northern blotting is usually looking at one or a small number of genes.

Disadvantages:

A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyrocarbonate). The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material, ethidium bromide, DEPC, and UV light are all harmful under certain exposures. Compared to RT- PCR, northern blotting has a low sensitivity, but it also has a high specificity, which is important to reduce false positive results. The advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, the quality and quantity of RNA can be measured on the gel prior to blotting, and the membranes can be stored and reprobed for years after blotting.

For northern blotting for the detection of acetylcholinesterase mRNA the nonradioactive technique was compared to a radioactive technique and found as sensitive as the radioactive one, but requires noprotection against radiation and is less time consuming.

Western blot:

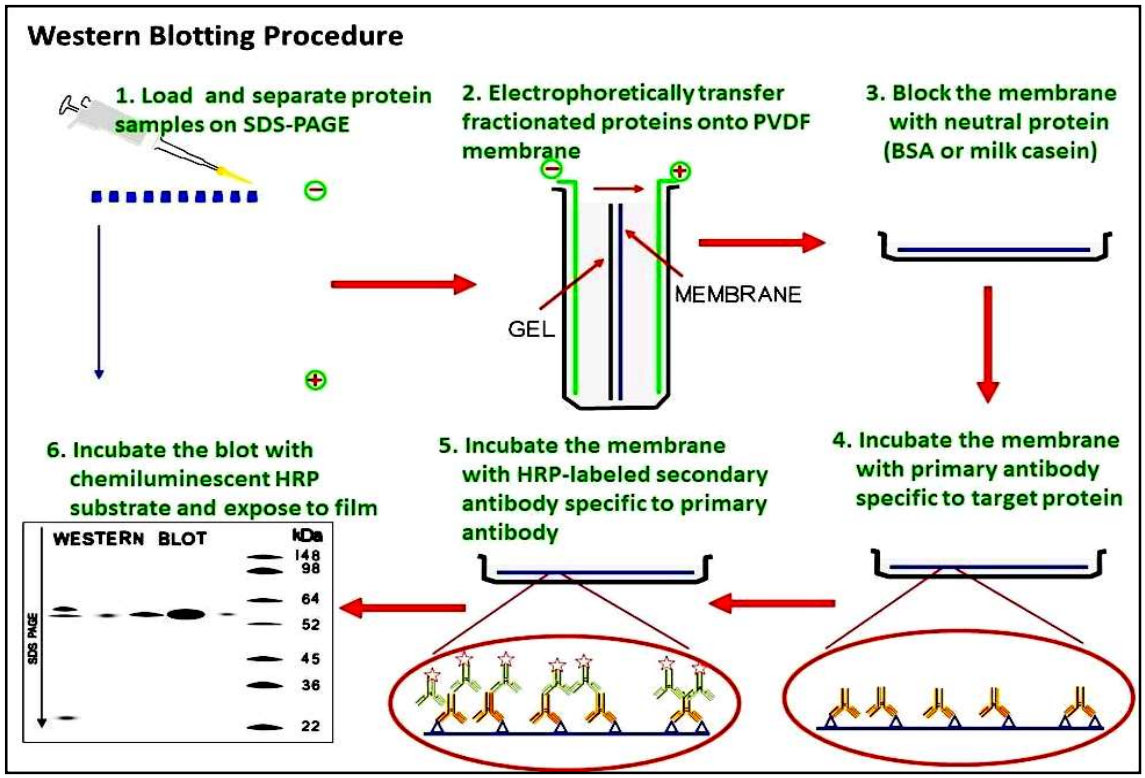
Western blotting is a common technique for separating proteins by size, but in straight columns. These parallel columns allow researchers to compare the amount of a protein across different samples that are run right next to each other, like bowling lanes. For example, if you were testing the effect of different amounts of a drug on cell growth, you would treat four different groups of cells with a different amount of drug. Then you could break the cells open and run the proteins of each group in separate lanes on a gel. Spreading the proteins out in this way allows you to see what an increasing concentration of drugs does to a certain protein.

Principle:

- Western blotting technique is used for identification of particular protein from the mixture of protein.
- In this method labelled antibody against particular protein is used identify the desired protein, so it is a specific test. Western blotting is also known as immunoblotting because it uses antibodies to detect the protein.

Procedure/Steps:

I. Extraction of protein
II. Gel electrophoresis: SDS PAGE
III. Blotting: electrical or capillary blotting
IV. Blocking: BSA
V. Treatment with primary antibody
VI. Treatment with secondary antibody(enzyme labelled anti Ab)
VII. Treatment with specific substrate; if enzyme is alkaline phosphatase, substrate is p-nitro phenyl phosphate which give color.



Step I: Extraction of protein

- ✓ Cell lysate is most common sample for western blotting.
- ✓ Protein is extracted from cell by mechanical or chemical lysis of cell. This step is also known as tissue preparation.
- ✓ To prevent denaturing of protein protease inhibitor is used.
- ✓ The concentration of protein is determined by spectroscopy.
- ✓ When sufficient amount of protein sample is obtained, it is diluted in loading buffer containing glycerol which helps to sink the sample in well.
- ✓ Tracking dye (bromothymol blue) is also added in sample to monitor the movement of proteins.

Step II: Gel electrophoresis

- ✓ The sample is loaded in well of SDS-PAGE Sodium dodecyl sulfate- poly-acrylamide gel electrophoresis.
- ✓ The proteins are separated on the basis of electric charge, isoelectric point,

molecular weight, or combination of these all.

- ✓ The small size protein moves faster than large size protein.
- ✓ Protein is negatively charged, so they move toward positive (anode) pole as electric current is applied

Step III: Blotting

- ✓ The nitrocellulose membrane is placed on the gel. The separated protein from gel gets transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may take 1-2 days
- ✓ For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.
- ✓ In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step IV: Blocking

- ✓ Blocking is very important step in western blotting.
- ✓ Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the primary antibody the membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA).

Step IV: Treatment with primary antibody

- ✓ The primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex

Step VI: Treatment with secondary antibody

- ✓ The secondary antibody is enzyme labelled. For example, alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody.
- ✓ Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

Step VII: Treatment with suitable substrate

- ✓ To visualize the enzyme action, the reaction mixture is incubated with specific substrate.
- ✓ The enzyme convert the substrate to give visible colored product, so band of color can bevisualized in the membrane.

- ✓ Western blotting is also a quantitative test to determine the amount of protein in sample.

Application of Southern blotting:

- * To determine the size and amount of protein in given sample.
- * Disease diagnosis: detects antibody against virus or bacteria in serum.
- * Western blotting technique is the confirmatory test for HIV. It detects anti HIV antibody inpatient's serum.
- * Useful to detect defective proteins. For example, Prions disease.
- * Definitive test for Creutzfeldt-Jacob disease, Lyme disease, Hepatitis B and Herpes.

Other blots:

The three main blotting techniques -- Western, Northern and Southern -- have been modified in different ways to detect slightly different molecules. The Western blot vs the Southern blot, for example, detects protein and DNA, respectively. Each modified technique is generally done the usual way, but uses a different method to detect the molecule that is being spread out into the parallel lanes. Southwestern blots detect molecules of protein stuck to DNA. North-western blots detect molecules of protein stuck to RNA. Farwestern blots detect molecules of protein stuck to other proteins.

DNA-fingerprinting:

It has been a subject of human interest since primitive times when man used to hunt for his food with the help of animal's foot prints. Science of fingerprinting was first used by Sir William Herschel as a method of identification in 1858. In India the science of fingerprints was discovered by chance during a murder investigation in Jalpaiguri in 1897.

Alec Jeffreys (1984) invented the DNA fingerprinting technique at Leicester University, United Kingdom. Dr. V.K. Kashyap and Dr. Lalji Singh started the DNA fingerprinting technology in Indiaat CCMB (Centre for Cell and Molecular Biology) Hyderabad.

What is DNA-fingerprinting?

DNA-fingerprinting (also called DNA typing or DNA profiling). It is a technique of determining nucleotide sequences of certain areas of DNA which are unique to each individual. Each person has a unique DNA fingerprint.

Unlike a conventional fingerprint that occurs only on the fingertips and can be altered by surgery, a DNA fingerprint is the same for every cell, tissue and organ of a person. It cannot be changed by any known treatment. The ideal way to distinguish an individual — from other people would be his or her entire genomic DNA sequence.

Principle of DNA Fingerprinting:

By their differences, about 0.1% or 3 x 106 base pairs (out of 3 x 109 bp) provide individuality to each human being. Human genome possesses numerous small noncoding but inheritable sequences of bases which are repeated many times. These sequences occur near telomere, centromeres, Y chromosome and heterochromatic area. The area with same sequence of bases repeated several times is called repetitive DNA.

They can be separated as satellite from the bulk DNA during density gradient centrifugation and hence called satellite DNA. In satellite DNA, repetition of bases is in tandem. Depending upon length base composition and numbers of tandemly repetitive units, satellite DNAs have subcategories like microsatellites and mini-satellites. Satellite DNAs show polymorphism. The term polymorphism is used when a variant at a locus is present with a frequency of more than 0.01 population.

Variations occur due to mutations. While mutations in genes produce alleles with different expressions, mutations in noncoding repetitive DNA have no immediate impact.

These mutations in the noncoding sequences have piled up with time and form the basis of DNA polymorphism (variation at genetic level arises due to mutations). DNA polymorphism is the basis of genetic mapping of human genome as well as DNA finger printing.

Short nucleotide repeats in the DNA are very specific in each individual and vary in number from person to person but are inherited. These are the 'Variable Number Tandem Repeats' (VNTRs). These are also called "minisatellites". Each individual inherits these repeats from his/her parents which are used as genetic markers in a personal identity test.

For example, a child might inherit a chromosome with six tandem repeats from the mother and the same tandem repeated four times in the homologous chromosome inherited from the father. One half of VNTR alleles of the child resemble that of the mother and other half with that of the father.

DNA Fingerprinting Steps

- **Extracting DNA from Cells**

To perform DNA fingerprinting, you must first have a DNA sample!

In order to procure this, a sample containing genetic material must be treated with different chemicals.Common sample types used today include blood and cheek swabs.

These samples must be treated with a series of chemicals to break open cell membranes, expose the DNA sample, and remove unwanted components – such as lipids and proteins – until relatively pure DNA emerges.

- **PCR Amplification (Optional)**

If the amount of DNA in a sample is small, scientists may wish to perform PCR – Polymerase Chain Reaction – amplification of the sample.

PCR is an ingenuous technology which essentially mimics the process of DNA replication carried out by cells. Nucleotides and DNA polymerase enzymes are added, along with "primer" pieces of DNA which will bind to the sample DNA and give the polymerases a starting point.

PCR "cycles" can be repeated until the sample DNA has been copied many times in the lab if necessary.

- **Treatment with Restriction Enzymes**

Once sufficient DNA has been isolated, and amplified if necessary, it must be cut with restriction enzymes to isolate the VNTRs.

Restriction enzymes are enzymes that attach to specific DNA sequences and create breaks in the DNAstrands.

Bacterial cells use restriction enzymes for protection against DNA viruses which may invade the cell and hijack its machinery, but scientists have taken advantage of these special enzymes and used them to make DNA profiling and even genetic engineering possible.

In genetic engineering, DNA is cut up with restriction enzymes and then "sewn" back together

by ligases to create new, recombinant DNA sequences.

In DNA profiling, however, only the cutting part is needed. Once the DNA has been cut to isolate the VNTRs, it's time to run the resulting DNA fragments on a gel to see how long they are.

- **Gel Electrophoresis**

Gel electrophoresis is a brilliant technology that separates molecules by size. The "gel" in question isa material that molecules can pass through, but only at a slow speed.

Just as air resistance slows a big truck more than it does a motorcycle, the resistance offered by the electrophoresis gel slows large molecules down more than small ones. The effect of the gel is so precise that scientists can tell exactly how big a molecule is by seeing how far it moves within a givengel in a set amount of time. In this case, measuring the size of the DNA fragments from the sample that has been treated with restriction enzyme will tell scientists how many copies of each VTNR repeat the sample DNA contains. It's called "electrophoresis" because, to make the molecules move through the gel, an electrical current is applied. Because the sugar-phosphate backbone of the DNA has a negative electrical charge, the electrical current tugs the DNA along with it through the gel

By looking at how many DNA fragments the restriction enzymes produced and the sizes of these fragments, the scientists can "fingerprint" the DNA donor.

- **Transfer onto Southern Blot**

Now that the DNA fragments have been separated by size, they must be transferred to a mediumwhere scientists can "read" and record the results of the electrophoresis.

To do this, scientists treat the gel with a weak acid, which breaks up the DNA fragments into individual nucleic acids that will more easily rub off onto paper.

They then "blot" the DNA fragments onto nitrocellulose paper, which fixes them in place.

- **Treatment with Radioactive Probe**

Now that the DNA is fixed onto the blot paper, it is treated with a special probe chemical that sticks to the desired DNA fragments. This chemical is radioactive, which means that it will create a visible record when exposed to X-ray paper.

This method of blotting DNA fragments onto nitrocellulose paper and then treating it with a radioactive probe was discovered by a scientist name Ed Southern – hence the name

"Southern blot." Amusingly, the fact that the Southern blot is named after a scientist has nothing to do with directions didn't stop scientists from naming similar methods "northern" and "western" blots in honor of theSouthern blot.

- **X-ray Film Exposure**

The last step of the process is to turn the information from the DNA fragments into a visible record.This is done by exposing the blot paper, with its radioactive DNA bands, to X-ray film.

X-ray film is "developed" by radiation, just like camera film is developed by visible light, resulting ina visual record of the pattern produced by the person's DNA "fingerprint."

To ensure a clear imprint, scientists often leave the X-ray film exposed to the weakly radioactiveSouthern blot paper for a day or more.

Once the image has been developed and fixed to prevent further light exposure from changing the image, this "fingerprint" can be used to determine if two DNA samples are the same or similar!

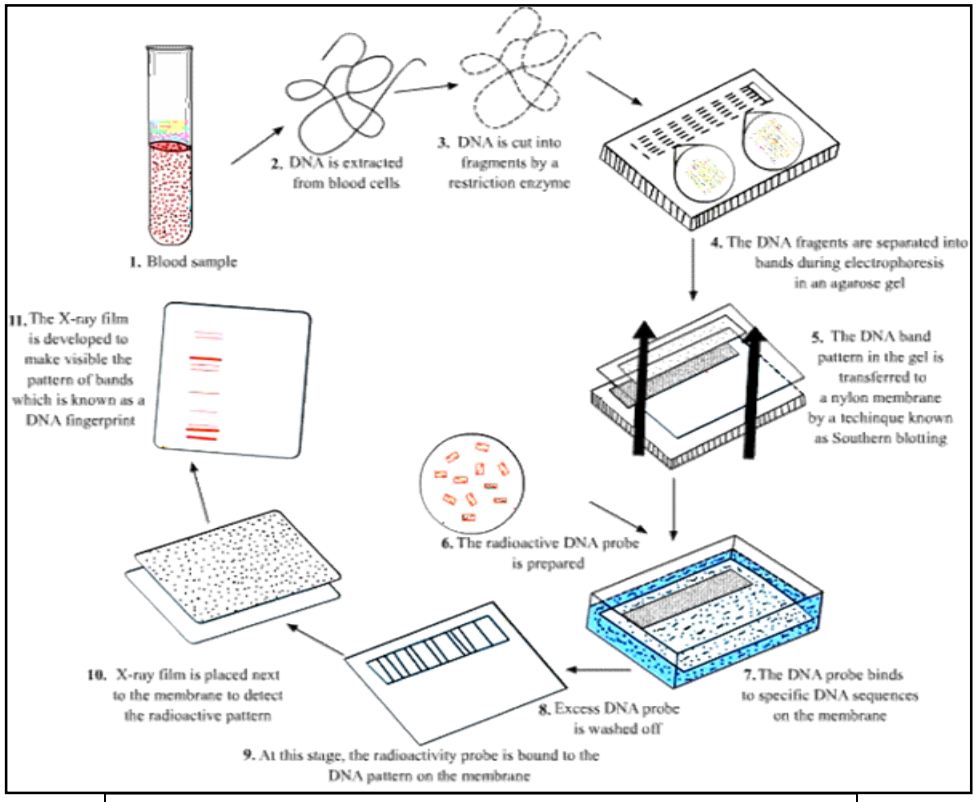


Fig.: Steps in DNA fingerprinting technique

Applications of DNA Fingerprinting:

- **Individuality:** Like skin finger printing (der- matoglyphics), DNA finger printing can help to distinguish one human being from another with exception of monozygotic twins.
- **Paternity/Maternity Disputes:** DNA finger print•ing can identify the real genetic mother, father and the offspring.
- **Human Lineage:** DNA from various probables is being studied to find out human lineage.
- **Hereditary Diseases:** The technique is being used to identify genes connected with hereditary diseases.
- **Forensics:** DNA finger printing is very useful in the detection of crime and legal pursuits. DNA fingerprinting has proved that Dhanu, the human bomb, was the real murderer of Shri Rajiv Gandhi, the former Prime Minister of India.
- **Sociology:** It can identify racial groups, their origin, historical migration and invasions. Genography is the study of migratory history of human species.

DNA footprinting:

DNA footprinting is a method of investigating the sequence specificity of DNA- binding proteins in vitro. This technique can be used to study protein-DNA interactions both outside and within cells.

The regulation of transcription has been studied extensively, and yet there is still much that is not known. Transcription factors and associated proteins that bind promoters, enhancers, or silencers to drive or repress transcription are fundamental to understanding the unique regulation of individual genes within the genome. Techniques like DNA footprinting help elucidate which proteins bind to these associated regions of DNA and unravel the complexities of transcriptional control.

In 1978, David Galas and Albert Schmitz developed the DNA footprinting technique to study the binding specificity of the lac repressor protein. It was originally a modification of the Maxam-Gilbert chemical sequencing technique.

Methods and Procedure:

The simplest application of this technique is to assess whether a given protein binds to a region of interest within a DNA molecule. Polymerase chain reaction (PCR) amplifies and label region of interest that contains a potential protein-binding site, ideally amplicon is between 50 and 200 base pairs in length. Add protein of interest to a portion of the labeled template DNA; a portion should remain separate without protein, for later comparison. Add a cleavage agent to both portions of DNA template. The cleavage agent is a chemical or enzyme that will cut at random locations in a sequence independent manner. The reaction should occur just long enough to cut each DNA molecule in only one location. A protein that specifically binds a region within the DNA template will protect the DNA it is bound to from the cleavage agent. Run both samples side by side on a polyacrylamide gel electrophoresis. The portion of DNA template without protein will be cut at random locations, and thus when it is run on a gel, will produce a ladder-like distribution. The DNA template with the protein will result in ladder distribution with a break in it, the "footprint", where the DNA has been protected from the cleavage agent. Note: Maxam-Gilbert chemical DNA sequencing can be run alongside the samples on the polyacrylamide gel to allow the prediction of the exact location of ligand binding site.

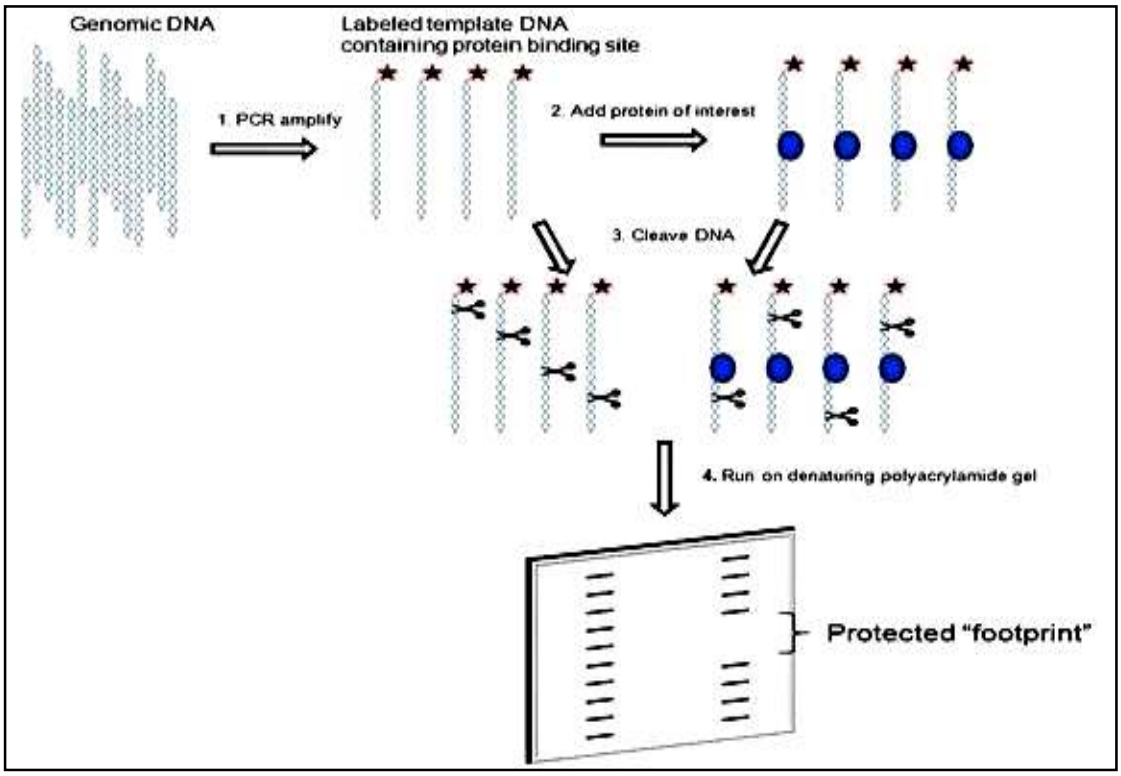
Labeling:

The DNA template labeled at the 3' or 5' end, depending on the location of the binding site(s). Labels that can be used are: radioactivity and fluorescence. Radioactivity has been traditionally used to label DNA fragments for footprinting analysis, as the method was originally developed from the Maxam- Gilbert chemical sequencing technique. Radioactive labeling is very sensitive and is optimal for visualizing small amounts of DNA. Fluorescence is a desirable advancement due to the hazards of using radio-chemicals. However, it has been more difficult to optimize because it is not always sensitive enough to detect the low concentrations of the target DNA strands used in DNA footprinting experiments. Electrophoretic sequencing gels or capillary electrophoresis have been successful in analyzing footprinting of fluorescent tagged fragments.

Cleavage agent:

A variety of cleavage agents can be chosen. a desirable agent is one that is sequence neutral, easy to use, and is easy to control. Unfortunately no available agents meet all of these standards, so an appropriate agent can be chosen, depending on your DNA sequence and ligand of interest. The following cleavage agents are described in detail:DNase I is a large protein that functions as a double- strand endonuclease. It binds the minor groove of DNA and cleaves the phosphodiester backbone. It is a good cleavage agent for footprinting because its size makes it easily physically hindered. Thus is more likely to have its action blocked by a bound protein on a DNA sequence. In addition, the DNase I enzyme is easily controlled by adding EDTA to stop the reaction. There are however some limitations in using DNase I. The enzyme does not cut DNA randomly; its activity is affected by local DNA structure and sequence and therefore results in an uneven ladder. This can limit the precision of predicting a protein's binding site on the DNA molecule. Hydroxyl radicals are created from the Fenton reaction, which involves reducing Fe^{2+} with H_2O_2 to form free hydroxyl molecules. These hydroxyl molecules react with the DNA backbone, resulting in a break. Due to their small size, the resulting DNA footprint has high resolution. Unlike DNase I they have no sequence dependence and result in a much more evenly distributed ladder. The negative aspect of using hydroxyl radicals is that they are more time consuming to use, due to a slower reaction and digestion time. Ultraviolet irradiation can be used to excite nucleic acids and create photoreactions, which results in damaged bases in the DNA strand. Photoreactions can include: single strand breaks, interactions between or within DNA strands, reactions with solvents, or crosslinks with proteins. The workflow for this method has an additional step, once both your protected and unprotected DNA have been treated, there is subsequent primer extension of the cleaved products. The extension will terminate upon reaching a damaged base, and thus when the PCR products are run side-by-side on a gel; the protected sample will show an additional band where the DNA was crosslinked with a bound protein. Advantages of using UV are that it reacts very quickly and can therefore capture interactions that are only momentary. Additionally it can be applied to in vivo experiments, because UV can penetrate cell membranes. A disadvantage is that the gel can be difficult to interpret, as the bound protein does not protect the DNA, it merely alters the photoreactions in the

vicinity.



Application:

- ***In vivo* footprinting**

In vivo footprinting is a technique used to analyze the protein-DNA interactions that are occurring in a cell at a given time point. DNase I can be used as a cleavage agent if the cellular membrane has been permeabilized. However the most common cleavage agent used is UV irradiation because it penetrates the cell membrane without disrupting cell state and can thus capture interactions that are sensitive to cellular changes. Once the DNA has been cleaved or damaged by UV, the cells can be lysed and DNA purified for analysis of a region of interest. Ligation-mediated PCR is an alternative method to footprint in vivo. Once a cleavage agent has been used on the genomic DNA, resulting in single strand breaks, and the DNA is isolated, a linker is added onto the break points. A region of interest is amplified between the linker and a gene-specific primer, and when run on a polyacrylamide gel, will have a footprint where a protein was bound. In vivo footprinting combined with immunoprecipitation can be

used to assess protein specificity at many locations throughout the genome. The DNA bound to a protein of interest can be immunoprecipitated with an antibody to that protein, and then specific region binding can be assessed using the DNA footprinting technique.

* **Quantitative footprinting**

The DNA footprinting technique can be modified to assess the binding strength of a protein to a region of DNA. Using varying concentrations of the protein for the footprinting experiment, the appearance of the footprint can be observed as the concentrations increase and the proteins binding affinity can then be estimated.

* **Detection by capillary electrophoresis**

To adapt the footprinting technique to updated detection methods, the labelled DNA fragments are detected by a capillary electrophoresis device instead of being run on a polyacrylamide gel. If the DNA fragment to be analyzed is produced by polymerase chain reaction (PCR), it is straightforward to couple a fluorescent molecule such as carboxyfluorescein (FAM) to the primers. This way, the fragments produced by DNaseI digestion will contain FAM, and will be detectable by the capillary electrophoresis machine.

Basic idea of Proteomics:

The entire protein component of a given organism is called 'proteome', the term coined by Wasinger in 1995. A proteome is a quantitatively expressed protein of a genome that provides information on the gene products that are translated, amount of products and any post translational modifications.

Proteomics is an emerging area of research in the post-genomic era, which involves identifying the structures and functions of all proteins of a proteome. It is sometimes also treated as structural based functional genomics.

Types of Proteomics:

(i) Structural Proteomics:

One of the main targets of proteomics investigation is to map the structure of protein complexes or the proteins present in a specific cellular organelle known as cell map or structural proteins. Structural proteomics attempt to identify all the proteins within a protein

complex and characterization all protein-protein interactions. Isolation of specific protein complex by purification can simplify the proteomic analysis.

(ii) Functional Proteomics:

It mainly includes isolation of protein complexes or the use of protein ligands to isolate specific types of proteins. It allows selected groups of proteins to be studied its chracteristics which can provide important information about protein signalling and disease mechanism etc.

(iii) Expression proteomics:

Expression proteomics includes the analysis of protein expression at larger scale. It helps identify main proteins in a particular sample, and those proteins differentially expressed in related samples— such as diseased vs. healthy tissue. If a protein is found only in a diseased sample then it can be a useful drug target or diagnostic marker. Proteins with same or similar expression profiles may also be functionally related. There are technologies such as 2D-PAGE and mass spectrometry that are used in expression proteomics.

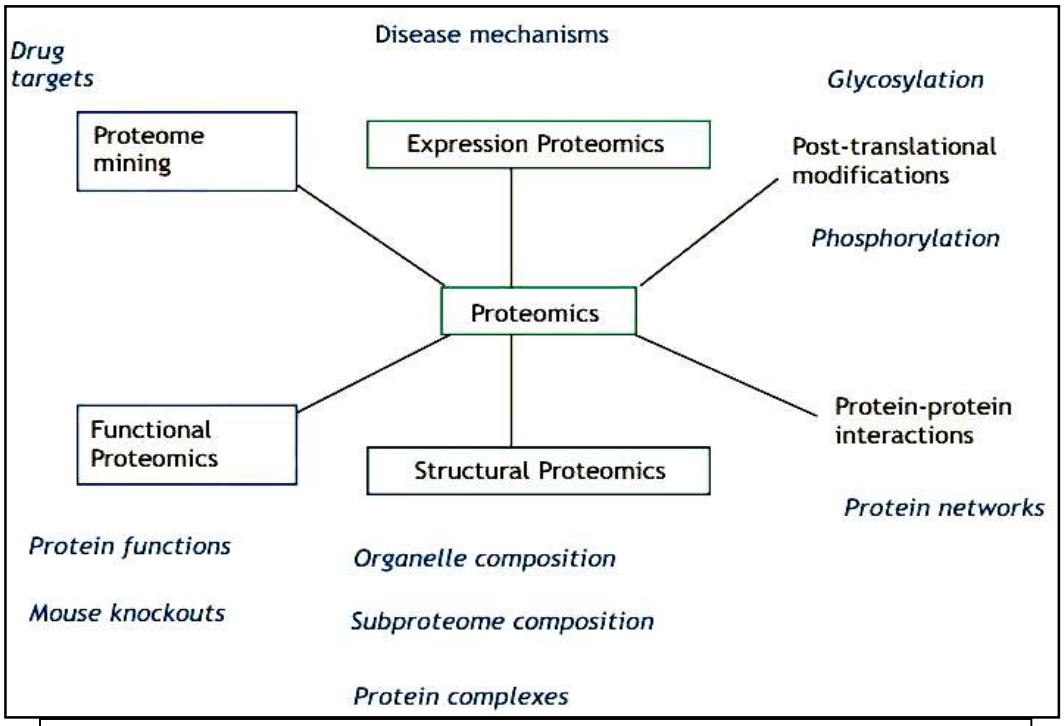


Fig.: Types of Proteomics and the scientific knowledge that can be gained from them.

Significance of Proteomics:

(i) Protein profiling

Bioinformatics has been widely employed in protein-profiling, where question of protein structural information for the purpose of protein identification, characterization and database is carried out. The spectrum of protein expressed in a cell type provides the cell with its unique identity. It explores how the protein complement changes in a cell type during development in response to environmental stress.

(ii) Protein arrays

Protein microarrays facilitate the detection of protein protein interaction and protein expression profiling. Several protein microarray examples indicate that protein arrays hold great promise for the global analysis of protein-protein and protein-ligand interaction.

(iii) Proteomics to a phosphorylation

In post-translational modification of protein, mass spectrometer (MS) can be used to identify novel phosphorylation. Measure changes in phosphorylation state of protein takes place in response to an effective and determining phosphorylation sites in proteins.

Identification of phosphorylation sites can provide information about the mechanism of enzyme regulation and protein kinase and phosphotases involved. A proteomics approach for this process has an advantage that one can study all the phosphorylating proteins in a cell at the same time.

(iv) Proteome mining

Proteome mining is a functional proteomic approach used to extract information from the analysis of specific sub-proteomics. In principle, it is based on the assumption. In principle, it is based oil the assumption that all drug like molecule selectively compete with a natural cellular ligand for a binding site on a protein target.

Basic idea of Genomics:

The term genomics was first used by Thomas Roderick in 1986. It refers to the study of structure and function of entire genome of a living organism. The complete genetic content of an organism is genome, and the DNA obtained is called genomic DNA. This genomic DNA of prokaryote contains the entire coding region and can be sequenced, whereas the DNA of

eukaryotes includes both intron and exon sequences (coding sequence) as well as noncoding regulatory sequences such as promo•ter, and enhancer sequences.

The subject genomics is the complete analysis of the entire genome of a chosen organism which involves the study of physical structure of the orga-nism's genome or the genetic makeup of an organism to know the number of genes present and the type of genes, i.e., to study the function of different genes.

Whole Genome Sequence Data:

Complete nucleotide sequences of nuclear, mitochondrial and chloroplast genomes have already beenworked out in large number of prokaryotes and several eukaryotes. By the year 2005, among prokaryotes, approx. 1400 viral genomes, 250 bacterial genomes (230 eubacteria and 20 archaea), 500 mitochondrial genomes, 35 chloroplast genomes have been fully sequenced.

Among the eukaryotes namely the whole genome of *Saccharomyces cerevisiae* (yeast), *Coenorhabditis elegans* (nematode), fruitfly (*Drosophila melanogaster*), Human (*Homo sapiens*), Crucifer weed (*Arabidopsis thaliana*) and rice (*Oryza sativa*) have been sequenced already and data available for annotation studies.

The sequence data of eukaryotic nuclear genome is an important source of identi•fication, discovery and isolation of important genes. This data is very much helpful in variety of application relevant to animal, plant and microbial biotechnology.

Types of Genomics:

The discipline of genomics consists of two parts, viz. structural genomics and functional genomics.These are defined as under –

1. **Structural Genomics:**

It deals with the study of the structure of entire genome of a living organism. In other words, it deals with the study of the genetic structure of each chromosome of the genome. It determines the size of the genome of a species in mega-bases [Mb] and also the genes present in the entire genome of a species.

2. **Functional Genomics:**

The study of function of all genes present in the entire genome is known as functional genomics. It deals with transcriptome and proteome. The transcriptome refers to complete set of RNAs transcribed from a genome and proteome refers to complete set of proteins encoded by a genome.

3. **Comparative Genomics:**

It deals with the study of multiple whole genomes for understanding the differences and similarities between all the genes of the multiple species. For example in the phylogenic tree analysis two specialized concepts are the rooted and unrooted trees.

Phylogenetic trees are designed to reveal evolutionary relationship among DNA or protein sequences. In unrooted trees, when distantly related sequence for comparison has not included when an unrooted tree is required.

Epigenomics:

Epigenomics is the study of the complete set of epigenetic modifications on the genetic material of a cell, known as the epigenome. Epigenetic modifications are reversible modifications on a cell's DNA or histones that affect gene expression without altering the DNA sequence. Two of the most characterized epigenetic modifications are DNA methylation and histone modification. Epigenetic modifications play an important role in gene expression and regulation, and are involved in numerous cellular processes such as in differentiation/development and tumorigenesis. The study of epigenetics on a global level has been made possible only recently through the adaptation of genomic high- throughput assays.

Metagenomics:

Environmental Shotgun Sequencing (ESS) is a key technique in metagenomics. (A) Sampling from habitat; (B) filtering particles, typically by size; (C) Lysis and DNA extraction; (D) cloning and library construction; (E) sequencing the clones; (F) sequence assembly into contigs and scaffolds.

Metagenomics is the study of metagenomes, genetic material recovered directly from

environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. While traditional microbiology and microbial genome sequencing rely upon cultivated clonal cultures, early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of diversity in a natural sample. Such work revealed that the vast majority of microbial biodiversity had been missed by cultivation-based methods. Recent studies use "shotgun" Sanger sequencing or massively parallel pyrosequencing to get largely unbiased samples of all genes from all the members of the sampled communities. Because of its power to reveal the previously hidden diversity of microscopic life, metagenomics offers a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world.

Role of Genomics in Crop Plants:

The discipline of genomics is ofrecent origin. The genome mappingwas first completed in free livingbacteria *Haemophillus influenza* in1995. Later on genome sequencingwork was intensified both in prokaryotes and eukaryotes. In plants, genome sequencing was first completed in Arabidopsis thaliana (a weedy relative of mustard followed by rice (*Oriza sativa*). Now genome sequencing work has been completed in more than 40 crop plants. The list of some field crops, fruit crops and other plant species in which genome sequencing work has been completed is presented below –

S.No.	Species	Botanical Name	Genome Size [Mb]	Gene No.
		A. FIELD CROPS		
1.	Arabidopsis	*Arabidopsis thaliana*	120	27416
2.	Rice	*Oryza sativa*	370	40577
3.	Corn	*Zea mays*	2500	>32000
4.	Sorghum	*Sorghum bicolor*	700	34496
5.	Cucumber	*Cucumis sativa*	243.5	26682
6.	Soybean	*Glycine max*	950	46430
7.	Caster bean	*Ricinus communis*	320	31237
8.	Pigeon pea	*Cajanus cajan*	833	48680
9.	Potato	*Solanum tuberosum*	844	39031
10.	Cannabis	*Cannabis sativa*	534	30,000

In plants, the smallest genome size has been reported in *Arabidopsis thaliana* (120 Mb) and the largest in corn (2500). Thus, in plants the genome size investigated so far varies from 120 Mb to 2500 Mb. After gene sequencing, function is assigned to individual gene through various molecular techniques.

Genes to be mapped:

In genome research, both types of genes, viz., major genes (oligogenes) and minor genes (polygenes) can be easily mapped. The mapping of polygenic traits is possible by genome mapping techniques which is not possible by conventional gene mapping techniques such recombination mapping and deletion mapping.

The genome mapping is done for morphological, productivity, resistance, quality, agronomic and some special traits as discussed below –

(i) **Morphological Characters:** It includes highly heritable traits such as shape, size and color of leaf, flower, calyx, corolla, etc. It also includes surface of leaf and stem (hairiness and smoothness).

(ii) **Productivity traits:** Such characters differ from species to species.

(iii) **Resistance Traits:** Such characters include resistance to diseases, insects, drought, soil salinity, soil alkalinity, soil acidity, heat, frost, water logging, cold, etc.

(iv) **Quality Traits:** Such traits include nutritional quality, market quality and keeping quality.

(v) **Agronomic Traits:** Such traits include earliness, plant height, plant type, etc.

(vi) **Special Characters:** Such characters include genes controlling male sterility, self-incompatibility, photo and thermo insensitivity, toxic substances, apomixes, adaptation, etc.

Genome Mapping Laboratories:

The genome mapping work is carried out by collaborative efforts of various International and National Research Laboratories of public domain. The list of some organizations where genome sequencing and mapping work on different crop species is carried out is presented in Table.

TABLE 35.2. List of some Genome Sequencing Organization

S.No.	Name of Crop	Scientific name	Name of Genome Project	In Collaboration with
1.	Arabidopsis	*Arabiddopsis thaliana*	Arabidopsis Genome Initiative	USA, China and Japan.
2.	Brassica	*Brassica* Spp.	Brassica Genome Gate way	UK, USA, France, Germany, Australia, South Korea, Poland and China.
3.	Wheat	*Tricum aestivum*	International Wheat genome	UK Scientists from Uni. of Liverpool, Bristol and John Innes.
4.	Rice	*Oriza* Spp.	International Rice Genome Sequencing Project	Mexico, USA, China and Japan
5.	Cotton	*Gossypium* Spp.	International Cotton Genome Initiative Project	USA, France, Australia
6.	Soybean	*Glycine max*	Uni. Purdue, North Carolina Uni, and 18 other centres	United States of America
7.	Potato	*Solanum tuberosum*	Potato Genome Sequencing Consortium	USA, UK, Russia, China, India, Poland, Chile,New Zealand, Nether land, Peru, etc.
8.	Tomato	*Lycopersicum esculentum*	Tomato Genome Consortium	China, japan, Germany,Korea, Spain, UK, USA, Israel, Netherland & Others
9.	Pigeon pea	*Cajanus cajan*	International Initiative for Pigeonpea Genomics	India (ICRISAT), China, USA, Mexico, etc.
10.	Apple	*Malus domesticus*	Apple Genome sequencing Initiative	Washington State Uni. USA and NRI COMPETITIVE GRANT

Genome Mapping in India:

In India, the functional genome research projects are looked after by the Depart of Biotechnology [DBT] and ICAR. The DBT has initiated such work on several crops such as rice, wheat, maize, chickpea, banana, tomato, *Brassica*, etc. The ICAR has created genome mapping facilities for rice at NRCPB, IARI, New Delhi. In India, the genome mapping work is carried out at the following centres.

i. National Research Centre for Plant Biotechnology, IARI, New Delhi.

ii. International Centre for Genetic Engineering and Biotechnology, New Delhi.

iii. Jawahar Lal Nehru University, New Delhi.

iv. National Botanical Research Institute, Lucknow.

Role of Genomics in Crop Improvement:

Genomics has several practical applications in crop improvement. Genome mapping is useful in several ways. It is useful or provides information about genome size, gene number, gene mapping, gene sequencing, evolution of crop plants, gene cloning, identification of DNA

markers, marker assisted selection, transgenic breeding, construction of linkage maps and QTL mapping.

All these aspects are briefly discussed as follows:

1. **Genome Size:**

Genome mapping is a very useful technique for determining the genome size in various plant species. In the plant species studied so far, the largest genome size has been reported in maize (2500 Mb) and the smallest in *Arabidopsis thaliana* (120 Mb).

2. **Gene Number:**

Genome mapping provide information about gene number in a species. In crop plants studied so far, the maximum number of genes has been reported in rice (56,000).

3. **Gene Mapping:**

Genome research is very much useful in mapping/tagging of genes on the different chromosomes of a genome. In other words, it helps in large scale discovery of new gene in a genome.

4. **Gene Sequencing:**

Genome mapping helps in determining the order of genes on the chromosomes. The order of genes is determined on each chromosome of a genome.

5. **Evolution:**

Genome mapping provide information about the evolution of different species. It measures the association between different genomes and thus provides information about the relatedness or evolutionary biology of crop plants.

6. **Gene Cloning:**

Genome research is very much useful in making multiple copies of a gene and transfer of the same from one genotype to another. Thus, it aids in specific gene transfer.

7. **Identification of DNA Markers:**

The genome mapping techniques are useful in identification of DNA markers which can be used in molecular breeding i.e. marker assisted selection. The mapping populations developed from inter- specific crosses have high polymorphism for DNA markers than those mapping populations derived from intra-specific crosses.

8. **Marker Assisted Selection:**

Marker assisted selection refers to indirect selection for a desired phenotype based on band pattern of linked DNA markers. The improvement of crop plants using such selection is cal molecular breeding. Various DNA markers used for such purpose include RFLP, AFLP, ISSR, etc.

The effect of DNA marker is correlated with morphological markers and then selection is made for particular trait. The selection based on DNA markers is more reliable because DNA markers are not influenced by environmental factors.

9. **Transgenic Breeding:**

Genome mapping is useful in gene cloning. The gene of interest can be cloned and used in developing transgenic plants (genetically engineered plants). Transgenic breeding permits direct gene transfer bypassing sexual process.

10. **Construction of Linkage maps:**

Genome mapping helps in construction of linkage groups. The linkage groups can be constructed from the information of gene mapping and gene sequencing.

11. **QTL Mapping:**

The genome mapping techniques is widely used for mapping of quantitative trait loci (QTL). The mapping of QTL or polygenic traits is not possible by conventional methods, viz., recombination mapping and deletion mapping techniques.

Limitations of Genomics:

These days, the genome mapping of crop plants is gaining increasing importance. It has several usefulapplications as discussed above. However, there are some limitations of genome mapping such high cost, high technical skill, laborious work, availability of limited genes and lake of proper markers.

These are briefly presented below:

(i) **Expensive Technique:** The genome research requires well equipped sophisticated laboratory with costly chemicals and glassware. Thus lot of funds is required for carrying genomic research. Lack of adequate funds sometimes becomes limiting factor in the progress of such project.

(ii) **High Technical Skill:** The genome mapping work requires high technical skill. It

requires training of scientists in the specialized field of genomics. It also requires International collaboration with other leading genome research laboratories which sometimes becomes limiting factor. The international collaboration is possible if the crop on which genome research work is to be carried out is of global significance.

(iii) **Laborious Work:** The genome mapping requires detection of various DNA markers (RFL, AFLP, RAPD, SSR, etc.) which is a laborious and time consuming work. Huge populations related to F2, RILs, NILs and doubled haploids need to be screened for such purpose. This limits the progress of the work.

(iv) **Limited Genes Available:** Firstly, limited number of genes and promoters are available for development of transgenic. Secondly, such genes are protected under Intellectual Property Rights and, therefore, cannot be used for developing transgenic plants.

(v) **Lack of Proper Markers:** Most of the useful agronomic traits are governed by polygenes and are complex in nature. Tightly linked DNA markers are yet to be identified for such characters.

Future Thrusts of Genomics:

Considerable research work on genome sequencing and mapping has been done on various crop plants so far. In future, for rapid progress of genome sequencing work several points need consideration. Important points include funding, training, material sharing, research priorities, important traits, selection of species, etc.

Difference between Genomics and Proteomics:

Genomics	Proteomics
Genomics is the study of genome of an organism. Genome represents the entire genes of an organism or a cell type	Proteomics is the study of proteome of an organism. Proteome refers to the entire protein set coded by the genome of an organism or a cell type
Genomics include mapping, sequencing and analysis of genome	Proteomics include characterization of all proteins of an organism or study of structure and function of proteins
Genomics can be broadly classified into structural and functional genomics **a)Structural genomics:** is the study of the structure of all genes and its relative position on the chromosome **b)Functional genomics:** study of function of all genes or the role of these genes in regulating metabolic activities of the cell	Proteomics can be classified into structural functional and expression proteomics **a)Structural proteomics:** is the study of the structure of proteins and their location in the cell **b)Functional proteomics:** study of function of all proteins which primarily include protein-protein interaction and interaction of proteins with other biomolecules **c) Expression proteomics:** is the study of identification and quantification or expression level of proteins of the cell at different developmental stages or at different environmental conditions
Techniques in genomics include a) gene sequencing strategies like directed gene sequencing, whole genome short gun sequencing, b)Construction of ESTs (expressed sequence Tags), c) Identification of single nucleotide polymorphisms (SNPs), d) Analysis and interpretation of sequenced data using different databases and software.	Techniques in proteomics include a) protein extraction, electrophoretic separation, digestion of separated proteins into small fragments using trypsin, mass spectroscopy to find out amino acid sequences and finally protein identification using standard databases. b) Protein 3D structure prediction using software. c) Protein expression study using protein microarray.
Thrust areas in Genomics: Genome Sequencing projects of many organisms including Human Genome Project	Proteome database development like SWISS-2D PAGE and software development for computer aided drug design

c-DNA:

In genetics, complementary DNA (cDNA) is DNA synthesized from a single stranded RNA (e.g., messenger RNA (mRNA) or microRNA) template in a reaction catalyzed by the enzyme reverse transcriptase. The cDNA is often used to clone eukaryotic genes in prokaryotes. When scientists want to express a specific protein in a cell that does not normally express that protein (i.e., heterologousexpression), they will transfer the cDNA that codes for the protein to the recipient cell. The cDNA is also produced naturally by retroviruses(such as HIV-1, HIV-2, simian immunodeficiency virus, etc.) and then integrated into the host's genome, where it creates a provirus. The term cDNA is also used, typically in a bioinformatics context, to refer to an mRNA transcript's sequence, expressed as DNA bases (GCAT) rather than RNA bases (GCAU). The cDNA is derived from mRNA, so it contains only exons, with no introns.

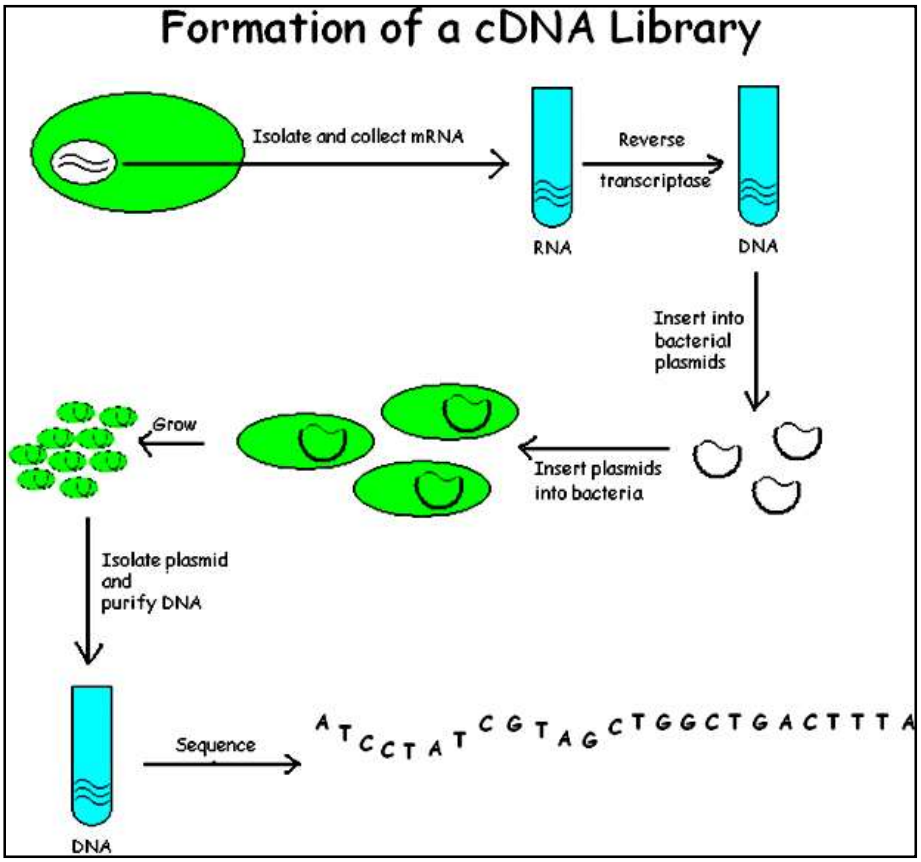
Synthesis:

Although there are several methods for doing so, cDNA is most often synthesized from mature (fully spliced) mRNA using the enzyme reverse transcriptase. This enzyme, which naturally occurs in retroviruses, operates on a single strand of mRNA, generating its complementary DNA based on the pairing of RNA base pairs (A, U, G and C) to their DNA complements (T, A, C and G, respectively). To obtain eukaryotic cDNA whose introns have been removed –

1. A eukaryotic cell transcribes the DNA (from genes) into RNA (pre-mRNA).
2. The same cell processes the pre-mRNA strands by removing introns, and adding a poly-A tail and 5’ Methyl-Guanine cap (this is known as post-transcriptional modification).
3. This mixture of mature mRNA strands is extracted from the cell. The poly-A tail of the post- transcriptional mRNA can be taken advantage of with oligo (dT) beads in an affinitychromatography assay.
4. A poly-T oligonucleotide primer is hybridized onto the poly-A tail of the mature mRNA template, or random hexamer primers can be added which contain every possible 6 base single strand of DNA and can therefore hybridize anywhere on the RNA (Reverse transcriptase requires this double-stranded segment as a primer to

start its operation.)

5. Reverse transcriptase is added, along with deoxynucleotide triphosphates (A, T, G, C). This synthesizes one complementary strand of DNA hybridized to the original mRNA strand.
6. To synthesize an additional DNA strand, traditionally one would digest the RNA of the hybrid strand, using an enzyme like RNase H, or through alkali digestion method.
7. After digestion of the RNA, a single stranded DNA (ssDNA) is left and because single stranded nucleic acids are hydrophobic, it tends to loop around itself. It is likely that the ssDNA forms a hairpin loop at the 3' end.
8. From the hairpin loop, a DNA polymerase can then use it as a primer to transcribe a complementary sequence for the ss cDNA.
9. Now, you should be left with a double stranded cDNA with identical sequence as the mRNA of interest.



Advantages of cDNA Library:

A cDNA library has two additional advantages. First, it is enriched with fragments from actively transcribed genes. Second, introns do not interrupt the cloned sequences; introns would pose a problem when the goal is to produce a eukaryotic protein in bacteria, because most bacteria have no means of removing the introns.

Disadvantages of cDNA Library:

The disadvantage of a cDNA library is that it contains only sequences that are present in mature mRNA. Introns and any other sequences that are altered after transcription are not present; sequences, such as promoters and enhancers, that are not transcribed into RNA also are not present ina cDNA library.

It is also important to note that the cDNA library represents only those gene sequences expressed in the tissue from which the RNA was isolated. Furthermore, the frequency of a particular DNA sequence in a cDNA library depends on the abundance of the corresponding mRNA in the given tissue. In contrast, almost all genes are present at the same frequency in a genomic DNA library.

Applications of cDNA Library:

Following are the applications of cDNA librar•ies:

* Discovery of novel genes.
* Cloning of full-length cDNA molecules for in vitro study of gene function.
* Study of the repertoire of mRNAs expressed in different cells or tissues.
* Study of alternative splicing in different cells or tissues.

Genomic library:

A genomic library is a collection of the total genomic DNA from a single organism. The DNA is stored in a population of identical vectors, each containing a different insert of DNA. In order to construct a genomic library, the organism's DNA is extracted from cells and then digested with a restriction enzyme to cut the DNA into fragments of a specific size. The fragments are

then inserted into the vector using DNA ligase. Next, the vector DNA can be taken up by a host organism - commonly a population of Escherichia coli or yeast - with each cell containing only one vector molecule. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis.

There are several kinds of vectors available with various insert capacities. Generally, libraries made from organisms with larger genomes require vectors featuring larger inserts, thereby fewer vector molecules are needed to make the library. Researchers can choose a vector also considering the ideal insert size to find a desired number of clones necessary for full genome coverage.

Genomic libraries are commonly used for sequencing applications. They have played an important role in the whole genome sequencing of several organisms, including the human genome and several model organisms.

The first DNA-based genome ever fully sequenced was achieved by two-time Nobel Prize winner, Frederick Sanger, in 1977. Sanger and his team of scientists created a library of the bacteriophage, phi X 174, for use in DNA sequencing. The importance of this success contributed to the ever- increasing demand for sequencing genomes to research gene therapy. Teams are now able to catalog polymorphisms in genomes and investigate those candidate genes contributing to maladies such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, and Type 1 diabetes.These are due to the advance of genome-wide association studies from the ability to create and sequence genomic libraries. Prior, linkage and candidate-gene studies were some of the only approaches.

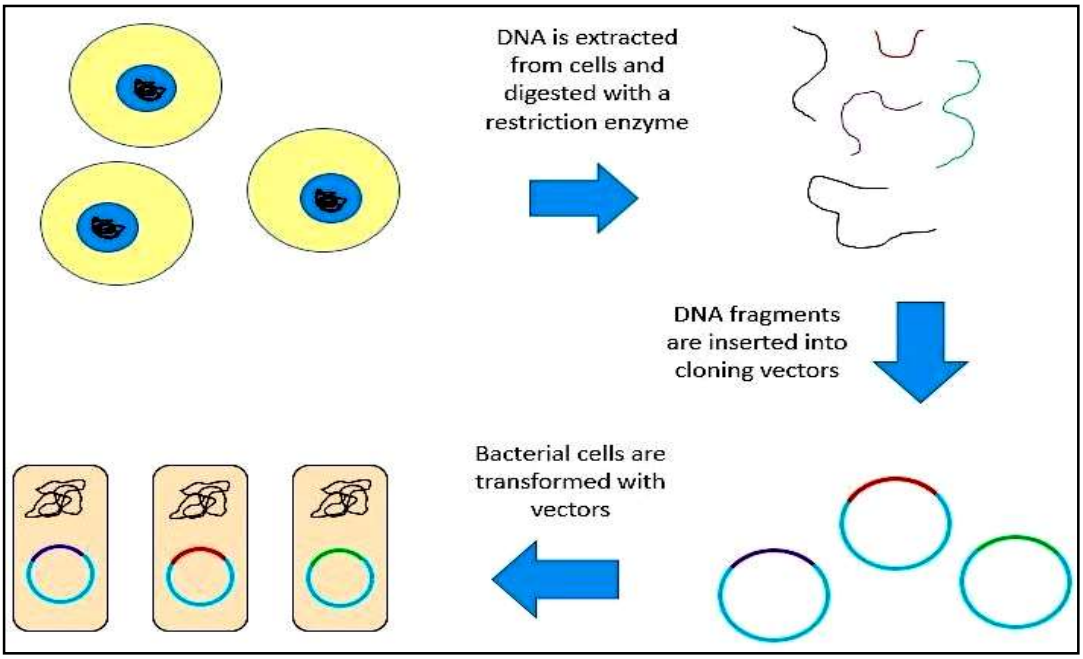
Genomic library construction:

Construction of a genomic library involves creating many recombinant DNA molecules. An organism's genomic DNA is extracted and then digested with a restriction enzyme. For organisms with very small genomes (~10 kb), the digested fragments can be separated by gel electrophoresis. The separated fragments can then be excised and cloned into the vector separately. However, when a large genome is digested with a restriction enzyme, there are far too many fragments to excise individually. The entire set of fragments must be cloned together with the vector, and separation of clones can occur after. In either case, the

fragments are ligated into a vector that has been digested with the same restriction enzyme. The vector containing the inserted fragments of genomic DNA can then be introduced into a host organism.

Below are the steps for creating a genomic library from a large genome –

1. Extract and purify DNA.
2. Digest the DNA with a restriction enzyme. This creates fragments that are similar in size, each containing one or more genes.
3. Insert the fragments of DNA into vectors that were cut with the same restriction enzyme. Use the enzyme DNA ligase to seal the DNA fragments into the vector. This creates a large pool of recombinant molecules.
4. These recombinant molecules are taken up by a host bacterium by transformation, creating a DNA library.



Applications of Genomic Library:

Genomic library has following applications –

* It helps in the determination of the complete genome sequence of a given organism.
* It serves as a source of genomic sequence for generation of transgenic animals

through geneticengineering.

* It helps in the study of the function of regulatory sequences *in vitro*.
* It helps in the study of genetic mutations in cancer tissues.
* Genomic library helps in identification of the novel pharmaceutical important genes.
* It helps us in understanding the complexity of genomes.

8. DNA sequencing: manual and automated methods, application as Bioinformatics tool.

The most important property of a DNA molecule is its nucleotide sequence. Until the late 1970s, determining the sequence of a nucleic acid containing even 5 or 10 nucleotides was difficult and very laborious. The development of two new techniques in 1977, one by Abn Maxam and Walter Gilbert and the other by Frederick Sanger, has it possible to sequence even larger DNA molecules with an ease which was unimagined just a few decades ago. The techniques depend upon the improved understanding of chemistry and DNA metabolism, and on electrophoretic methods that allow the separation of DNA strands differing in size by only one nucleotide. Electrophoresis of DNA is similar to the electrophoresis of protein. Polyacrylamide gel is often used as the gel matrix for short DNAs. Agarose is generally used as the gel matrix for separating larger DNAs.

Maxam and Gilbert Chemical Degradation Method:

By this method, sequencing of DNA fragments can be prepared upto 500 nucleotides.

Principle – Maxam and Gilbert method, also called chemical degradation method, which depends on certain chemicals to selectively degrade particular based within a molecular. This

technique is less frequently used today, partly because the chemicals used are toxic and partly because this method is laborious. For selective degradation, different regents are used, which react in two steps firstly modify (i) the bases and secondly detaches it from the remaining part of DNA.

Step 1: The double stranded fragment to be sequenced is labeled at the 5′ ends with ^{3L}P. The label is removed from one end by RE.

Step 2: The fragments are then denatured.

Step 3: Divide the mixture in 4 samples, each is treated with a different reagent having a property of selectively destroying either only G or only C or 'A' and 'G' or T and C. The reactions are controlled so that each labeled chain is likely to be broken only once. The labeled subfragmegists created by all 4 reactions have the label at one end and are of different sizes.

Step 4: The 4 samples mixtures are then subjected to gel electrophoresis, each of the 4 samples in 4 different lanes of the same gel electrophoretic plates.

Step 5: Autogradiography of each mixture yield one radioactive band for each nudeotide in the original fragment, each is separated according to their length. The bands appearing in the G and C lanes can be read directly. Bands A+G lane that are not duplicated in the G lane are read as A Bands in T+C lane that are not duplicated in the C lane are read as T.

The sequence is read from the bottom of the gel up.

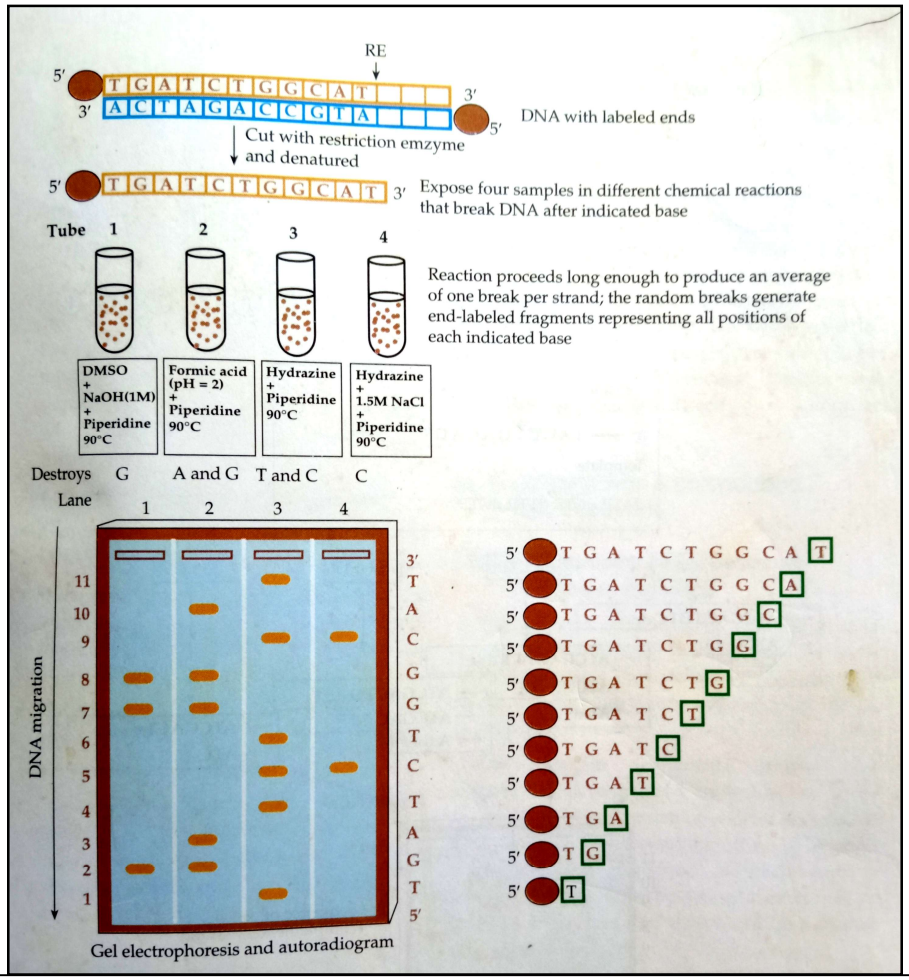


Fig.: Maxam-Gilbert method for sequencing DNA fragment

Terminal green boxes indicated bases.

Sanger's dideoxynucleotide synthetic method:

- Fyed Sanger (who won Nobel Prize twice) and his colleagues developed a method of DNA sequencing which utilized DNA polymerase to extend DNA chain length. This was termed plus-minus method.
- He used single strandard DNA template which has been sequenced and also DNA polymerases are used. It requires both a primer, to which nucleotides are added and a template strand to guide selection of each new nucleotide. The 3′-OH group of the

primer reacts with the incoming deoxynucleoside triphosphate (dNTP), forming a new phosphor diester bond.

- The Sanger method is also called dideoxy sequencing because this procedure uses dideoxy nucleoside triphosphate (ddNTP) analog to interrupt DNA synthesis. When dNTP is replaced by ddNTP, strand elongation is halted after the analogue is added because it lacks the 3′ OH group needed for the next step.

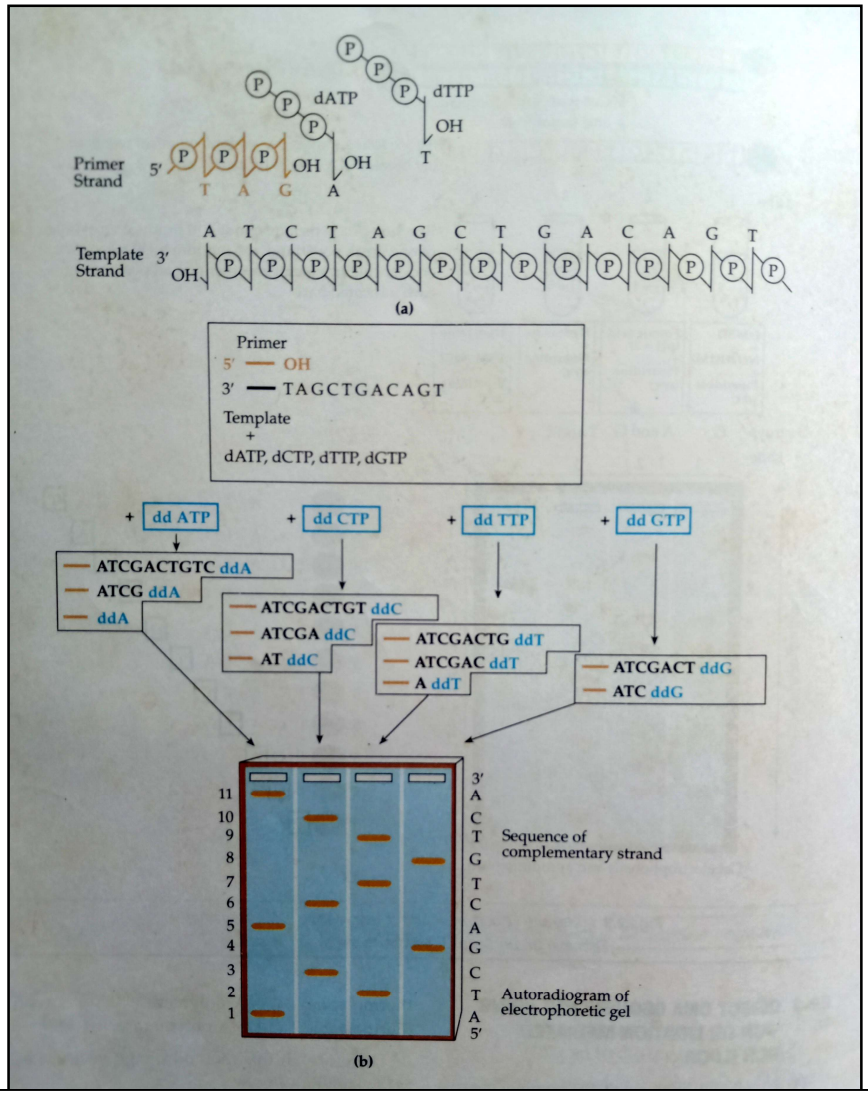


Fig.: DNA sequencing by the Sanger's dideoxynucleotide method (a) 3′ –OH group of the primer reacts with incoming dNTP, (b) Schematic diagram of the reactions.

Step 1: Four reaction tubes are set up, each containing single stranded DNA samples (cloned in M13 phage) to be sequenced, all 4 dNTPs synthetic oligonucleotide primers (radioactively labeled at the 5′ ends) and the enzyme for DNA synthesis (DNA polymerase 1). Each tube contains a small amount of one of the 4 ddNTP so that 4 tubes have different each a different ddNTP, bringing about termination at a specific base A, C, G or T. Because there is much more e.g. dCTP than ddCTP, there is only a small chance that the analog will be incorporated whenever a dc is to be added, but there is generally enough ddCTP that each new strand has a high probability of acquiring one ddC at some point during synthesis.

Step 2: The fragments generated by random incorporation of ddNTP leading to termination of reaction are then subjected to high resolution polyacrylamide gel electrophoresis and are separated on the same gel plate taking four reaction mixtures on the side by side 4 lanes separately.

Step 3: The gel is then used for autoradiography so that position of different bands in each lane can be visualized.

Step 4: The sequences can be read directly from an auto diagram of the gel. Because shorter DNA fragments migrate faster, the fragments near the bottom represent the nucleotide position closest to the primer (5′ end) and the sequence is read from bottom to the top.

It is to be noted that the sequence obtained is that of the short strand complementary to the strand being analyzed.

Direct DNA sequencing using PCR or ligation method PCR (LPCR):

- PCR has also been used for sequencing the amplified DNA product. The process involves I. Generation of sequencing template using PCR. II. Sequencing the PCR product either with thermolabile DNA polymerase or with thermastable Taq DNA polymerase.
- Therefore in this method phage vector MB is not required. In this method, Sanger's synthetic method involving incorporation of ddNTP for chain termination is used for sequencing.

Steps of reactions are as follows:

- Genomic or cloned DNA: Isolation of DNA fragments.
- DNA amplification (PCR): Generation of sequencing templates from cloned fragments using PCR and primers to vector site flanking the insert.
- Pipetting robots: Annealing of sequencing primers, labeling reactions, termination reaction and stop.
- Automated analysis of sequencing reactions. Electrophoresis for the separation of sequencing reaction and computer registration of the sequencing data.

Automated DNA sequencing method:

- DNA sequencing is now automated; using variation of Sanger's sequencing method. In this method a primer is used for each reaction is labeled differently with a coloured fluorescent tag. This method allows sequences of thousands nucleotides to be obtained in a few hours.
- The short oligatide used as a primer for DNA synthesis in the Sanger's method which can be linked to a fluorescent molecular that gives the DNA strand a colour. If each nucleotide is assigned to a different colour, the nucleotide on the end of each fragment will be coloured. The dialoxy method is used with a different ddNTP added to each of the 4 tubes according to the colour assignments. The resulting coloured DNA fragments are mixed and then separated by size in a single electrophoretic gel lane. The fragments of a given length migrate through the gel in a peak and the colour is associated with each successive peak is detedted using a laser beam. The DNA sequence is read by determining the sequence of colours in the peak as they pass the detector and the information is fed directly to a computer.

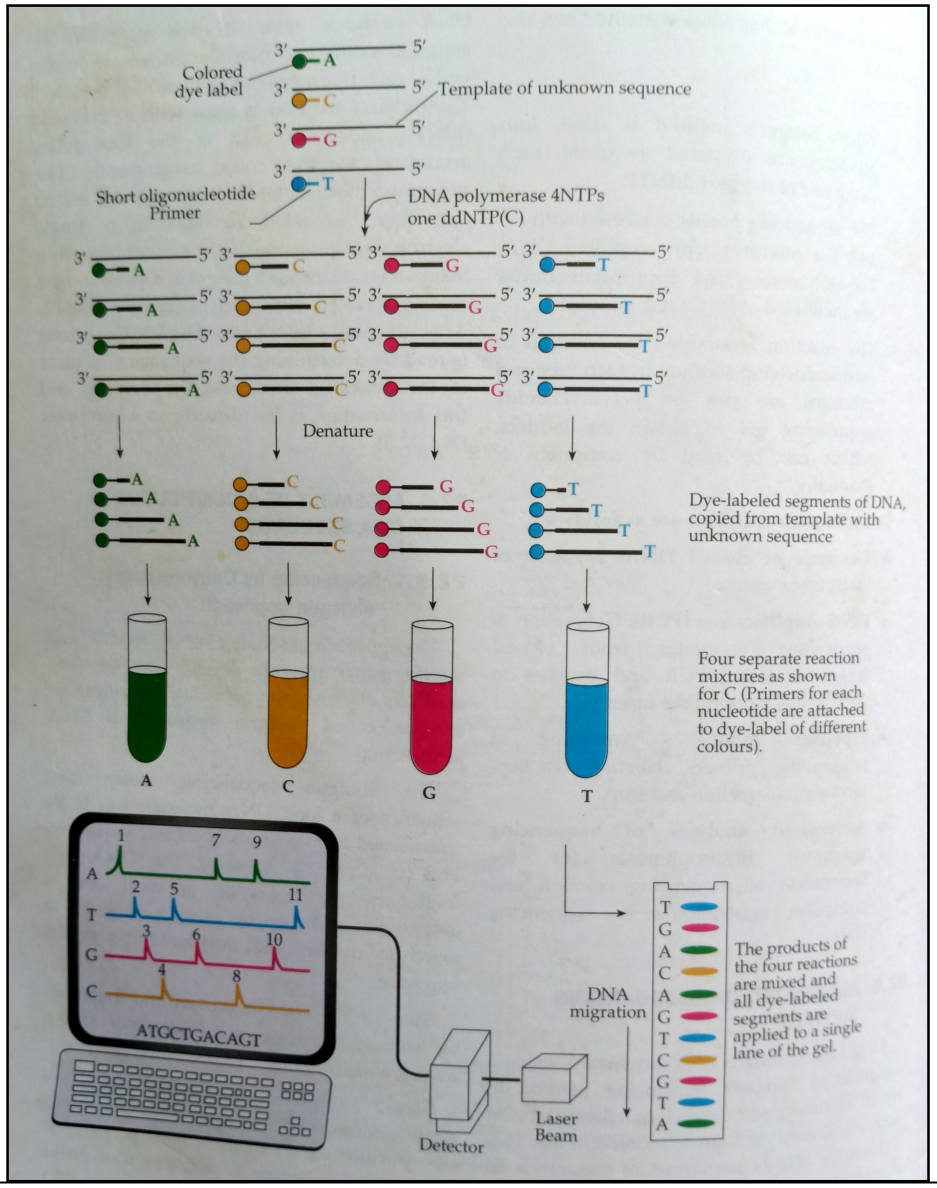


Fig.: A prototype strategy for automated DNA sequencing reactions.

Sequencing by conventional shot gun approach:

- The approach of sequencing which is to buildup the master sequence directly from the short sequences obtained from individual sequencing for overlaps, called shotgun approach of DNA sequencing.

- The shotgun sequencing means that sequence of a long DNA molecule has to be constructed from a series of shorter sequences. This is done by breaking the DNA into fragments, the sequence of each one is determined and then by using a computer to search for overlaps and thus build up a master sequence.

Step 1: The DNA is broken into fragments by sonication, using high frequency sound waves, which cut the DNA molecule randomly.

Step 2: The fragments are then electrophoresed in agarose gel. These fragments are purified and ligated into plasmid vector. Thus a library is prepared from these fragments.

Step 3: All the sequences were obtained from clones taken from this library and a computer is used to identity overlaps between sequences. This resulted in a number of sequence contigs which were assembled into the complete genome sequences. In practice, an overlap to 10 base pair would be needed to establish the two sequences should be linked together.

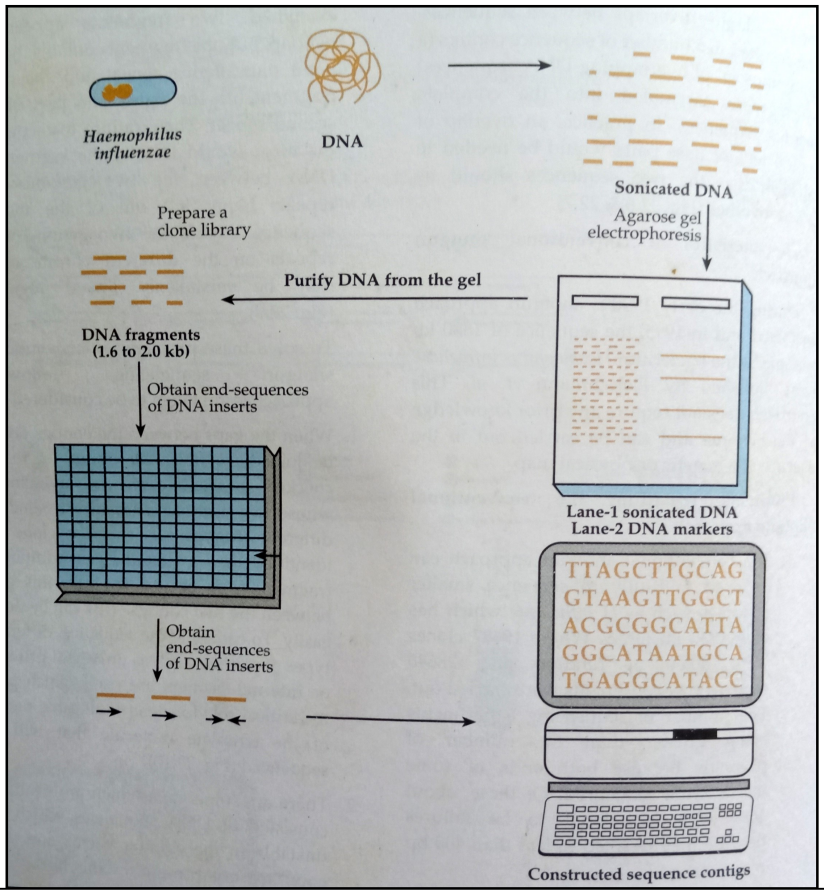


Fig.: Shortgun approach to obtain DNA sequence of *H. Influenza*.

Next-Generation Sequencing Technologies:

New methods, called next-generation sequencing technologies, have made sequencing hundreds of times faster and less expensive than the traditional Sanger sequencing method. Most next-generation sequencing technologies do sequencing in parallel, which means that hundreds of thou- sands or even millions of DNA fragments are simultaneously sequenced. One type, called pyrosequencing, is based on DNA synthesis: nucleotides are added one at a time in the order specified by template DNA and the addition of a particular nucleotide is detected with a flash of light, which is generated as the nucleotide is added.

To carry out pyrosequencing, DNA to be sequenced is first fragmented. Adaptors, consisting of a short string of nucleotides, are added to each fragment. The adaptor provides a known sequence to prime a PCR reaction. The DNA fragments are then made single stranded. In one version of pyrosequencing, each fragment is then attached to a separate bead and surrounded by a droplet of solution. The bead is used to hold the DNA and later deposit it on a plate for the sequencing reaction. Within the droplet, the fragment is then amplified by PCR and the copies of DNA remain attached to the bead. After amplification by PCR, the beads are mixed with DNA polymerase and are deposited on a plate containing more than a million wells (small holes). Each bead is deposited into a different well on the plate.

The sequencing reaction takes place in each well and is based on DNA synthesis. Recall from Chapter 13 that the substrate for DNA synthesis is a deoxynucleoside triphosphate, consisting of a deoxyribose sugar attached to a base and three phosphates. In the process of DNA synthesis, two phosphates (pyrophosphate) are cleaved off, and the resulting nucleotide is attached to the 3' end of the growing DNA chain. A solution containing one particular type of deoxynucleoside triphosphatesay, deoxyadenosine triphosphate is passed across the wells. If the template within a particular well specifies an adenine nucleotide in the next position of the growing chain, then pyrophosphate is cleaved from the nucleoside triphosphate and the adenine nucleotide is added. A chemical reaction uses the pyrophosphate produced in the reaction to generate a flash of light, which is measured by an optical detector. The amount of light emitted in each well is proportional to the number of nucleotides added: if the template in a well specifies three successive adenine nucleotides (As), then three nucleotides are added and three times more light is emitted than if a single A were added. If the position in the

template specifies a base other than adenine, no nucleotide is added, no pyrophosphate is produced, and no light is emitted.

As mentioned, the first solution passed over the plate contains adenosine triphosphate and allows adenine nucleotides to be added to the template. Each well with a template that specifies adenine in the next position will generate a flash of light. Then, a different type of nucleo-side triphosphate say, deoxyguanosine triphosphate is passed across the wells. Any fragment that specifies a G in the next position of its growing chain will add a guanine nucleotide and emit a flash of light. The nucleotide triphosphates are passed across the well in a predetermined order, and the light emitted by each well is measured. In this way, hundreds of thousands or millions of fragments of DNA are sequenced simultaneously on the basis of the order in which nucleotides are added to the 3 end of the growing chain. Pyrosequencing determines only the sequence of the fragments in each well; it does not, by itself, allow the sequences of these fragments to be reassembled into the sequence of the entire original piece of DNA.

Most next-generation sequencing techniques read short- er DNA fragments than do the Sanger sequencing reactions but, because hundreds of thousands or millions of fragments are sequenced simultaneously, these methods are much faster than traditional Sanger sequencing technology. Even more rapid sequencing technologies, typically called third-generation sequencing, are currently under development.

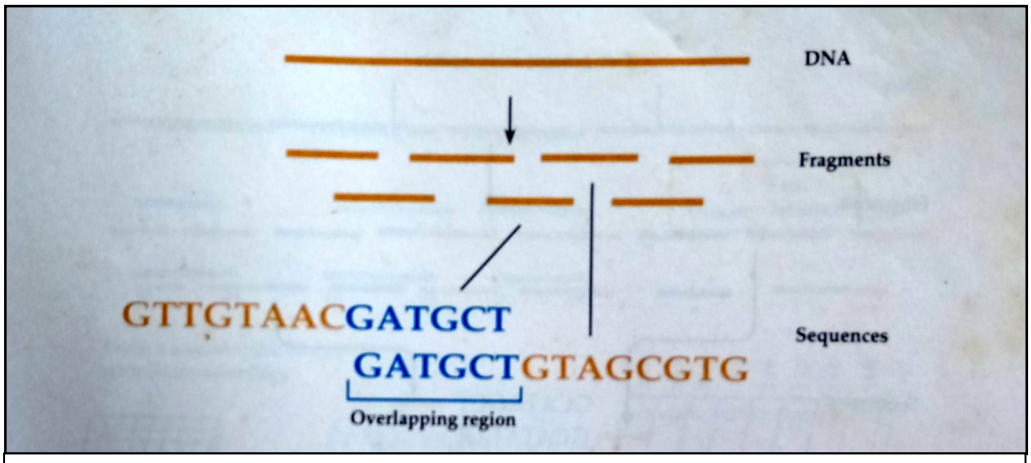


Fig.: Shortgun approach to sequence assembly

Application as Bioinformatics tool:

Bioinformatics is currently defined as the study of information content and information flow in biological systems and processes.

Major public domain bioinformatics facilities are:

(a) NCBI – National Centre for Biotechnology information, USA.

(b) EBI – European Bioinformatics Institute, UK.

(c) SIB – Swiss Institute of Bioinformatics, Switzerland.

(d) Genome – NET (KEGG @ DDBJ), Japan

- The method for sequencing gene or DNA segments through Maxamand Gilberts chemical degradation method or Sanger's dideoxy nucleotide synthetic method have been discussed. The method for automated DNA sequencing has also been discussed before.
- The sequence data of eukaryotic nuclear genome is an important source of identification, discovery and isolation of important gene. This data is very much helpful in variety of application with relevant to plant, animal and microbial biotechnology.

Table: List of sequence alignment tools and their specific features

Name of tool	Features
(i) Pair-wise sequence alignment tools	
FASTA family	(i) [fastaa] and [ssearch], compare a protein sequence against a protein database or a DNA sequence against a DNA database. (ii) [fastx/fasty], translates a DNA sequence into amino acid sequence of a protein and compares such a protein against a protein database. (iii) [tfastx/tfasty], compares a protein sequence against a DNA sequence translated in three forward and three reverse frames.
PatMatch	Searches for short nucleotide or peptide sequences (3-30 nt or amino acid).
BLAST (earlier	(i) BLASTp compares protein sequence against a protein sequence;

versions)	(ii) BLASTn compares a nucleotide sequence; (iii) BLASTx compares a nucleotide sequence translated in all reading frames against a protein sequence; (iv) tBLASTn compares a protein sequence against a nucleotide sequence translated in all reading frames; (v) tBLASTx compares the six frame translations of a nucleotide sequence against the six frame translation of a nucleotide sequence; (vi) BLASTz compares long stretches of nucleotides (>2 kb).
BLAST (later version)	(i) VecScreen (for match with sequence of plasmids, phase, cosmids, BACs, PACs and YACs). (ii) IgBLAST (for analysis of immunoglobulin sequences), (iii) MegaBLAST (for comparing a set of ESTs with a set of genes, and grouping them in clusters), (iv) SNP BLAST (for match query sequence with sequence carrying SNPs available in the SNP database, dbSNP), (v) PowerBlast (for alignment of query sequence with all sequences rather than one to one alignment).
ParAlign	Exploits parallelism involving division of a major task into small task that are performed in parallel to perform a very rapid computation.
Protein Engine and Transeq	Used first for translating DNA sequences into proteins, which are then used for further similarity searches and for the study of 3-D structures encoded.
(ii) Multiple sequence alignment tools	
ClustalW	Multiple sequence alignments of divergent sequences; evolutionary relationships can be seen via viewing Cladograms or Phylograms.
Clustal X	New window interface for the ClustalW multiple sequence alignment program.
MSA	Multiple Sequence Alignment: for alignment of several protein or nucleic acid sequences.
SAGA	Sequence Alignment by Genetic Algorithm for multiple sequence alignments.
PHYLIP	Following are some of the 30 programmes, which PHYLIP carries for phylogenetic analysis: (i) PROTPARS and PROTDIST use protein

	sequences; (iii) DNAPARS uses DNA sequence; (iv) NEIGHBOR uses distance matrix prepared using raw data, (v) DRAWGRAM draws a tree based on output from other phylogeny programme.

9. Polymerase Chain reaction: Types and their application.

Polymerase chain reaction:

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis, who was an employee of the Cetus Corporation, and also the winner of Nobel Prize in Chemistry in 1993, it is an easy, cheap, and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences. PCR is probably the most widely used technique in molecular biology. This technique is used in biomedical research, criminal forensics, and molecular archaeology.

PCR is now a common and often indispensable technique used in clinical and research laboratories for a broad variety of applications. These include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis; construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; amplification of ancient DNA; analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

Components of Polymerase Chain Reactions (PCR):

- DNA template (the sample DNA that contains the target sequence to amplify)
- Deoxyribonucleoside triphosphates (dNTPs)
- PCR buffer
- Primers (forward and reverse)
- Taq polymerase

Placing a strip of eight PCR tubes into a thermal cycler:

The vast majority of PCR methods rely on thermal cycling, which involves exposing the reactants to cycles of repeated heating and cooling, permitting different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication—to quickly proceed many times in sequence. Primers (short DNA fragments) containing sequences complementary to the target region, along with a DNA polymerase (e.g. Taq polymerase), after which the method is named, enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified. The simplicity of the basic principle underlying PCR means it can be extensively modified to perform a wide array of genetic manipulations. PCR is not generally considered to be a recombinant DNA method, as it does not involve cutting and pasting DNA, only amplification of existing sequences.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the thermophilic bacterium *Thermus aquaticus*. If heat-susceptible DNA polymerase is used, it will denature every cycle at the denaturation step. Before the use of Taq polymerase, DNA polymerase had to be manually added every cycle, which was a tedious and costly process. This DNA polymerase enzymatically assembles a new DNA strand from free nucleotides, the building blocks of DNA, by using single-stranded DNA as a template and DNA oligonucleotides (the primers mentioned above) to initiate DNA synthesis.

In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the

target DNA. The selectivity of PCR results from the use of primers that are complementary to sequence around the DNA region targeted for amplification under specific thermal cycling conditions.

PCR, like recombinant DNA technology, has had an enormous impact in both basic and diagnostic aspects of molecular biology because it can produce large amounts of a specific DNA fragment from small amounts of a complex template. Recombinant DNA techniques create molecular clones by conferring on a specific sequence the ability to replicate by inserting it into a vector and introducing the vector into a host cell. PCR represents a form of "in vitro cloning" that can generate, as well as modify, DNA fragments of defined length and sequence in a simple automated reaction. In addition to its many applications in basic molecular biological research, PCR promises to play a critical role in the identification of medically important sequences as well as an important diagnostic one in their detection.

Procedure:

Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of three discrete temperature steps. The cycling is often preceded by a single temperature step at a very high temperature (>90°C (194°F)), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers. The individual steps common to most PCR methods are as follows –

Initialization: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96°C (201–205°F), or 98°C (208°F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.

Denaturation: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98°C (201–208°F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between

complementary bases, yielding two single-stranded DNA molecules.

Annealing: In the next step, the reaction temperature is lowered to 50–65°C (122–149°F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind only to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5°C below the Tm of the primers used. Stable hydrogen bonds between complementary bases are formed only when the primer sequence very closely matches the template sequence. During this step, the polymerase binds to the primer-template hybrid and begins DNA formation.

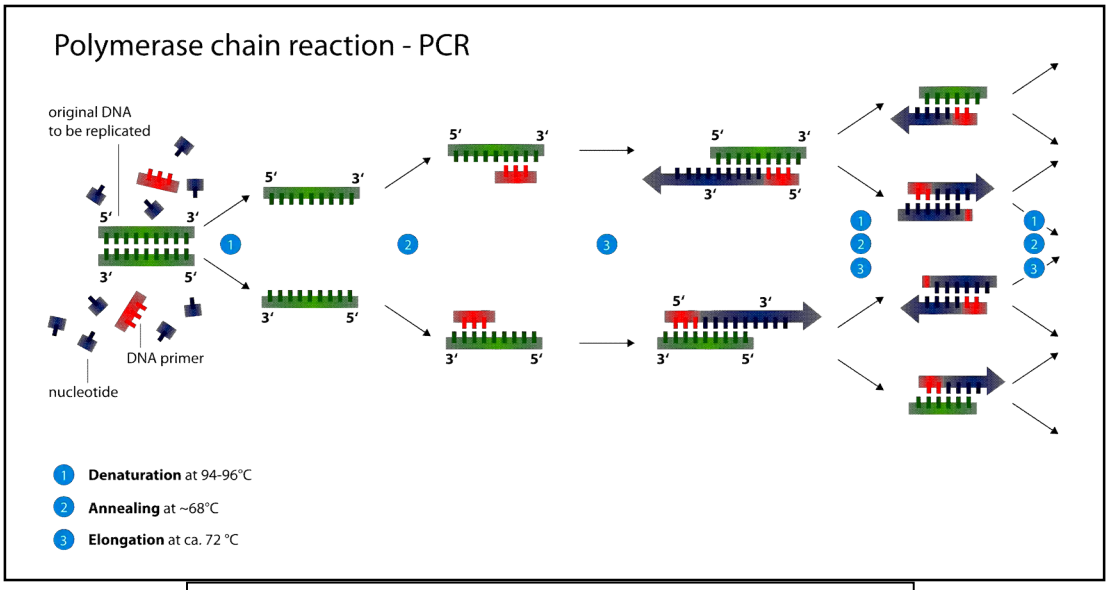


Fig.: Schematic drawing of a complete PCR cycle

Extension/elongation: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of Taq (*Thermus aquaticus*) polymerase is approximately 75–80°C (167–176°F), though a temperature of 72°C (162°F) is commonly used with this enzyme. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand. The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a rule of thumb, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute. Under optimal conditions (i.e., if there are no limitations due to limiting substrates or reagents), at each extension/elongation step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2n, where n is the number of cycles. Thus, a reaction set for 30 cycles results in 230, or 1073741824, copies of the original double-stranded DNA target region.

Final elongation: This single step is optional, but is performed at a temperature of 70–74°C (158–165°F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

Final hold: The final step cools the reaction chamber to 4–15°C (39–59°F) for an indefinite time, and may be employed for short-term storage of the PCR products.

Once the first round is completed, the process is repeated by cycling back to the first reaction

temperature and the next round of denaturation, annealing, and extension is started *(an automatic process in thermocycler)*. This 3 steps temperature cycle is repeated approximately 30 times which results in exponential amplification of the target gene sequence.

Types of PCR:

1. Reverse Transcriptase PCR (RT- PCR): This technique allows the detection of RNA in the sample. The RNA molecule is reverse transcribed into complementary DNA (cDNA) using the enzyme reverse transcriptase. This is followed by the amplification of cDNA using standard PCR.

2. Real time-PCR or quantitative PCR (qPCR): Detects fluorescent reporter dyes like SYBR Green, used to quantify DNA amplification at each PCR cycle. The fluorescence signal increases proportionally to the amount of replicated DNA and hence the DNA is quantified in "real time". The fluorescence grows to a point where it becomes quantifiable during the log linear phase of amplification, which is known as the Threshold cycle (CT).

3. RT-PCR/qPCR combined: This involves the quantitative detection of RNA expression using both RT-PCR for cDNA synthesis and q-PCR for real time amplification.

4. Multiplex PCR: is used for the simultaneous amplification multiple targets in a single reaction with a specific set of primers pair of each target. Two or more probes that can be distinguished from each other and detected simultaneously using this technique.

5. Nested PCR: involves the two consecutive amplification reactions with two distinct primer set. The first amplification reaction product serves as a template for second reaction. This variant of PCR minimizes the non-specific amplification and increases the sensitivity and specificity of PCR.

6. High-fidelity PCR: Unlike standard DNA polymerases (such as Taq DNA polymerase), high-fidelity PCR enzymes generally provide a 3' to 5' exonuclease activity for removing incorrectly incorporated bases. High-fidelity PCR enzymes are ideally suited to applications requiring a low error rate, such as cloning, sequencing, and site-directed mutagenesis. However, if the

enzyme is not provided in a hot-start version, the 3' to 5' exonuclease activity can degrade primers during PCR setup and the early stages of PCR. Nonspecific priming caused by shortened primers can result in smearing on a gel or amplification failure — especially when using low amounts of template. It should be noted that the proofreading function often causes high-fidelity enzymes to work more slowly than other DNA polymerases. In addition, the A-addition function required for direct UA- or TA-cloning is strongly reduced, resulting in the need for blunt-end cloning with lower ligation and transformation efficiency.

7. Fast-cycling PCR: Faster PCR amplification enables increased PCR throughput and allows researchers to spend more time on downstream analysis. The demand for reducing time-to-result is met by the recent development of faster PCR techniques. Fast PCR can be achieved using new thermal cyclers with faster ramping times or through innovative PCR chemistries that allow reduced cycling times due to significantly shortened DNA denaturation, primer annealing, and DNA extension times. Fast-cycling PCR reagents must be highly optimized to ensure amplification specificity and sensitivity.

8. Hot-start PCR: When amplification reaction setup is performed at room temperature, primers can bind nonspecifically to each other, forming primer–dimers. During amplification cycles, primer–dimers can be extended to produce nonspecific products, which reduces specific product yield. For more challenging PCR applications, the use of hot-start PCR is crucial for successful specific results. To produce hot-start DNA polymerases, Taq DNA polymerase activity can be inhibited at lower temperatures with antibodies or, more effectively, with chemical modifiers that form covalent bonds with amino acids in the polymerase. The chemical modification leads to complete inactivation of the polymerase until the covalent bonds are broken during the initial heat activation step. In contrast, in antibody-mediated hot-start procedures, antibodies bind to the polymerase by relatively weak non-covalent forces, which leave some polymerase molecules in their active state. This sometimes leads to nonspecific primer extension products that can be further amplified during PCR. These products appear as smearing or incorrectly sized fragments when run on an agarose gel.

9. Long-range PCR: PCR products of up to 4 kb can be routinely amplified using standard PCR protocols using Taq DNA polymerase. However, amplification of PCR products longer than 4 kb often fails without lengthy optimization. Reasons for failure include nonspecific primer annealing, secondary structures in the DNA template, and suboptimal cycling conditions — all factors which have a greater effect on the amplification of longer PCR products than on shorter ones. Preventing DNA damage, such as DNA depurination, is of particular importance for amplification of long PCR products, as a single DNA lesion within the template is sufficient to stall the PCR enzyme. DNA damage during PCR cycling can be minimized with specific buffering substances that stabilize the pH of the reaction. Commercial PCR kits are available that are specifically designed to overcome the challenges of long-range PCR, for example, by using an optimized mixture of Taq DNA polymerase and proofreading enzymes, and it is recommended that, where possible, such a kit is used.

10. Asymmetric PCR: It amplifies only one strand of the target DNA molecule by using unequal primer concentrations. The technique is mainly applied in sequencing or hybridization probing where only one strand of DNA is required.

11. Colony PCR: It rapidly screens the colonies of bacteria or yeast that are grown on the selective media after cloning to verify whether segment of interest is successfully transformed or to amplify the section of insert.

12. Degenerate PCR: It amplifies the unknown DNA sequences, mainly coding gene sequences using degenerate set of primers. The primers are constructed based on the known sequences of gene homologs.

13. Inverse PCR: It amplifies DNA with only one known sequence. It is used to determine the location of the insert.

14. Touchdown PCR (TD-PCR): It is a modification of PCR in which the initial annealing temperature is higher than the optimal Tm of the primers and is gradually reduced over subsequent cycles until the Tm temperature or "touchdown temperature" is reached. This is used to increase the specificity of PCRs.

15. Amplification Refractory Mutation System (ARMS) PCR: It is used to detect a single base change or SNP using sequence-specific primers. Here two different set of primers are constructed, mutant and wild type. The 3' end each primer is modified so that normal primer can amplify only normal allele and mutant primer can amplify only mutant allele.

16. Multiplex Ligation-dependent Probe Amplification (MLPA) PCR: It allows amplification of multiple targets using the single pair. It's used for the molecular detection of variation in copy numbers. It serves as an important molecular diagnostic tool for identification of genetic diseases.

Applications of PCR:

- Identification and characterization of infectious agents
  - ✓ Direct detection of microorganisms in patient specimens
  - ✓ Identification of microorganisms grown in culture
  - ✓ Detection of antimicrobial resistance
  - ✓ Investigation of strain relatedness of a pathogen of interest
- Genetic fingerprinting (forensic application/paternity testing)
- Detection of mutation (investigation of genetic diseases)
- Cloning genes
- PCR sequencing

10. DNA-protein interactions: methods for detection and analysis.

Introduction:

It has been known since the second half of the last century that the binding of a protein to a DNA molecule has a very important role in the function of a living cell and in lif's sustainability itself. For many decades representing a big segment of molecular biology research conducted, scientists tried to unravel how DNA links to proteins, forming complex and vital interactions. In the early beginning of these studies, even before the publication of the DNA molecular structure, Stedman and Stedman (1950) already referred to histones as potential regulator of the DNA biological activity. Since then scientists are not abandoned this research field, having unraveled many details about the crucial interaction between proteins and DNA. This interaction is responsible for essential molecular and cellular mechanisms, such as transcription, transcriptional regulation, recombination, replication, DNA repair, viral infection, DNA packing, DNA modifications (ofan, Mysore, Rost, 2007). The studies usually performed either from purely chemical perspective, analyzing the structure of the complex formed, or from a transcriptomic level, investigating if a certain protein does bind to a particular DNA or gene and the interference of this interaction in gene expression, existing great interaction between both approaches.

This topic intends to compile and briefly describe the majority of existing techniques that enable to access imformation related to DNA-protein interaction. The methods to study DNA protein interactions are as follows:

(i) Electrophoretic mobility shieft assay technique:

The interaction between nucleic acids and proteins was not yet totally described when Yarus and Berg developed the filter binding assay that relies in the fact that a major part of proteins can be retained in a nitrocellulose membrane. In case the protein under study does bind to a nucleic acid, subsequently the complex may also be held in the nitrocellulose filter. This method is quite inexpensive, simple and relatively rapid, starts by the extraction and purification of the protein of interest and radio-lebeling of the nucleic acid, followed by the binding reaction and ends in the filtering technique, which consists in applying vacuum to a

porous plastic disc placed bellow a nitrocellulose filter that is impregnated with a solution containing the binding reaction. Then, the results are revealed and the quantities of bound nucleic acid are calculated using a phosphorimager (Fig. 1).

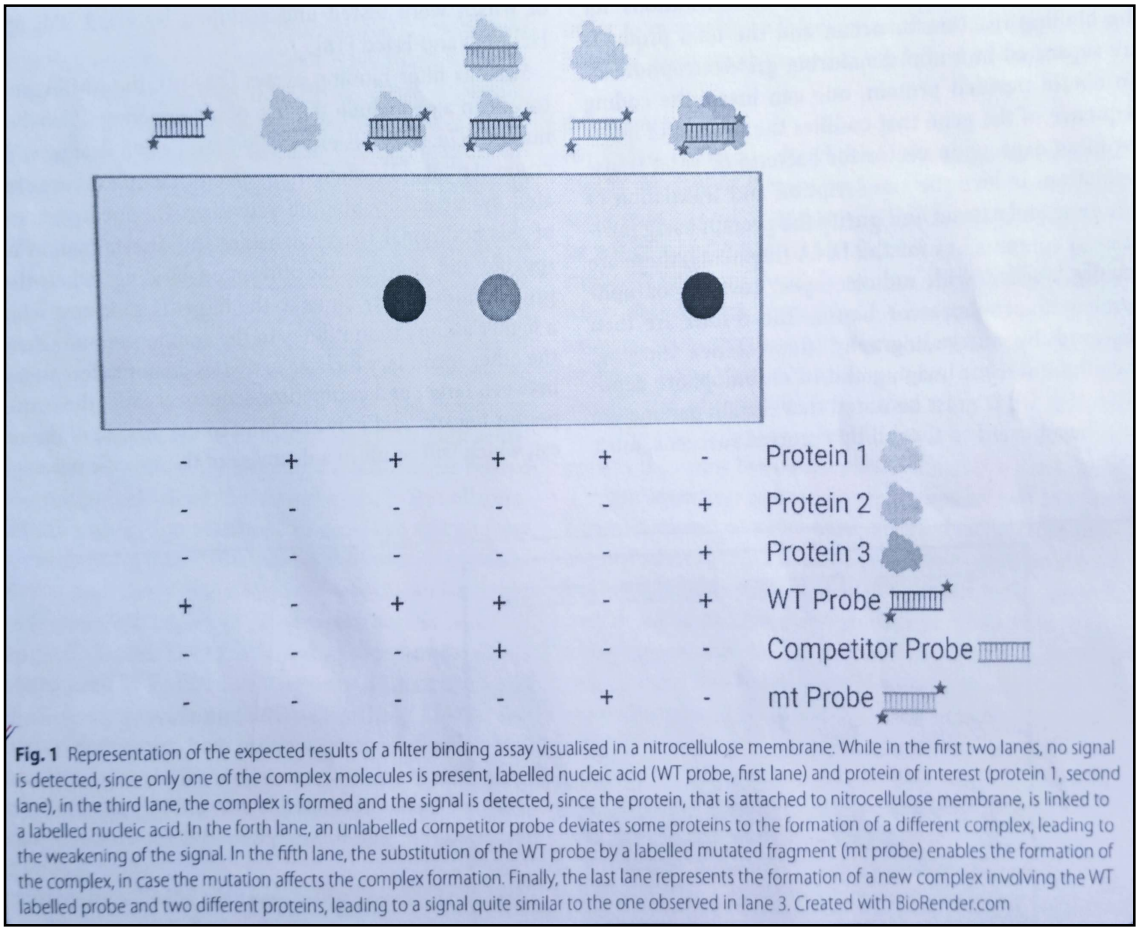


Fig. 1 Representation of the expected results of a filter binding assay visualised in a nitrocellulose membrane. While in the first two lanes, no signal is detected, since only one of the complex molecules is present, labelled nucleic acid (WT probe, first lane) and protein of interest (protein 1, second lane), in the third lane, the complex is formed and the signal is detected, since the protein, that is attached to nitrocellulose membrane, is linked to a labelled nucleic acid. In the forth lane, an unlabelled competitor probe deviates some proteins to the formation of a different complex, leading to the weakening of the signal. In the fifth lane, the substitution of the WT probe by a labelled mutated fragment (mt probe) enables the formation of the complex, in case the mutation affects the complex formation. Finally, the last lane represents the formation of a new complex involving the WT labelled probe and two different proteins, leading to a signal quite similar to the one observed in lane 3. Created with BioRender.com

However, this procedure presents several drawbacks that have led to its disuse. Firstly, not all the proteins bind to nitrocellulose membranes and some even denature when clinging to these filters. Moreover, if the interaction between the molecules of the complex is not strong enough, it may not withstand the filtration process. Furthermore, using this method one cannot recover and analyse the composition of the binding reaction resulting products, making it impossible to determine if a DNA molecule binds to more than one protein, since the DNA only needs to interact to a single protein in order to be retained in the filter (Fig. 1). Additionally, if single stranded nucleic acids adhere randomly to the filter, which happens

under certain solution conditions, it may result in undesirable interferences that might obscure the true binding signal.

Finally, a technique was developed that uses gel electrophoresis and that surpasses these inconveniences. Fried and Crothers and Garner Revzin created the electrophoretic mobility shift assay (EMSA), which consists in evaluating if a protein causes retardation in the electrophoretic run of a nucleic acid fragment, when bound to it in a complex compared to the run of the same nucleic acid not bound to a protein. Like the filter binding assay mentioned above, EMSA tests the nucleic acid-protein interaction qualitatively, but unlike the first one, EMSA also does it quantitatively, since the mobility of the nucleic acid fragment in the gel decreases as the number of proteins bound to it increases. So, this method assesses not only the stoichiometric ratio of protein linked to the nucleic acid, but also the relative binding affinities of a certain protein for two different nucleic acids.

Usually, the technique starts by mixing the protein, present in a crude cell extract or purified, with the labeled nucleic acid and an appropriate buffer, under the right specific conditions and concentrations for the binding reaction to occur, and the final products are separated in a non-denaturing gel electrophoresis. To obtain purified protein, one can insert the coding sequence of the gene that codifies the protein of interest in an expression vector for bacteria or yeast transformation, induce the transcription and translation of this gene and extract and purify the protein using purification columns. As for the DNA fragments, these are usually labeled with radioisotopes, covalent or non-covalent fluorophores or biotin. The results are then observed by autoradiography, fluorescene imaging, chemiluminescent imaging and/or chromophore deposition (Fig. 2).

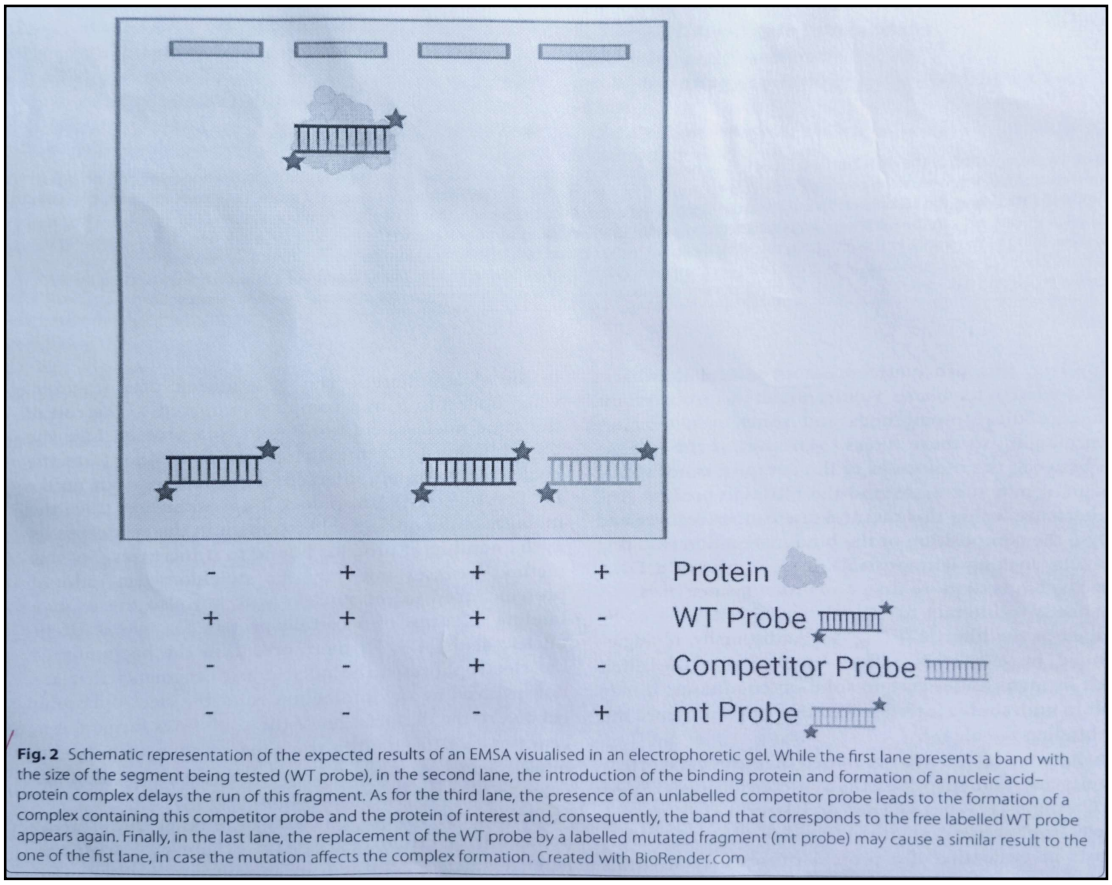


Fig. 2 Schematic representation of the expected results of an EMSA visualised in an electrophoretic gel. While the first lane presents a band with the size of the segment being tested (WT probe), in the second lane, the introduction of the binding protein and formation of a nucleic acid–protein complex delays the run of this fragment. As for the third lane, the presence of an unlabelled competitor probe leads to the formation of a complex containing this competitor probe and the protein of interest and, consequently, the band that corresponds to the free labelled WT probe appears again. Finally, in the last lane, the replacement of the WT probe by a labelled mutated fragment (mt probe) may cause a similar result to the one of the fist lane, in case the mutation affects the complex formation. Created with BioRender.com

Filter binding assay and EMSA are two important techniques that can help in identifying nucleic acid-protein interactions quite easily and rapidly. Advantages, disadvantages and applications of these two methods are resumed in Table 1.

Cross-linking

An EMSA technique drawback is the dependence of its results on several factors besides the protein size. This assay does not allow identifying nor revealing the molecular weights of the proteins present in the complex being studies. In the seventies, quaternary structures related to interaction between proteins were analysed using cross-linking techniques. Generally, each distinct procedure starts by forming or isolating the DNA-protein complexes, excluding free probes and nonspecific complexes. A cross-linker agent is then applied and the specific comples is removed and precipitated, being finally analysed (Fig. 3a).

Table 1: Advantages and disadvantages of techniques that study DNA-binding proteins

Technique (References)	Technique		Applications	Case studies
	Pros	Cons		
Filter bindings assay	Inexpensive and easy	The interaction may withstand the filtration process. Impossible to recover the resulting products.	Identify nucleic acid protein interactions	Prabu et al.
EMSA	Fast and easy Powerful and sensitive. Semi-quantitative.		Identify nucleic acid-protein interactions Identify complexes in a cell/tissue extract.	
Cross-linking	UV cross-linking is not invasive and does not practically disturb the molecular structures. Laser cross-linking is simple and fast and decreases the probability of damaging the molecular structures.	Formaldehyde, glutaraldehyde and standard cross-linking methods are non-specific.	Idendifies the molecules that participate in a DNA-protein complex, even though some of them may not be directly in contact with the DNA.	Liu et al
EMSA combined with Western Bloting techniques	Immunoblotting analysis combined with EMSA performs only one diffusion blotting. Electrophoretic	Electrophoretic 'supershift' assay requires the purification of the antibody preparation Shift-Western Blotting	Detect if a certain protein is present in a nucleic acid-protein complex (electrophoretic 'supershift' assay) and	Wang et al

	'supershift' assay only uses one gel and does not need any diffusion blotting. All variants are relatively fast and easy.	involves two blotting membranes.	estimate its size (shift-Western Blotting and immunoblotting analysis combined with EMSA). Identify the nucleic acid-binding proteins that link to a certain nucleic acid and estimate their molecular weight (2D electrophoresis (EMSA+SDS+PAGE) and South-Western Blotting).	
In vivo analysis:Y1H and PTA	EMSA takes in vitro binding with a cell extract using mutated probes are quick and easy Y1H and PTA provide reliable results and are relatively direct and sensitive. Y1H is quick.	PTA takes a long time to be performed and involves several steps.	Determine if a certain TF binds to a given sequence *in vivo*.	Lia et al

(ii) DNA foot printing:

When transcription factor binds to a DNA sequence, it protects that sequence from digestion by nucleases Researchers take the advantage of this properly by isolating chromatin from cells and treating it with DNA-digesting enzymes, such as DNA ase-1, that are not protected by

bound transcription factors. Once the chromatin has been digested, the bound protein is removed and the DNA sequences that had been protected are identified. This method is called DNA foot printing. This is also used to locate the binding sites of proteins on RNA.

Method:

1. A pure DNA fragment that is labeled at one end with ^{32}P is isolated.
2. The molecule is then cleaved with a nuclease or a chemical that makes random single – stranded cuts in the DNA.
3. The DNA molecules are then denatured to separate into two strands.
4. The resultant fragments from the labeled stand are separated on a gel and detected by autoradiography.

The pattern of bands from DNA cut in the presence of a DNA-binding protein is then compared with that from DNA cut in its absence. The protein covers the nucleotides at the binding site and proteins it from DNAase. The labeled fragments that shows no cleavage will represent an area which is missing in the electrophoretic gel, is leaving a gap is called footprint.

(iii) DNA-Binding Proteins:

Much of gene regulation in bacteria and eukaryotes is accomplished by proteins that bind to DNA sequences and affect their expression. These regulatory proteins generally have discrete functional parts—called domains, typically consisting of 60 to 90 amino acids—that are responsible for binding to DNA. Within a domain, only a few amino acids actually make contact with the DNA. These amino acids (most commonly asparagines, glutamine, glycine, lysine, and arginine) often form hydrogen bonds with the bases or interact with the sugarphosphate backbone of the DNA. Many regulatory proteins have additional domains that can bind other molecules such as other regulatory proteins. By physically attaching to DNA, these proteins can affect the expression of a gene. Most DNA binding proteins bind dynamically, which means that they are transiently binding and unbinding DNA and other regulatory proteins. Thus, although they may spend most of their time bound to DNA, they are never permanently attached. This dynamic nature means that other molecules can compete with DNA-binding proteins for regulatory sites on the DNA.

DNA-binding proteins can be grouped into several distinct types on the basis of a characteristic structure, called a motif, found within the binding domain. Motifs are simple structures, such as alpha helices, that can fit into the major groove of the DNA. For example, the helix-turn-helix motif (Fig. 3), consisting of two alpha helices connected by a turn, is common in bacterial regulatory proteins. The zinc-finger motif, common to many eukaryotic regulatory proteins, consists of aloop amino acids containing a zinc ion. The leucine zipper (Fig. 3) is another motif found in a variety of eukaryotic binding proteins. These common DNA-binding motifs and others are summarized in (Table 2).

Table 2: Common DNA-binding motifs

Motif	Location	Characteristics	Binding Site in DNA
Helix-turn-helix	Bacterial regulatory proteins; related motis in eukaryotic proteins	Two alpha helices	Major groove
Zinc-finger	Eukaryotic regulatory and other proteins	Loop amino acids with zinc at base	Major groove
Steroid receptor	Eukaryotic proteins	Two perpendicular alpha helices with zinc surrounded by four cyteine residues	Major groove and DNA backbone
Leucine-zipper	Eukaryotic transcription factors	Helix of leucine residues and a basic arm; two leucine residues interdigitate	Two adjacent major grooves
Helix-loop-helix	Eukaryotic proteins	Two alpha helices separated by a loop of amino acids	Major groove
Homeodomain	Eukaryotic regulatory proteins	Three alpha helices	Major groove

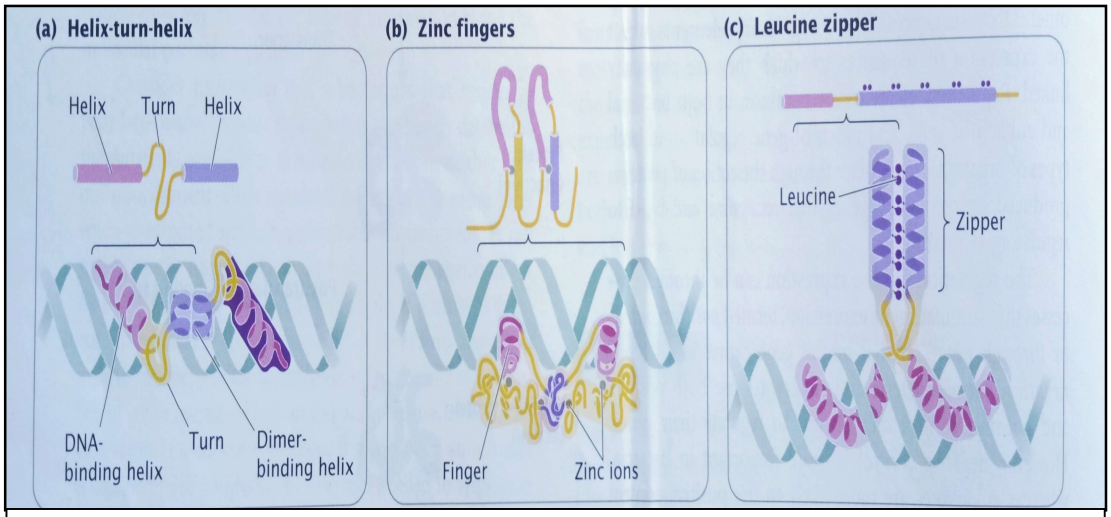


Fig. 3: DNA-binding proteins can be grouped into several types on the basis of their structure, or motifs.

11. Let's sum up

- Recombinant DNA technology is a technology which allows DNA to be produced via artificial means. The procedure has been used to change DNA in living organisms and may have even more practical uses in the future.
- Naturally occurring restriction endonucleases are categorized into four groups (Types I, II III, andIV) based on their composition and enzyme cofactor requirements, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence.
- In all prokaryotic and eukaryotic organisms, three main classes of RNA molecules exist-1) Messenger RNA(m RNA) 2) Transfer RNA (t RNA) 3) Ribosomal RNA (r RNA)
- Large-scale changes take place in precursor mRNA. These changes are called

processing of mRNA. Both 5′-end 3′-end of mRNA are modified. Non-coding regions are removed by splicing. The changes lead to the formation of mature mRNA which takes part in protein synthesis.

- An exogenous dsRNA or endogenous pre-miRNA can be processed by dicer and incorporated into the RNA-induced silencing complex (RISC), which targets single-stranded messenger RNA molecules and triggers translational repression; incorporation into the RNA-induced transcriptional silencing complex (RITS) induces genome maintenance activities such as histone methylation and chromatin reorganization.

- In molecular biology, a riboswitch is a regulatory segment of a messenger RNA molecule that binds a small molecule, resulting in a change in production of the proteins encoded by the mRNA.

- Blotting technique is an extremely powerful tool for analyzing gene structure and used to study gene expression, once cloned cDNA is isolated. There are three important types of blotting techniques are: a) Southern Blotting b) Northern Blotting c) Western Blotting. DNA fingerprinting is a laboratory technique used to establish a link between biological evidence and a suspect in a criminal investigation.

- Genomics provides an overview of the complete set of genetic instructions provided by the DNA, while transcriptomics looks into gene expression patterns. Proteomics studies dynamic protein products and their interactions, while metabolomics is also an intermediate step in understanding organism's entire metabolism.

- A genomic library is a collection of independently isolated vector linked DNA fragments derived from a single organism. Complementary DNA (cDNA) libraries can also be prepared by isolating mRNAs from tissues which are actively synthesizing proteins, like roots and leaves in plants, ovaries or reticulocytes in mammals, etc

- Polymerase Chain Reaction is a technique used to amplify a specific region of a DNA molecule to generate multiple copies.

- The applications of PCR are: (1) PCR in Clinical Diagnosis (2) PCR in DNA Sequencing (3) PCR in Gene Manipulation and Expression Studies (4) PCR in Comparative Studies of Genomes (5) PCR in Forensic Medicine and (6) PCR in Comparison with Gene Cloning.

12. Suggested Readings

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13. Assignments

1. What are the criteria of an ideal vectors?
2. What is the full form of TEMED?
3. How does a miRISC function in posttrancriptional gene silencing?
4. What is IEF?
5. Discuss RNA processing in eukaryotes to produce mRNA from pre mRNA.
6. Briefly describe pathways for protein targeting into and across thylakoid membranes.
7. Write a short note on sorting of proteins in mitochondria.
8. Mention the characteristic features of Watson and Crick model of DNA.
9. What are different types of restriction enzymes?
10. Enumerate the steps of recombinant DNA construction.
11. What is cosmid?
12. Differentiate between BAC abd YAC.
13. What is shuttle vector?
14. Write a short note on t-RNA.
15. What is riboswitch? Mention its mechanism of action.
16. What are the principles of Southern blotting? Explain the procedure of its.
17. How c-DNA library is constructed?
18. Enumerate the steps of DNA fingerprinting. Mention its application.
19. What is meant by expression proteomics?
20. Write a short note on genomics.
21. Differentiate between genomics and proteomics

22. What is RT-PCR? Write the application of PCR.
23. Briefly discuss about types of PCR.

All the materials are self writing and collected from e-book, journals and web sites

COURSE – BOTCOR T310

Plant Molecular Biology and Biotechnology

Core Theory Paper **Credit: (Groups A+B) = 3**

Group B (Biotechnology)

Content Structure

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1. Introduction
2. Course Objectives
3. Plant Tissue Culture: Cellular totipotency; organogenesis, somatic embryogenesis, Role of SERK and LEC genes during SE; haploidy and DH populations in crop improvement.
4. Biotechnological Applications for Crop Management: Approaches to improve shelf life of fruits and vegetables; herbicide resistance; insect and pest management.
5. Micropropagation: Production of virus free plants, virus free assessment methods, assessment by RAPD and ISSR markers, certification for quality plants.
6. Fermentation Technology: Application of fermentation; batch, fed batch & continuous cultures of microbes; Bioreactors: Principles & their design; microbial strain improvement.
7. Immobilization of microbial enzymes & whole cells and their applications in industries.
8. Microbes as Food & in food processing, single cell protein.
9. Biofertilizers and Biopesticides in agriculture.
10. Environmental Biotechnology: Treatment of waste & waste water; bioremediation.
11. Let's sum up
12. Suggested Readings
13. Assignments

1. Introduction

Biotechnology is technology based on biology, especially when used in agriculture, food science, and medicine. The UN Convention on Biological Diversity has come up with one of many definitions of biotechnology: "Biotechnology means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use." Traditional pharmaceutical drugs are small chemicals molecules that treat the symptoms of a disease or illness - one molecule directed at a single target. Biopharmaceuticals are large biological moleculesknown as proteins and these target the underlying mechanisms and pathways of a malady; it is a relatively young industry. They can deal with targets in humans that are not accessible with traditionalmedicines. Small molecules are manufactured by chemistry but large molecules are created by livingcells: for example, - bacteria cells, yeast cell. Biotechnology has applications in four major industrial areas, including health care (medical), crop production and agriculture, non-food (industrial) uses of crops and other products (e.g. biodegradable plastics, vegetable oil, biofuels), and environmental uses.This course is very important for students.

2. Course Objectives

This course aims to:

- To acquire knowledge and abilities to apply the biotechnological tools necessary in the development of products and services involving biological organisms or components, from genetic engineering techniques to the use of bioreactors and development of bioprocesses, along with applications to healthcare.
- Knowledge about the single cell protein, uses of biofertilizers and biopesticides in agriculture.

3. Plant Tissue Culture: Cellular totipotency; organogenesis, somatic embryogenesis, Role of SERK and LEC genes during SE; haploidy and DH populations in crop improvement.

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatlyreduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating andgrowing, i.e.: orchids and Nepenthes.
- To clear particular plants of viral and other infections and to quickly multiply these plants as'cleaned stock' for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

What conditions do plant cells need to multiply *in vitro*?

Tissue culture has several critical requirements:

- Appropriate tissue (some tissues culture better than others).
- A suitable growth medium containing energy sources and inorganic salts to supply cell growth needs. This can be liquid or semisolid.
- Aseptic (sterile) conditions, as microorganisms grow much more quickly than plant and animal tissue and can overrun a culture.
- Growth regulators - in plants, both auxins & cytokinins.
- Frequent subculturing to ensure adequate nutrition and to avoid the build-up of waste metabolites.

Cellular Totipotency:

Unlike an animal cell, a plant cell, even one that highly maturated and differentiated, retains the ability to change a meristematic state and differentiate into a whole plant if it has retained an intact membrane system and a viable nucleus. 1902 Haberlandt raised the totipotentiality concept of plant totipotency in his Book "Kulturversuche mit isolierten Pflanzenzellen" (Theoretically all plant cells are able to give rise to a complete plant).

Totipotency is the genetic potential of a plant cell to produce the entire plant. In other words, totipotency is the cell characteristic in which the potential for forming all the cell types in the adult organism is retained.

However, all living plant cells retain the potential to revert back to the meristematic state and form new plants on exposure to favorable conditions, irrespective of their specialization and ploidy level (haploid, diploid or triploid). It has been a routine horticultural practice to use leaf, stem, and root cuttings as source material to regenerate new individuals for vegetative propagation of some plant species. Plant tissue culture has considerably enlarged the scope of regeneration of plants from highly differentiated and structurally and functionally specialized cells of leaves, roots, stem, floral parts, andendosperm. In vitro regeneration of plants is also possible from isolated gametic cells. The potentiality of differentiated and specialized cells to form complete plants like the zygote is referred to as Cellular Totipotency. The term was probably coined by T.H. Morgan (1901). However, it was the famous German plant physiologist, Göttlieb Haberlandt, who in his famous address to the German Academy in 1902 introduced the concept of cellular totipotency and suggested that the terminally differentiated

plant cells, as long as they contain the entire complement of chromosomes, should be capable of regenerating whole plants. In tissue cultures, cellular totipotency may be expressed via organogenesis (shoot differentiation) or embryogenesis (adventive embryony).

Organogenesis:

In plant tissue culture, organogenesis is a process of differentiation by which plant organs viz. roots, shoots, bud flowers, stem etc. are formed while adventitious refers to the development of organs (roots, buds, shoots, flowers etc.) or embryos (embryo like structures) from unusual points of origin ofan organized explant where a preformed meristem is lacking. Adventitious shoots and roots are induced on tissues that normally do not produce these organs. Adventitious shoots are stem and leaf structures that arise naturally on plant tissues located in

sites other than at the normal leaf axil regions. Plant development through organogenesis is the formation of organs either de novo or adventitious in origin. Whole plant regeneration via organogenesis is a monopolar structure. Plant production through organogenesis can be achieved by two modes: (i) Organogenesis through callus formation with de novo origin and emergence of adventitious organs directly from the explant.

Organogenesis through callus formation:

Plant regeneration from cultured explants involves the initiation of basal callus and then shoot bud differentiation. Establishment of callus growth with subsequent organogenesis has been obtained from many species of plants and from numerous explants viz. cotyledons, hypocotyl, stem, leaf, shoot apex, root, young inflorescence, flower petals, petioles, embryos etc. cultured in vitro. For any given speciesor variety a particular explants may be necessary for successful plant regeneration. Explants from both mature and immature organs can be induced to form callus and then plant regeneration. However, explants with mitotically active cells are generally good for callus initiation. Immature tissues and organs are invariably more mopho-genetically plastic in vitro than mature tissues and organs. The sizeand shape of the explant is also crucial. It has been seen that a small percentage of cells in a given explant contribute to the formation of callus. Callus is produced on explants in vitro as a result of

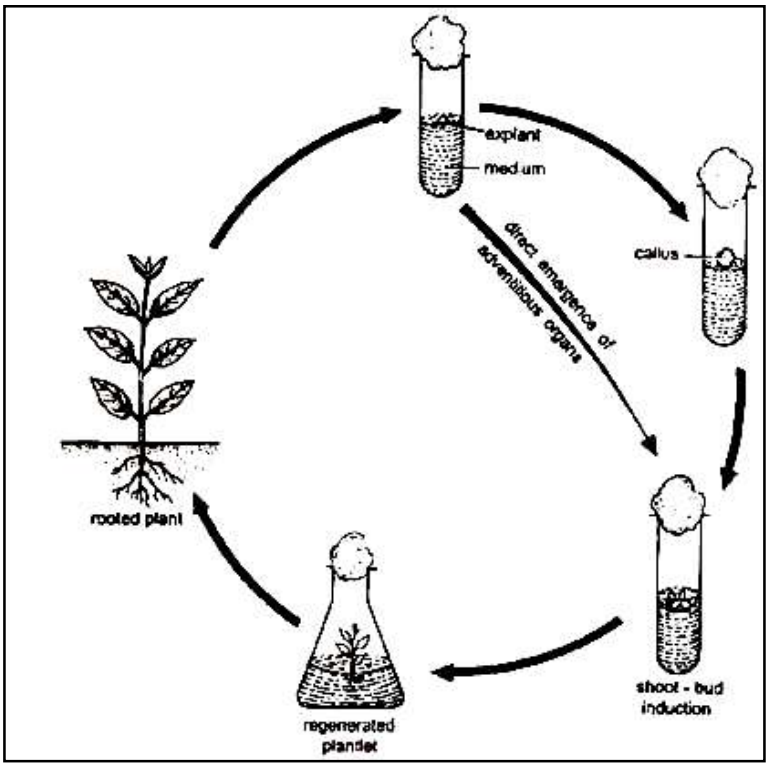
wounding and in response to hormones either endogenous or exogenously supplied in the medium.

Two modes of cell culture are generally used for organogenic path:

(i) the cultivation of cell cultures on a solid medium and

(ii) the cultivation of cell suspensions in liquid medium.

Suspension culture should be subcultured at leat once a week while callus culture should be subcultured after3-4 weeks. Plant growth regulators (PGRs) concentration in the culture medium is critical for morphogenesis. Auxin at a moderate to high concentration is the primary hormone used to produce callus. Often, 2, 4-D , a verypotentauxin, is used alone to imitate callus. In some species a high concentration of auxinand a low concentration of cytokinin in the medium promoteabundant cell proliferation with the formation of callus. Cytokinin if supplied are kinetic or benzyladenine. Callus tissuecomprises a wide range of cell types andcharacteristically consists of irregularly differentiated, vacuolated cells interspersed with smaller more meristematic cells. The nature of any callus will depend on the explant tissue or tissues form which it has arisen and also on the composition of the medium used to induce and maintain it.



Direct adventitious organ formation:

The somatic tissues of higher plants are capable under certain conditions of regenerating adventitious buds. Adventitious buds are those which arise directly from a plant organ or a piece thereof without any intervening callus phase. Induction of adventitious shoots directly on roots and leaves, bulb scales and other organs of intact plants is a common method of propagation. The requirement of exogenous auxin and cytokinin in the process varies with the tissue system, apparently depending on the endogenous levels of the hormones in the tissue. Adventitious in vitro regeneration may give a much higher rate of shoot production than axillary shoots. Adventitious shoots proliferation is the most frequently used multiplication technique in micropropagation system.

What is Somatic Embryogenesis?

In plant tissue culture, the developmental pathway of numerous well-organised, small embryoids resembling the zygotic embryos from the embryo genic potential somatic plant cell of the callus tissueor cells of suspension culture is known as somatic embryogenesis.

The process was discovered for the first time in *Daucas carota* L. (carrot) by **Steward (1958), Reinert (1959)**.

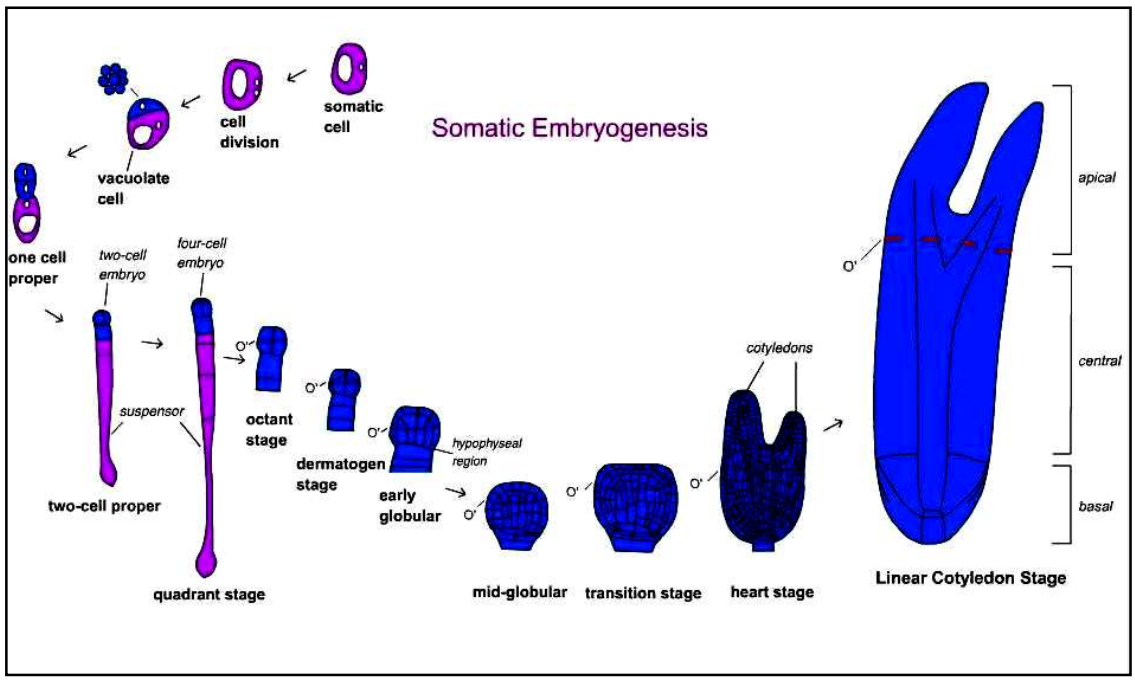
The most basic requirement for embryo development is the physical and chemical environment which is available only inside the '**Magic Bath'** of embryo sac. In vitro embryo can be developed if we provide the nutritional conditions same as in magic bath.

What is Embryo genic Potential?

The capability of the somatic plant cell of a culture to produce embryoids is known as embryo genic potential.

What is Embryoid?

Embryoid is a small, well-organised struc•ture comparable to the sexual embryo, which is produced intissue culture of dividing embryo.



Stages of somatic embryogenesis:

1. **Induction**
   - An auxin, particularly 2, 4-D, is generally necessary to induce embryogenesis.
   - Requirement of exogenous auxin for induction of SEs depends on nature of explants used with relative concentration of auxin.
2. **Development**
   - After reinitiation of cell division and a period of cell proliferation in presence of auxin embryogenic cells are released into auxin free medium. These cells are in the clusters of cytoplasmic cells called Proembryonic mass of cells (PEMs).
3. **Maturation**
   - The quality of SEs with regard to their germinability or conversion into plants is very poor. This is because the apparently normal looking SEs is actually incomplete in their development.

- Unlike seed embryos, SEs do not go through the final phase of embryogenesis, called embryo maturation which is characterised by accumulation of embryo specific reserve food materials and proteins which impart desiccation tolerance to the embryos; embryo size does not increase during this phase.
- ABA, which prevents precocious germination and promotes normal development of embryogenesis by triggering expression of genes which normally express during dryingdown stage of seeds (Dure et al, 1981).

Sharp et. al. (1980) described mainly two routes for somatic embryogenesis:

1. Direct Embryogenesis:

The embryos initiate directly from the explant without callus formation and here some cells which are called as 'Pre-embryonic determined cells' (PEDC) initiates embryonic development, only those cells need to be released. Such cells are found mostly in embryonic tissues, certain tissues of young in vitrogrown plants, hypocotyl, nucellus, embryo-sac, etc.

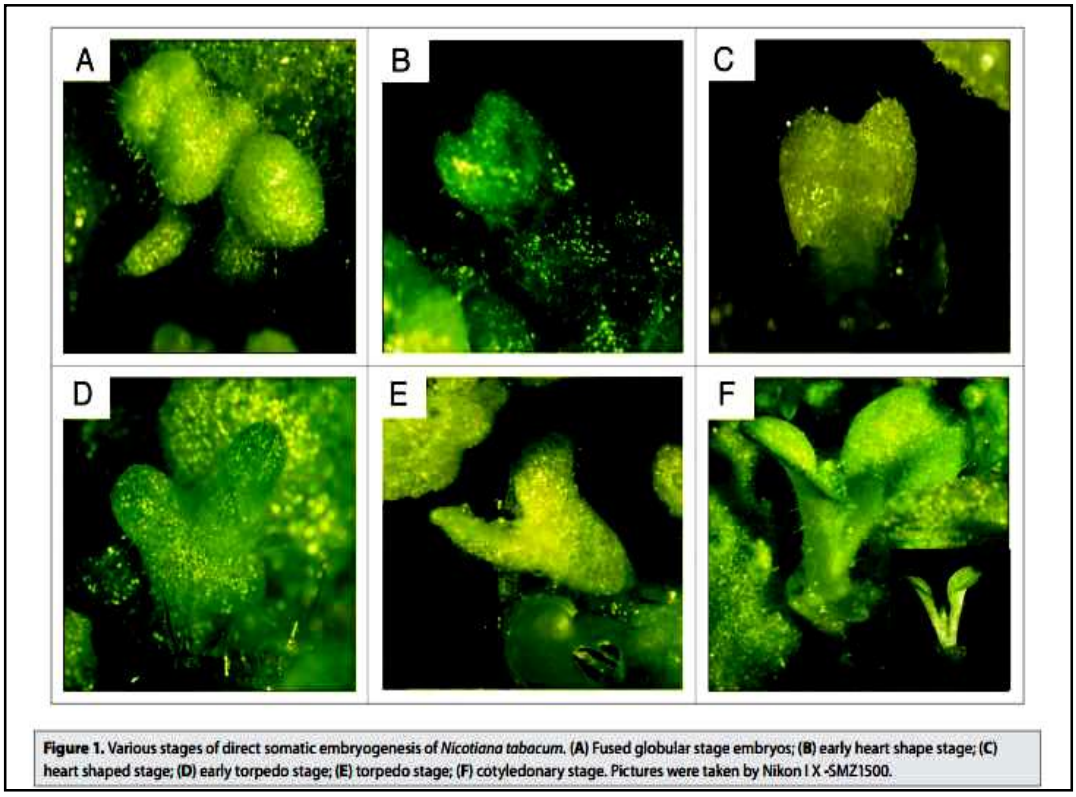


Figure 1. Various stages of direct somatic embryogenesis of *Nicotiana tabacum*. **(A)** Fused globular stage embryos; **(B)** early heart shape stage; **(C)** heart shaped stage; **(D)** early torpedo stage; **(E)** torpedo stage; **(F)** cotyledonary stage. Pictures were taken by Nikon I X -SMZ1500.

2. Indirect Embryogenesis:

Here, the embryos are developed through cell proliferation i.e., callus formation. The cells from which embryos arise are called as 'Induced embryogenic determined cells' (IEDC).Here growth regulators with specific cul•tural conditions are required for initiation of callus and then redetermination of those cells into the embryo development.

Figure 1 - Primary callus induction, somatic embryogenesis and plant regeneration from immature embryos of maize cv. Gaurav (A) Inoculation of immature embryos in MS with 5 mg/L 2,4-D and 2 mg/l NAA + 1 mg/l BAP, (B) Callus proliferation in MS with 5 mg/L 2,4-D and 2 mg/l NAA + 1 mg/l BAP, (C-D) Globular shape observed during somatic embryogenesis (arrows) (E) Regenerating calli in MS medium with 5.0 mg/L 2,4-D. Culture showing mature green somatic embryos (arrow) (F) Root induction in MS medium with 5.0 mg/L 2,4-D (Joshi et al., 2010).

Difference between Somatic embryos and Zygotic embryos:

Somatic embryos	Zygotic embryos
1. SEs are formed by sporophytic cells. **2**. SEs store less amount of embryo specific reserves. **3**. A distinct suspensor is absent in SEs even if it is present it may not be functional as in seed embryos. **4**. Embryos have no vascular connections with the cultured explant. **5**. SEs generally lack a dormant phase and often show secondary embryogenesis and pluricotyledony. **6**. SEs show high rate of propagation .	**1**. Formed by fusion of gametic cells. **2**. Seed storage proteins, carbohydrates are the characterstic features. **3**. A well developed distinct suspensor is present. **4**. Embryos have vascular connections with the explant. **5**. They do not show secondary embryogenesis and pluricotyledony. **6**. Low rate of propagation than SEs.

Advantages:

- It is observable, as its various culture conditions can be controlled.
- Lack of material is not a limiting factor for experimentation.
- High propagation rate.
- Somaclonal variations.

- Germplasm conservation.
- Labour saving.
- Elimination of diseases and viruses

Disadvantages:

- Confined to few species.
- The somatic embryos show very poor germination because of their physiological andbiochemical immaturity.
- Instability of cultured cells in long-term cultures is a major limitation in commercialexploitation and mass propagation of SEs.

Applications of Somatic Embryogenesis:

1. **Large Scale Propagation Compared to Zygotic Embryos:**

Induction of somatic embryogenesis forms the ultimate goal in free cell suspension cultures relying onthe totipotency of the cell and could reasonably be exploited for micro-propagation.

Each cell of the suspension cultures can be induced to produce somatic embryos which can be maintained in an arrested state of development by cold storage or using mitotic inhibitors until thetime of sowing. Somatic embryogenesis is highly desirable and holds out promise for rapid multiplication in a shorter time, with a shoot-root axis.

2. **More Useful than Organogenesis:**

The mass production of adventitious embryos in cell culture is still regarded by many as the ideal propagation system. The adventitious embryo is a bipolar structure that develops directly into a complete plantlet and there is no need for a separate rooting phase as with shoot culture.

3. **Useful for Mutagenic Studies and Mutant Production:**

The somatic embryos generally arise from single cells, so it may be advantageous for mutagenic studies. Also the plantlets arising from such somatic embryos are more homogeneous in nature, so themutant gene expression can be studied well.

4. **Useful for Genetic Manipulation Technique:**

In plant biotechnological application, during foreign gene transfer if the transformed cell gives

rise toplantlet via somatic embryogenesis then there is least possibility of chimera formation. So for transgenic plant production this method of multiplication system is very much useful.

5. Useful for Pathogen-Free Plant Production:

Plants derived from this kind of somatic embryos may be free from viral or other pathogens. So it maybe an alternative approach of disease free plant production.

6. A Good Source of Protoplast Culture:

Embryogenic cultures are specially valuable in providing a source of regenerable protoplasts in the graminaceous and coniferous plants. Protoplasts from these cultures were induced to divide to form a cell mass from which the embryoids, even plantlets are regenerated on a suitable nutrient medium.

7. Conservation of Genetic Resources:

Somatic embryos which originate from single cells and subsequently regenerate mostly genetically uniform plants are good materials for genetic resource conservation. Embryogenic cultures as well as somatic embryos remain viable upon storage at ambient temperature, cold storage or cryostorage.

Role of SERK and LEC genes during SE:

In plant biology, zygotic embryogenesis (ZE) is a model to study the expression of genes and translated proteins in the signal response during embryo development. However, SE has been found to be a useful tool as well, and can probe different questions surrounding these phenomena, and in a more controlled way. Due to the advantages of SE for the culturing of thousands of crop plants of economic importance, a field of study has been opened to understand the regulatory processes involved during the initiation and progress of different embryo developmental stages.

SERK:

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) is a transmembrane receptor that has been implicated in triggering embryogenesis. It was isolated for the first time from Daucus carota suspension cells, where expression was up regulated in the globular stage of embryogenesis. SERK is activated by auto-phosphorylation, specifically in the residue

threonine 468, glutathione S- transferee fusion and in vivo phosphorylation assays. SERK transduces the signal from cell membrane to obtain site, regulating the subsequent downstream proteins in the signal transduction pathway. To date, different experiments suggest the pivotal role of this protein in the initiation of embryogenic competence. For instance, the ortholog SERK of Arabidopsis enhances the ability to undergo the somatic embryo response. The histochemical and immunochemical techniques using β-glucoronidase detection have revealed the special localization of gene expression during indirect somatic embryogenesis. The expression was detected in four places: in embryos, in the outer layers of cotyledons and in the provascular and vascular strands of developing somatic embryos.

LEC:

The LEAFY COTYLEDON (LEC) genes are clasiified into two classes. The first class is HAP3-related transcription factors, represented in Arabidopsis by LEC1 and L1L. The second class encodes B3 domain transcription factors, which are represented by LEC2, FUS3, and AB13 in Arabidopsis. Among the LEC genes LEC1 and LEC2 have been suggested to have complementary a partial redundancy to induce somatic embryos. They have similar but not identical functions, as lec1lec2 double mutants have synergistic phenotypes. Overexpressing phenotypes of LEC1 seedlings arrest embryo-like seedlings and fail to develop; cotyledon-like organs sometimes form in place of the first leaves. LEC1 has been associated with the maturation of embryos. In LEC2, embryo-like seedlings continued to proliferate, producing callus, cotyledon-like structures and leaf-like organs in addition to the somatic embryos. Transgenic plants expressing LEC2 ectopically develop somatic embryos, which have cotyledon-like and leaf-like structures.

Haploidy and DH populations in crop improvement:

What do you mean by doubled haploids?

Doubled haploids refer to the diploid lines which are obtained by doubling the chromosome number of a haploid line by colchicine treatment. Such condition is referred to as doubled haploids.

What are main features doubled haploids?

Main features of doubled haploids are briefly presented below –

1) Doubled haploids are represented as DH.
2) Double haploids are developed from haploids by doubling the chromosome number of haploids by colchicine treatment.
3) Double haploids are completely homozygous lines.
4) Doubled haploids can be produced in both self pollinated and cross pollinated species.
5) Double haploid method allows to fix recombinant gametes directly as fertile homozygous lines.
6) This is a rapid method of producing pure lines. The pure lines can be produced from a heterozygous parent in one generation. The development of pure lines or inbred lines by conventional methods takes 3-5 years.
7) Doubled haploids can be released as cultivars after testing or can be used as parents of hybrids.
8) Doubled haploid method reduces the time needed to develop and release new cultivars by 3-5 years in comparison with conventional breeding techniques.
9) Doubled haploids are homozygous and homogeneous populations and hence their produce is highly uniform.
10) The doubled haploids obtained from recombinant lines generally have broad genetic base due to gene combination from two different sources.
11) The doubled haploid method opens a unique way for the fixation hybrid vigour or heterosis in homozygous lines, which would avoid all problems associated with the production of hybrid seed. For example, from a cross between AAbb x aaBB genotypes we can get a haploid with AB genotype which can be fixed as doubled haploid (AABB).
12) Doubled haploids segregate much simpler in the progeny of crosses being true breeding individuals.

Table 20.1 Differences between doubled Haploid and conventional methods of producing pure lines

Particulars	Doubled Haploid Method	Conventional Method
1. Time required for developing pure lines.	One year or one crop season	3-5 years.
2. Time required for developing cultivars	Two to three years	Seven-eight years
3. Fixation of heterosis	Possible	Not possible
4. Expenditure/cost involved	More than conventional method	Lesser than DH method.
5. Identification of recessive mutants	Very easy	Difficult

Application in Crop Improvement:

Doubled haploids (DH) are presently being used in breeding of a number of crop species. Doubledhaploid cultivars have been developed in different countries such as Canada, USA, UK, Spain, China, Hungry, and Italy etc. The maximum DH cultivars have been developed in Barley followed by Rapeseed, wheat, melon, pepper, Rice, *Asparagus*, Tobacco and Eggplant. Moreover, maximum doubled haploid cultivars have been released by anther culture.

Table 20.2 Number of doubled haploid cultivars released for commercial cultivation in different crops.

Name of Crop	Number of Cultivars Released	Developed
Barley (Hordeum vulgare)	115	Anther culture
Rapeseed (Brassica campestris)	47	Anther culture
Wheat (Triticum aestivum)	21	Anther culture
Melon (Cucurbita marchata)	9	Irradiated pollen
Pepper (Capsicum annum)	8	Anther culture
Rice (Oriza sativa)	8	Anther culture
Asparagus (Asparagus officinalis)	7	Irradiation
Tobacco (Nicotiana tabacum)	6	Irradiation
Eggplant (Solanum melongena)	5	Anther/Irradiation

What are uses of doubled haploids?

The use of doubled haploid method is gaining increasing importance these days looking to its variousadvantages.

Doubled haploids are used in the followings –

(i) Development of purelines

(ii) Development of cultivars

(iii) Development of hybrids as parents

(iv) Construction of genetic maps

(v) Gene tagging/locating genes

(vi) Identification of molecular markers for trait selection.

(vii) Doubled haploids will be useful to trace the evolutionary origin of various plants. The close evolutionary relationship between tomato and potato has been evaluated by this approach.

What do you mean by haploid?

An individual having gametic chromosome number in the somatic cells is known as haploid and suchcondition is referred to as haploidy.

Main features of haploids are presented below:

(i) Haploids occur spontaneously as well as can be induced artificially. The spontaneous haploids have been reported in 10 families covering 26 genera and 36 species. Main crops in which spontaneous haploids have been reported include tomato, cotton, coffee, beets, barley, flax, pearl millet, rapeseed, asparagus and wheat.

(ii) Haploids are sterile due to lack of pairing partners of chromosomes,

(iii) Haploids are represented by n, Haploids differ from monoploids. Monoploids have single copy of the genome and are represented by x. In a true diploid species both monoploid and haploid chromosome number is the same (n = x). Thus a monoploid is haploid but all haploids cannot be monoploids.

(iv) Haploids are of different types; their detailed description is beyond the scope of this discussion.

How would you induce haploids?

Haploids can be induced in four principal ways, viz. by

(i) Wide crossing,

(ii) Irradiation and – chemical treatment,

(iii) Selection of twins, and

(iv) By anther and pollen culture.

These are briefly discussed below –

(i) Wide Crossing: It includes interspecific and intergeneric hybridization and is also known as distant hybridization. Interspecific crosses have resulted in development of haploids in wheat and barley. Use of maize as pollen parent for wheat has produced high frequency of haploids in wheat.

(ii) Irradiation and Chemical Treatment: The pollination with irradiated pollen stimulates the unfertilized eggs to parthenogenic development. Such haploids have been developed in tabacco, wheat, snapdragon and oenothera. Application of a dye toluene blue (TB) to the pistils has induced haploids in Vinca rosea, tomato, maize and poplar.

(iii) Selection of Twins: In some species haploids can be obtained from the screening of poly-embryonic seeds. Poly- embryonic seeds can produce three types of seedlings viz. haploid-haploid; diploid-diploid and haploid-diploid. This has been reported in capsicum and in some horticultural species.

(iv) Anther and Pollen Culture: This method is widely used for production of haploids. Haploids develop directly from pollen grains in culture, either through direct formation of embryos from pollen grains or formation of callus and subsequent plant regeneration.

Application in Crop Improvement:

1) Development of homozygous lines:

It is now possible to develop homozygous lines within a span of few months or a year by

employing anther/pollen culture. This is in contrast to the conventional plant breeding programme that might take several years (6-10 yrs). In this way, production of haploids is highly useful for research related to plant genetics and breeding.

2) Generation of exclusive male plants:

By the process of androgenesis to produce haploids, followed by chromosome doubling, it is possible to develop exclusive male plants. The male plants are particularly useful when their productivity and applications are much more than female plants.

3) Induction of mutations:

In general, majority of induced mutations are recessive and therefore are not expressed in diploid cells (due to the presence of dominant allele). Haploids provide a convenient system for the induction of mutations and selection of mutants with desired traits. In fact, the haploid cells can be cultured and handled in a fashion similar to microorganisms.

Mutants from several plant species that are resistant to antibiotics, toxins, herbicides etc. have been developed. When the haploid cells of tobacco plant (Nicotiana tabacum) were exposed to methionine sulfoximine (a mutagen), mutants which showed lower level of infection to Pseudomonas tabaci were produced.

4) Production of disease resistance plants:

Disease resistance genes can be introduced while producing haploids. The so developed haploids are screened for the desired resistance, and then diploidized. Some examples of disease resistance plants are listed –

- ✓ Hwansambye, a rice variety resistant to leaf blast, bacterial leaf blight and rice stripe tenui virus.
- ✓ Barley accession Q21681 resistant to stem rust, leaf rust and powdery mildew. More examples of disease resistance crops are given in Table.

TABLE 45.1 A selected list of improved varieties of crops developed by using anther culture

Crop	*Varieties*	*Improvements made*
Wheat (*Triticum aestivum*)	Lunghua 1, Zing Hua 1, Zing Hua 2 Huapei 1, Florin, Ambitus, Jingdan 2288	High yield, rust resistance, cold resistance, large spikes, more tillers.
Rice (*Oryza sativa*)	Tanglong 1, Xin Xiu, Zhog Hua 8 Zhong Hua 9, Hua yu 1, Hua Yu 2, Huapei Shanyou 63, Zhe keng 66, Ta Be 78, Nonhua 5, Hirohikari, Hirohonami.	High yield, good quality, disease resistance
Tobacco (*Nicotiana tabacum*)	Tanyu 1, Tanyu 2, Tanyu 3, F 211 Hai Hua 19, Hai Hua 30	Mild smoking, disease resistance
Brassica napus	Jai kisan	Low erucic acid

5) **Production of insect resistance plants:**

Some varieties of rice resistant to insects have been developed e.g. Hwacheongbyeo resistant to brown plant hopper. Other varieties of rice that are resistant to pests have also been produced.

6) **Production of salt tolerance plants:**

The plant species with salt tolerance are needed for their cultivation in some areas. Anther cultures have resulted in some varieties of rice and wheat with good salt tolerance e.g. wheat Hua Bain 124-4.

7) **Cytogenetic research:**

Haploids are useful in several areas of cytogenetic research. These include –

- ✓ Production of aneuploids
- ✓ Determination of the nature of ploidy
- ✓ Determination of basic chromosome number
- ✓ Evaluation of origin of chromosomes.

8) **Induction of genetic variability:**

Besides the development of haploid mutants, it is also possible to produce plants with various ploidylevels through androgenesis.

4. Biotechnological Applications for Crop Management: Approaches to improve shelf life of fruits and vegetables; herbicide resistance; insect and pest management.

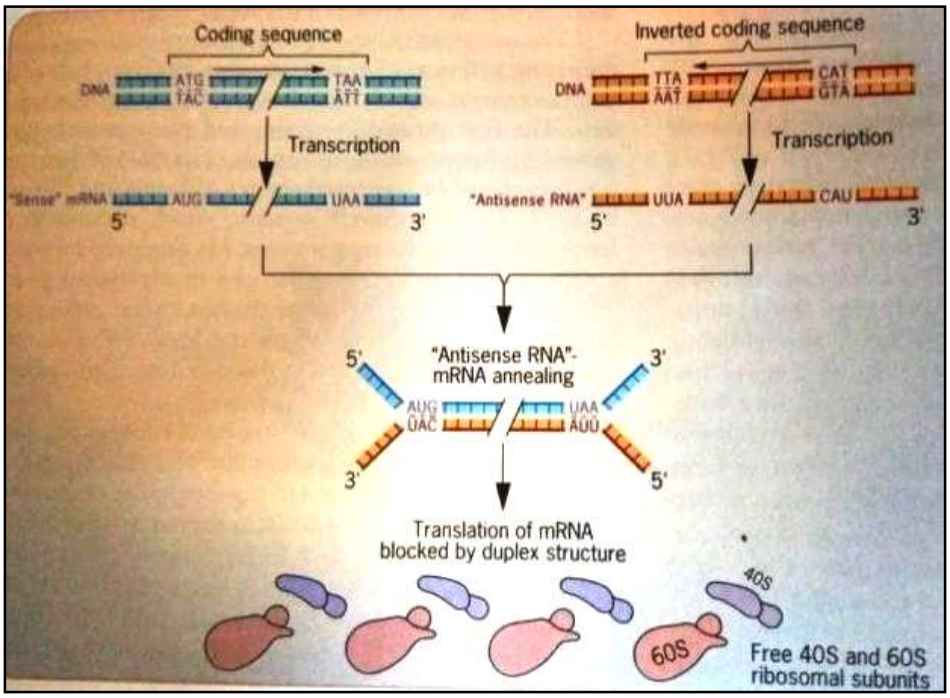
Approaches to improve shelf life of fruits and vegetables:

The importance of fruits and vegetables in the diet of mankind cannot be over emphasized. Typically, biotechnology technique such as genetic modification is used in fruits and vegetables to enable plants tolerate the biotic and abiotic stresses, and plant resistances to

problematic pests and disease, which may provide higher nutritional contents, and extend the shelf life of the produce. Recently, recombinant DNA technology has been used by scientists to delay ripening in fruits and vegetables in order for farmers to have the flexibility in marketing their produce and ensure consumers good qualityproduce from their farms. Genes related to ethylene biosynthesis and cell wall degradation are the primary targets for the extension of the shelf life of the fruits and vegetables. The levels of endogenous gene expression can be reduced by the following 4 approaches: (i) Antisense RNA approach (ii) ribozyme (iii) gene disruption (iv) Over transcription leading to co-suppression.

Antisense RNA approach has proven useful to improve shelf life of fruits and vegetables. It has also been a commercial success. The antisense RNA method involves the synthesis of the RNA molecules that are complementary to the mRNA molecules produced by transcription of a given gene. The antisense is produced by inverting the orientation of the protein encoding region of a gene in relation to its promoter. A s result, the reference sense strand of the gene becomes oriented 3'-5' direction with reference to its promoter and is transcribed. The RNA produced by this gene has the same sequence asthe antisense strand of the normal gene and is therefore known as antisense RNA.

A major problem in agriculture is perishing ability of its products. Various methods have been initiated and developed to increase the shelf life of crops especially for fruits and vegetables. One such successful technique is delaying the ripening of fruits and vegetables by manipulating the genes through genetic engineering.



Flavr Savr is a genetically modified tomato, was the first commercially grown genetically engineered food to be granted a license for human consumption. It was produced by Californian Company Calgene 1992. Calgene introduced a gene in plant which synthesizes a complementary mRNA to PG gene and inhibiting the synthesis of PG enzyme. On May 21, 1994, the genetically engineered Flavr Savr tomato was introduced.

Fruit ripening is an active process characterized by increased respiration accompanied by a rapid increase in ethylene synthesis. As the chlorophyll gets degraded, the green color of fruit disappears and a red pigment, lycopene is synthesized. The fruit gets softened as a result of the activity of cell wall degrading enzymes namely polygalacturonase (PG) and methyl esterase. The phyto hormone ethylene production is linked to fruit ripening as the same is known to trigger the ripening effect. The breakdown of starch to sugars and accumulation of large number of secondary products improves the flavor, taste and smell of the fruits.

Genes involved in tomatoes ripening:

1. **pTOM5** encodes for phytoene synthase which promote lycopene synthesis that gives red coloration
2. **pTOM6** gene encodes for polygalacturonase. This enzyme degrades the cell wall, resulting in fruitsoftening.
3. **pTOM** gene encodes for ACC oxidase. This enzyme catalyzes the ethylene formation that triggersthe fruit ripening.

Development of flavr savr tomato:

Softening of fruits is largely due to degradation of cell wall (pectin) by enzyme polygalacturonase(PG). The gene encoding PG has been isolated and cloned (pTOM6).

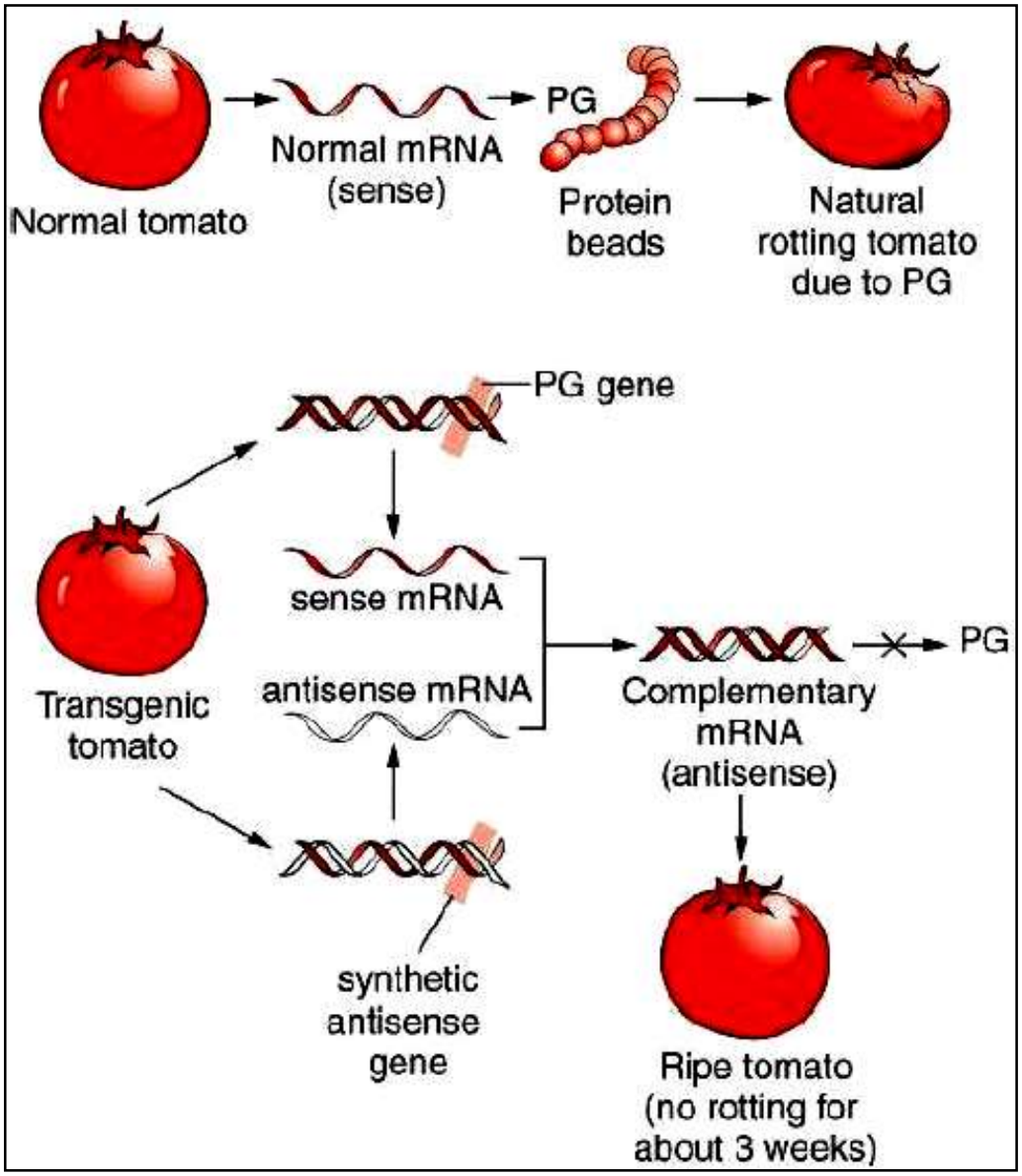
Procedure involves:

1) Isolation of DNA from tomato plant that encodes the enzyme polygalacturonase (PG).
2) Transfer of PG gene to a vector bacteria and production of complementary DNA (cDNA)molecules.
3) Introduction of cDNA into a fresh tomato plant to produce transgenic plant.

Mechanism of pg antisense RNA approach:

In normal plants, PG gene encodes a normal or sense mRNA that produce the enzyme PG and it isactively involved in fruit ripening.

- The cDNA of PG encodes for antisense mRNA, which is complementary to sense mRNA.
- The hybridization between sense and antisense mRNA render the sense mRNA ineffective.Consequently, no polygalacturonase is produced hence fruit ripening is delayed.



Advantages:

1. Slower ripen rate
2. Ripen longer on vine
3. Fully developed flavours
4. Increase the shelf life

The rise and fall of Flavr Savr Tomato:

The genetically engineered tomato, known as Flavr Savr (pronounced flavour saver) by employing PC antisense RNA was approved by U.S. Food and Drug Administration on 18th May 1994.

The FDA ruled that Flavr Savr tomatoes are as safe as tomatoes that are bred by conventional means, and therefore no special labeling is required. The new tomato could be shipped without refrigeration too far off places, as it was capable of resisting rot for more than three weeks (double the time of a conventional tomato).

Although Flavr Savr was launched with a great fanfare in 1995, it did not fulfill the expectation forthe following reasons:

i. Transgenic tomatoes could not be grown properly in different parts of U.S.A.
ii. The yield of tomatoes was low.
iii. The cost of Flavr Savr was high.

It is argued that the company that developed Flavr Savr, in its overenthusiasm to become the first Biotech Company to market a bioengineered food had not taken adequate care in developing the transgenic plant. And unfortunately, within a year after its entry, Flavr Savr was withdrawn, and it is now almost forgotten!

Herbicide Resistance:

Weed infestations destroy about 10 percent of crops worldwide. To combat weeds, farmers often apply herbicides before seeding a crop and between rows after the crops are growing. As the most efficient broad-spectrum herbicides also kill crop plants, herbicide use may be difficult and limited. Farmers also use tillage to control weeds; however, tillage damages soil structure and increases erosion.

A good or an ideal herbicide is expected to possess the following characteristics –

i. Capable of killing weeds without affecting crop plants.
ii. Not toxic to animals and microorganisms.
iii. Rapidly translocated within the target plant.
iv. Rapidly degraded in the soil.

Glyphosate Resistance:

Glyphosate, is a glycine derivative. It acts as a broad-spectrum herbicide and is effective against 76 of the world's worst 78 weeds. Glyphosate is less toxic to animals and is rapidly degraded by microorganisms. In addition, it has a short half-life. The American chemical company Monsanto markets glyphosate as **Round up**.

Mechanism of action of glyphosate:

Glyphosate is rapidly transported to the growing points of plants. It is capable of killing the plants even at a low concentration. Glyphosate acts as a competitive inhibitor of the enzyme 5- enoylpyruvylshikimate 3-phosphate synthase (EPSPS). This is a key enzyme in shikimic acid pathwaythat results in the formation of aromatic amino acids (tryptophan, phenylalanine and tyrosine), phenols and certain secondary metabolites.

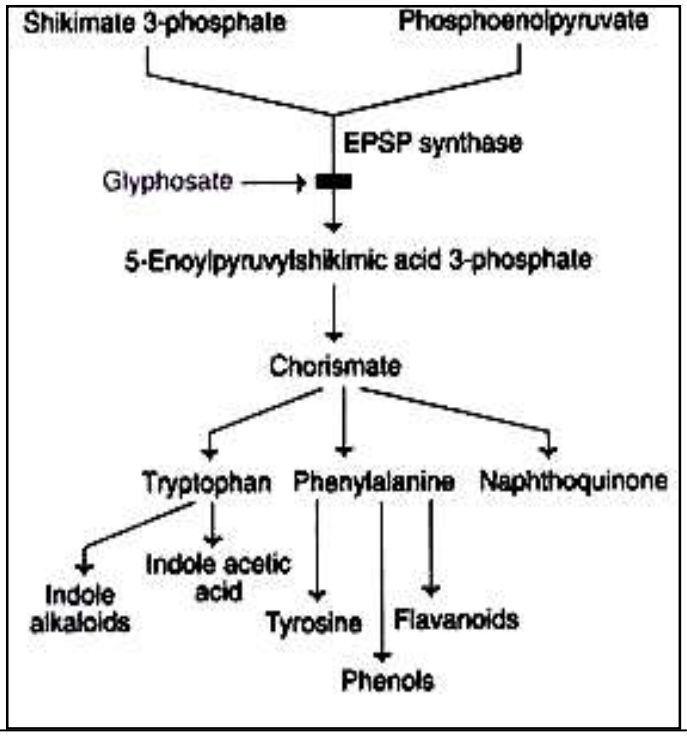


Fig.: Shikimate pathway indicating the action of the herbicide glyphosate (EPSP synthase – 5-enolpyruvylshikimate-3-phosphate synthase)

The enzyme EPSPS catalyses the synthesis of 5-enoylpyruvylshikimate-3-phosphate from shikimate 3-phosphate and phosphoenoylpyruvate. Glyphosate has some structural similarly with the substrate phosphoenol pyruvate. Consequently, glyphosate binds more tightly with EPSPS and blocks the normal shikimic acid pathway. Thus, the herbicide glyphosate inhibits the biosynthesis of aromatic amino acids and other important products.

This results in inhibition of protein biosynthesis (due to lack of aromatic amino acids). As a consequence, cell division and plant growth are blocked. Further, the plant growth regulator indole acetic acid (an auxin) is also produced from tryptophan. The net result of glyphosate is the death of the plants. Glyphosate is toxic to microorganisms as they also possess shikimate pathway.

Glyphosate is non-toxic to animals (including humans), since they do not possess shikimate pathway.

Strategies for glyphosate resistance:

There are three distinct strategies to provide glyphosphate resistance to plants –

1. ***Overexpression of crop plant EPSPS gene:***

An overexpressing gene of EPSPS was detected in *Petunia*. This expression was found to be due to gene amplification rather than an increased expression of the gene. EPSPS gene from *Petunia* was isolated and introduced into other plants. The increased synthesis of EPSPS (by about 40 fold) in transgenic plants provides resistance to glyphosate. These plants can tolerate glyphosate at a dose of 2-4 times higher than that required to kill wild-type plants.

2. ***Use of mutant EPSPS genes:***

An EPSPS mutant gene that conferred resistance to glyphosate was first detected in the bacterium Salmonella typhimurium. It was found that a single base substitution (C to 7) resulted in the change ofan amino acid from proline to serine in EPSPS. This modified enzyme cannot bind to glyphosate, and thus provides resistance.

The mutant EPSPS gene was introduced into tobacco plants using *Agrobacterium* Ti plasmid vectors. The transgene produced high quantities of the enzyme EPSPS. However, the transformed tobacco plants provided only marginal resistance to glyphosate. The reason for this was not immediately identified.

3. Detoxification of glyphosate:

The soil microorganisms possess the enzyme glyphosate oxidase that converts glyphosate to glyoxylate and aminomethylphosponic acid. The gene encoding glyphosate oxidase has been isolated from a soil organism *Ochrobactrum anthropi*. With suitable modifications, this gene was introduced into crop plants e.g. oilseed rape. The transgenic plants were found to exhibit very good glyphosate resistance in the field.

Use of a combined strategy:

More efficient resistance of plants against glyphosate can be provided by employing a combined strategy. Thus, resistant (i.e. mutant) EPSPS gene in combination with glyphosate oxidase gene are used. By this approach, there occurs glyphosate resistance (due to mutant EPSPS gene) as well as its detoxification (due to glyphosate oxidase gene).

Phosphinothricin Resistance:

Phosphinothricin (or glufosinate) is also a broad spectrum herbicide like glyphosate. Phosphinothricin is more effective against broad-leafed weeds but least effective against perennials.

Phosphinothricin – a natural herbicide:

Phosphinothricin is an unusual herbicide, being a derivative of a natural product namely bialaphos.

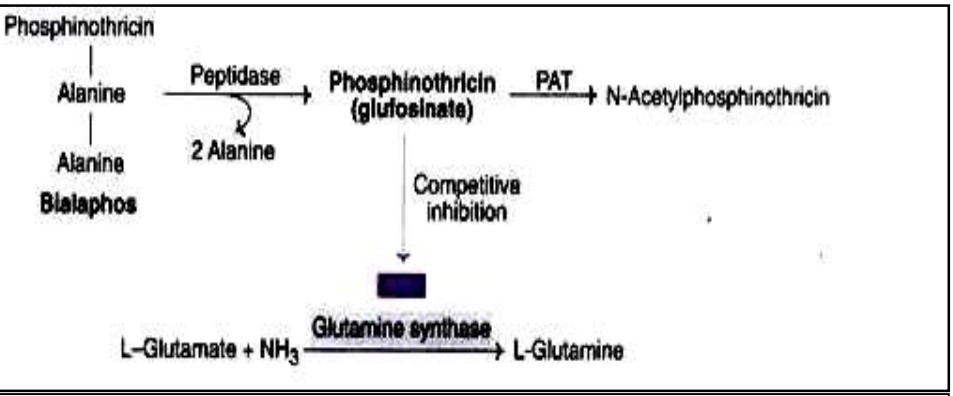


Fig.: The formation, mode of action and detoxification of phosphinothricin (PAT-Phosphinothricin acetyl transferase)

Certain species of *Streptomyces* produce bialaphos which is a combination of phosphinothricin bound to two alanine residues, forming a tripeptide. By the action of a peptidase, bialaphos is converted to active phosphinothricin.

Mechanism of action of phosphinothricin:

Phosphinothricin acts as a competitive inhibitor of the enzyme glutamine synthase. This is possible since phosphinothricin has some structural similarity with the substrate glutamate. As a consequence of the inhibition of glutamine synthase, ammonia accumulates and kills the plant cells. Further,disturbance in glutamine synthesis also inhibits photosynthesis. Thus, the herbicidal activity ofphosphinothricin is due to the combined effects of ammonia toxicity and inhibition of photosynthesis.

Strategy for phosphinothricin resistance:

The natural detoxifying mechanism of phosphinothricin observed in Streptomyces sp has prompted scientists to develop resistant plants against this herbicide. The enzyme phosphinothricin acetyl transferase (of *Streptomyces* sp.) acetylates phosphinothricin, and thus inactivates the herbicide.

The gene responsible for coding phosphinothricin acetyl transferase (**bar gene**) has been identified in *Streptomyces hygroscopicus*. Some success has been reported in developing transgenic maize and oilseed rape by introducing bar gene. These plants were found to provide resistance to phosphinothricin.

Bromoxynil

Tobacco plants have been engineered to be resistant to the herbicide bromoxynil.

Glufosinate

Crops have been commercialized that are resistant to the herbicide glufosinate, as well. Crops engineered for resistance to multiple herbicides to allow farmers to use a mixed group of two, three, or four different chemicals are under development to combat growing herbicide resistance.

2,4-D

In October 2014, the US EPA registered Dow's Enlist Duo maize, which is genetically modified to be resistant to both glyphosate and 2, 4-D, in six states. Inserting a bacterial aryloxyalkanoate dioxygenase gene, aad1 makes the corn resistant to 2, 4-D. The USDA had approved maize and soybeans with the mutation in September 2014.

Insect (Pest) Resistance GM crops:

It is estimated that about 15% of the world's crop yield is lost to insects or pests. The damage to cropsis mainly caused by insect larvae and to some extent adult insects.

The majority of the insects that damage crops belong to the following orders (with examples) are –

i. Lepidoptera (bollworms).
ii. Coleoptera (beetles).
iii. Orthoptera (grasshoppers).
iv. Homoptera (aphids).

Till some time ago, chemical pesticides are the only means of pest control. Transgenic plants with insect resistance transgenes have been developed. About 40 genes obtained from microorganisms of higher plants and animals have been used to provide insect resistance in crop plants.

Resistance Genes from Microorganisms:

***Bacillus thuringiensis* (Bt) toxin:**

Bacillus thuringiensis was first discovered by Ishiwaki in 1901, although its commercial importance was ignored until 1951. *B. thuringiensis* is a Gram negative, soil bacterium. This bacterium produces a parasporal crystalline proteinous toxin with insecticidal activity. The protein produced by *B. thuringiensis* is referred to as insecticidal crystalline protein (ICP). ICPs are among the endotoxins produced by sporulating bacteria, and were originally classified as δ-endotoxins (to distinguish them from other classes of α-, β- and *y*-endotoxins).

Bt toxin genes:

Several strains of B. thuringiensis producing a wide range of crystal (cry) proteins have been

identified. Further, the structure of cry genes and their corresponding toxin (δ-endotoxin) products have been characterized. The cry genes are classified into a large number of distinct families (about 40) designated as cry 1…….cry 40, based on their size and sequence similarities. And within each family, there may be sub-families. Thus, the total number of genes producing Bt toxins (Cry proteins) is more than 100.

There are differences in the structure of different Cry proteins, besides certain sequence similarities. The molecular weights of Cry proteins may be either large (~130 KDa) or small (~70KDa). Despite the differences in the Cry proteins, they share a common active core of three domains.

Mode of action of Cry proteins:

Most of the Bt toxins (Cry proteins) are active against *Lepidopteran* larvae, while some of them are specific against Dipteran and Coleopteran insects. The pro-toxin of Cry I toxin group has a molecular mass of 130 kilo Daltons (130 KDa).

When this parasporal crystal is ingested by the target insect, the pro-toxin gets activated within its gut by a combination of alkaline pH (7.5 to 8.5) and proteolytic enzymes. This results in the conversion ofpro-toxin into an active toxin with a molecular weight of 68 KDa. The active form of toxin protein gets itself inserted into the membrane of the gut epithelial cells of theinsect. This result in the formation of ion channels through which there occurs an excessive loss of cellular ATP. As a consequence, cellular metabolism ceases, insect stops feeding, and becomes dehydrated and finally dies.

Some workers in the recent years suggest that the Bt toxin opens cation-selective pores in the membranes, leading to the inflow of cations into the cells that causes osmotic lysis and destruction of epithelial cells (and finally the death of insect larvae). The Bt toxin is not toxic to humans and animals since the conversion of pro-toxin to toxin requires alkaline pH and specific proteases.

The usage Bt is commonly used for a transgenic crop with a cry gene e.g. Bt cotton. In the same way, Cry proteins are also referred to as Bt proteins. It may also be stated here that the

authors use four different names for the same group of proteins-δ-endotoxin, insecticidal crystal protein (ICP), Cry andnow Bt.

Bt-based genetic transformation of plants:

It has been possible to genetically modify (GM) plants by inserting Bt genes and provide pest resistance to these transformed plants. For an effective pest resistance, the bacterial gene in transgenic plants must possess high level expression. This obviously means that the transgene transcription should be under the effective control of promoter and terminator sequences. The early attempts to express cry 1A and cry 3A proteins under the control of CaMV 35S or *Agrobacterium* T-DNA promoters resulted in a very low expression in tobacco, tomato and potato plants.

In March 1995, the first Bt crop deregulated in the U.S. were seven lines of Colorado Potato Beetle Resistant Bt Potato by Monsanto. Since then, many more Bt crops have been deregulated, engineered to produce a variety of different Bt proteins from various subspecies of Bt. Bt crops include:

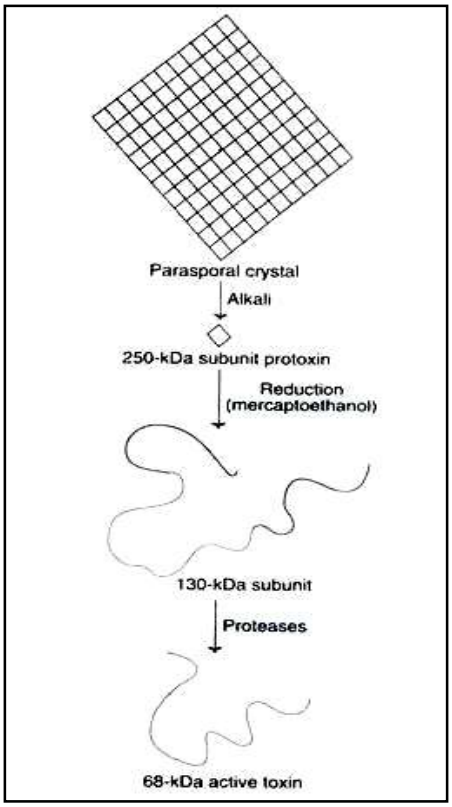
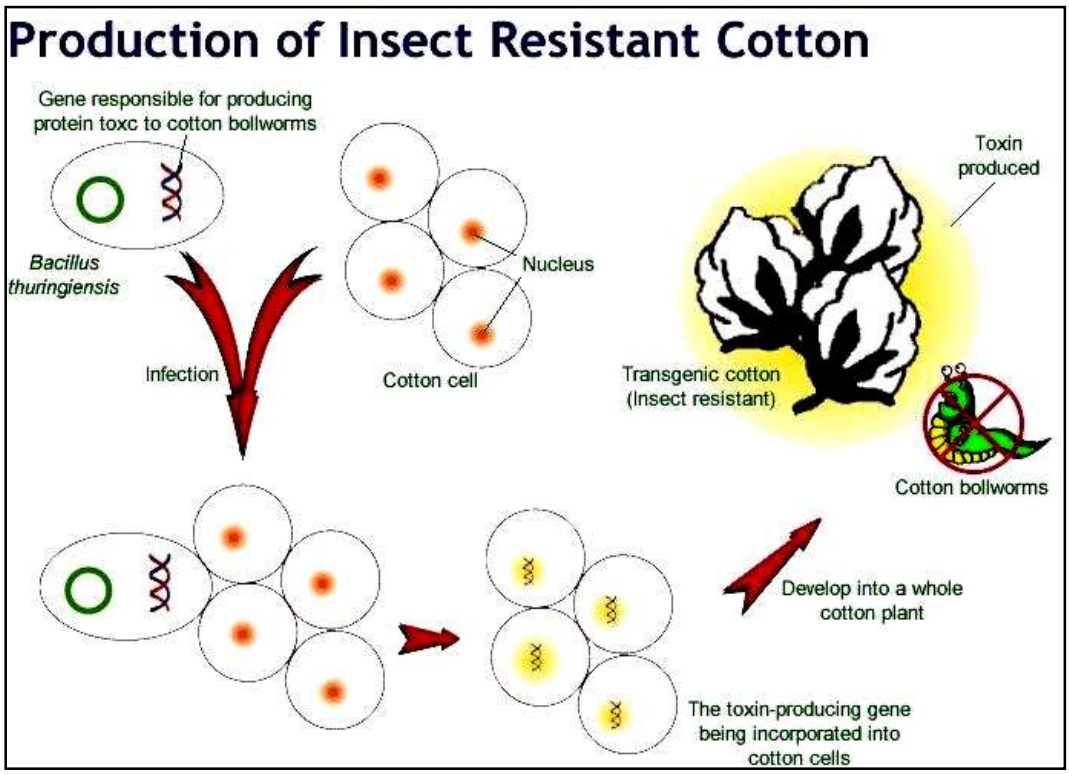


Fig.: A Diagrammatic representation of the formation of active toxin from the parasporal



Corn:

European Corn Borer Resistant Corn (first deregulated in the U.S. in May 1995)

Corn Rootworm Resistant Corn (first deregulated in the U.S. in October 2002)

Cotton:

Lepidopteran Resistant Cotton (first deregulated in the U.S. in June 1995)

Potato:

Colorado Potato Beetle Resistant Bt Potato (first deregulated in the U.S. in March 1995)

Potato Tuber Moth Resistant Bt Potato (being developed in South Africa)

Soybean:

Bt Soybean (first deregulated in the U.S. in October 2011, not yet sold commercially)

Tomato:

Lepidopteran Resistant Tomato (first deregulated in the U.S. in March 1998, not yet sold commercially)

Golden Rice —The Provitamin A Enriched Rice:

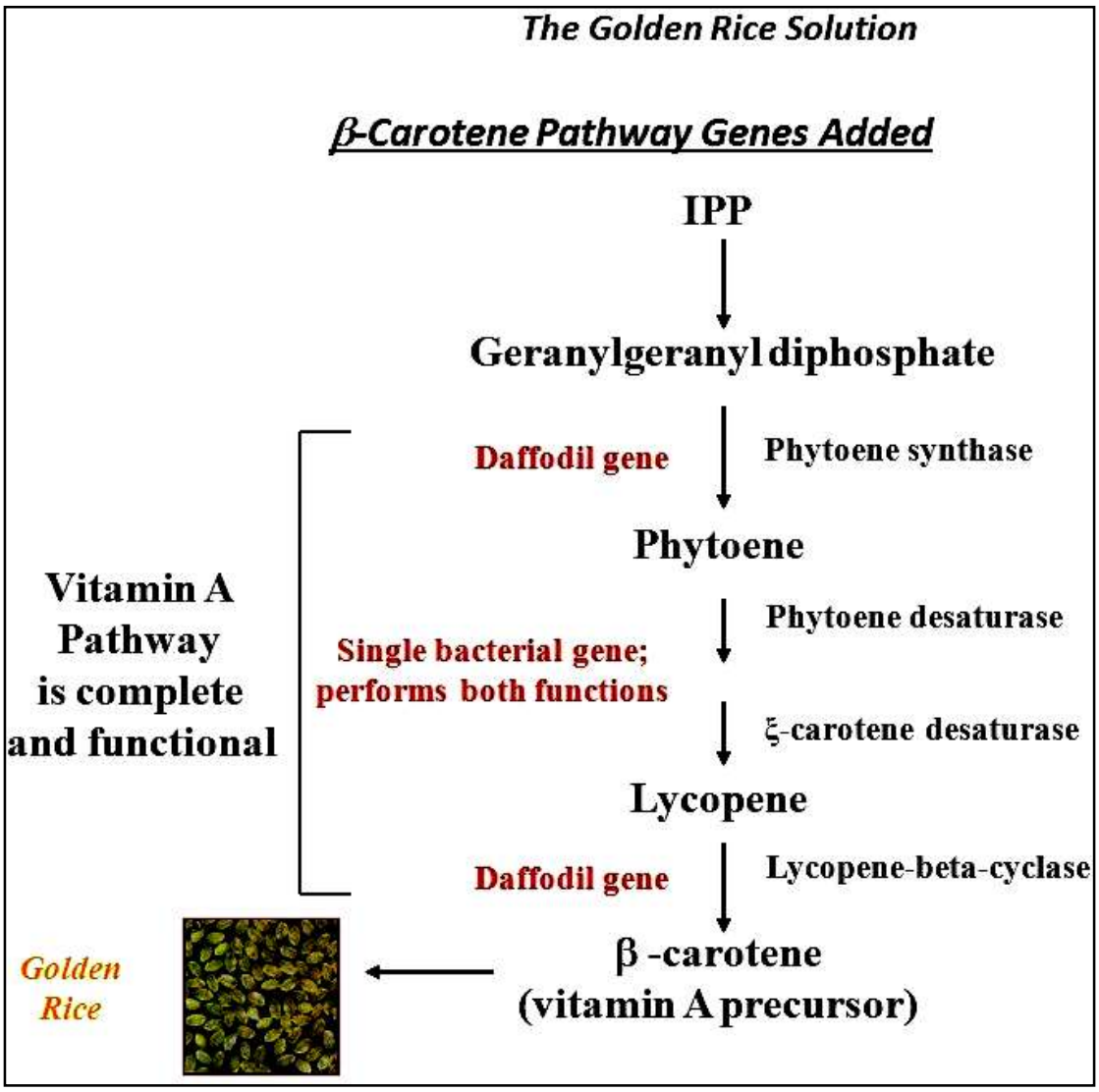
About one-third of the world's population is dependent on rice as staple food. The milled rice that is usually consumed is almost deficit in P-carotene, the pro-vitamin A. As such, vitamin A deficiency (causing night blindness) is major nutritional disorder world over, particularly in people subsisting on rice.

To overcome vitamin A deficiency, it was proposed to genetically manipulate rice to produce β-carotene, in the rice endosperm. The presence of β-carotene in the rice gives a characteristic yellow/orange colour, hence the pro-vitamin A-enriched rice are appropriately considered as Golden Rice.

The genetic manipulation to produce Golden Rice required the introduction of three genes encoding the enzymes phytoene synthase, carotene desaturase and lycopene β-cyclase. It took about 7 years to insert three genes for developing Golden Rice.

Golden Rice has met almost all the objections raised by the opponents of GM foods. However, many people are still against the large scale production of Golden Rice, as this will open door to the entry ofmany other GM foods. Another argument put forth against the consumption of Golden Rice is that it can supply only about 20% of daily requirement of vitamin A. But the proponents justify that since rice is a part of a mixed diet consumed (along with many other foods), the contribution of pro-vitamin A through Golden Rice is quite substantial.

Recently (in 2004), a group of British scientists have developed an improved version of Golden Rice. The new strain, Golden Rice 2 contains more than 20 times the amount of pro-vitamin A than its predecessor. It is claimed that a daily consumption of 70 g rice can meet the recommended dietary allowance for vitamin A.


The Golden Rice Solution
β-Carotene Pathway Genes Added
IPP
Geranylgeranyl diphosphate
Daffodil gene
Phytoene synthase
Phytoene
Vitamin A Pathway is complete and functional
Single bacterial gene; performs both functions
Phytoene desaturase
ξ-carotene desaturase
Lycopene
Daffodil gene
Lycopene-beta-cyclase
β -carotene (vitamin A precursor)
Golden Rice

5. Micropropagation: Production of virus free plants, virus free assessment methods, genetic assessment by RAPD and ISSR markers, certification for quality plants.

Micropropagation:

Micropropagation is the practice of rapidly multiplying stock plant material to produce many progenyplants, using modern plant tissue culture methods.

Micropropagation is used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

Cornell University botanist Frederick Campion Steward discovered and pioneered micropropagation and plant tissue culture in the late 1950s and early 1960s.

Stages:

Stage 0: Mother plant selection, maintenance and preparation for culture initiation

Stage I: Initiation and establishing an aseptic culture

Stage II: Multiplication of suitable propagules

Stage III: Preparation for growth in the natural environment

Stage IV: Transfer to the natural environment

Applications of Micro propagation:

Micro propagation has become a suitable alternative to conventional methods of vegetative propagation of plants. There are several advantages of micro propagation.

1. ***High Rate of Plant Propagation:***

Through micro propagation, a large number of plants can be grown from a piece of plant tissue within a short period. Another advantage is that micro propagation can be carried out throughout the year, irrespective of the seasonal variations. Further, for many plants that are highly resistant to conventional propagation, micro propagation is the suitable alternative.

The small sized propagules obtained in micro propagation can be easily stored for many years (germplasm storage), and transported across international boundaries.

2. ***Production of Disease-free Plants:***

It is possible to produce disease-free plants through micro propagation. Meristem tip cultures are generally employed to develop pathogen-free plants .In fact, micro propagation is successfully used for the production of virus-free plants of sweet potato (*Ipomea batatus*), cassava (*Manihot esculenta*) and yam (*Discorea rotundata*).

3. ***Production of Seeds in Some Crops:***

Micro propagation, through axillary bud proliferation method, is suitable for seed production in some plants. This is required in certain plants where the limitation for' seed production is high degree of genetic conservation e.g. cauliflower, onion.

4. ***Cost-effective Process:***

Micro propagation requires minimum growing space. Thus, millions of plant species can be maintained inside culture vials in a small room in a nursery. The production cost is relatively low particularly in developing countries (like India) where the manpower and labour charges are low.

5. ***Automated Micropropagation:***

It has now become possible to automate micro propagation at various stages. In fact, bio-reactors have been set up for large scale multiplication of shoots and bulbs. Some workers employ robots (in place of labourers) for micro- propagation, and this further reduces production cost of plants.

Disadvantages of Micro propagation:

1. **Contamination of Cultures:**

During the course of micro propagation, several slow-growing microorganisms (e.g. *Eswinia* sp, *Bacillus* sp) contaminate and grow in cultures. The microbial infection can be controlled by addition of antibiotics or fungicides. However, this will adversely influence propagation of plants.

2. ***Brewing of Medium:***

Micro propagation of certain plants (e.g. woody perennials) is often associated with

accumulation of growth inhibitory substances in the medium. Chemically, these substances are phenolic compounds, which can turn the medium into dark colour. Phenolic compounds are toxic and can inhibit the growth of tissues. Brewing of the medium can be prevented by the addition of ascorbic acid or citric acid orpolyvinyl pyrrolidone to the medium.

3. ***Genetic Variability:***

When micro propagation is carried out through shoot tip cultures, genetic variability is very low. However, use of adventitious shoots is often associated with pronounced genetic variability.

4. ***Vitrification:***

During the course of repeated in vitro shoot multiplication, the cultures exhibit water soaked or almosttranslucent leaves. Such shoots cannot grow and even may die. This phenomenon is referred to as vitrification. Vitrification may be prevented by increasing the agar concentration (from 0.6 to 1%) in the medium. However, increased agar concentration reduces the growth rate of tissues.

5. ***Cost Factor:***

For some micro propagation techniques, expensive equipment, sophisticated facilities and trained manpower are needed. This limits its use.

Production of virus free plants:

Viruses are very small (submicroscopic) infectious particles (virions) composed of a protein coat and a nucleic acid core. They carry genetic information encoded in their nucleic acid, which typically specifies two or more proteins. Translation of the genome (to produce proteins) or transcription and replication (to produce more nucleic acid) takes place within the host cell and uses some of the host's biochemical "machinery".

Viruses cause many important plant diseases and are responsible for huge losses in crop production and quality in all parts of the world. Infected plants may show a range of symptoms ranging from leaf yellowing (either of the whole leaf or in a pattern of stripes or blotches), leaf distortion (e.g. curling) and/or other growth distortions (e.g. stunting of the whole plant, abnormalities in flower or fruit formation). Viral diseases occur in virtually all seed propagated crops.

There is no commercially available treatment to cure virus-infected plants.

To produce disease free plants a healthy nucleus stock is needed. However, when the whole population is infected then the only way to obtain a pathogen free plant is through tissue culture.

Virus replication is unable to keep pace with cell proliferation.

Apical meristems in the infected plants are generally either free or carry a very low concentration of the viruses. However, the titer of the viruses increases in the older tissues corresponding to the increase in the distance from the meristem tips.

Reasons attributed to the escape of the meristems by virus invasions are –

- Viruses **move rapidly in a plant body** through the vascular system which in meristems isabsent.
- A high **metabolic activity** in the in the actively dividing meristematic cells does not allowvirus replication.
- A **high endogenous auxin** level in shoot apices may inhibit virus multiplication.
- A meristem-tip culture has also enabled plants to be freed from other pathogens includingviroids, mycoplasmas, bacteria and fungi.

Methods of Virus Elimination:

A general term 'virus free' is used by commercial horticulturists for plants free of any type of virus. Virus free material can be re-infected if proper precautionary measures are not adopted.

1. Heat treatment
2. Meristem-tip culture
3. Chemical treatment
4. Other in vitro methods

1. Heat treatment

Before the advent of the meristem cultures the in vivo eradication of viruses from plants can be achieved by **heat treatment (thermotherapy)** of whole plants.

At temperature higher than optimum for many viruses in plant tissues are partially or completely inactivated with little or no injury to the host tissues. Heat treatment is given through hot water or hotair.

The hot water treatment effectively eliminates viruses in dormant buds, where as hot air treatment is recommended for elimination of viruses from actively growing shoots. The survival rate of the host tissue is better in hot air treatment.

Hot Air Treatment

I. Temperature: **35-40°C [few minutes to several months]**
II. Adequate supply of **humidity and light**
III. Plants must have **ample carbohydrate** reserves to withstand the heat treatment
IV. The **temperature** of the air should be **gradually raised** during the first few days until thedesired temperature is raised.
V. **Small cuttings are taken** from the shoot tips **immediately** after heat treatment and graftedonto healthy rootstocks.
VI. Generally the **percentage** of plants that survive after heat treatment is **small.**

2. Meristem-tip culture

For purposes of virus elimination the chance are better if culture are initiated with shoot tips ofsmaller size comprising mostly meristematic cell.

Apical meristem: The portion of the shoot lying distal to the youngest leaf primordium measuring upto about 100 mm in diameter and 250 mm in length.

Shoot apex: The apical meristem together with one to three young leaf primordia measuring 100-500mm.

In most published work explants of larger size (100-1000 mm long) have been cultured to raisevirus free plants.

Under a stereoscopic microscope (8-40 magnification) with a suitable light source explants are placedon a Petri plate lined with a sterile moist filter paper to avoid desiccation.

Meristem tips taken from terminal buds observed to give better result than those from axillary buds. The percentage of virus free plants can also depend on season, especially with crops which displayperiodic growth.

Although the apical meristems are often virus free, there may be exceptions. Some viruses are knownto actually invade the meristimatic region of the growing tips in certain plants. i.e. TMV. In such cases it has also been possible to obtain virus free plants by combining meristem tip culture with thermotherapy. The duration of heat treatment has to be decided carefully since excessive exposure can damage the plant tissues. To avoid deterioration of meristem tip cultures by continuous exposure to high temperature, the treatment with diurnal or daily cycle of high and low temperature can be tried. i.e. CMV in tissue cultures of *Nicotina rustica* and *Stellaria indica* can be inactivated by following the diurnal cycles of 40°C (16hrs) and then 22°C (8 hrs) per day.

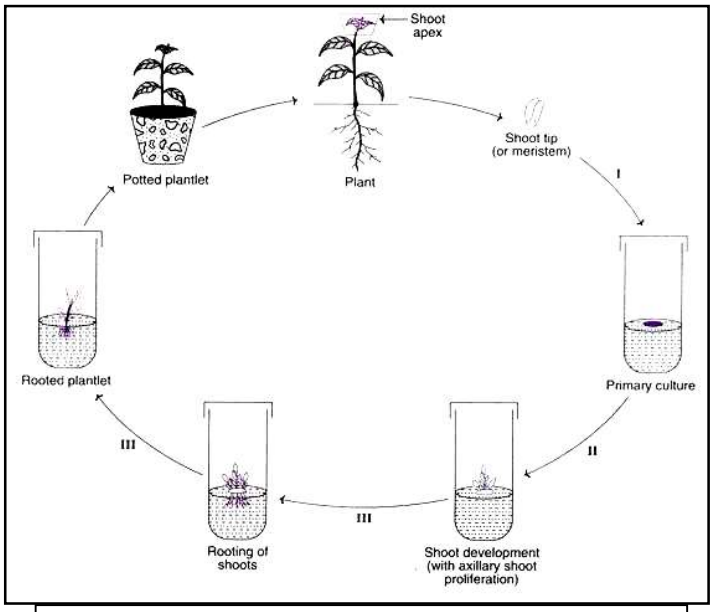


Fig.: A diagrammatic representation of shoot tip (or meristem) culture in micropropagation

Meristem-tip cultures are influenced by the following factors:

i. **Physiological condition of the explant** — actively growing buds are more effective.
ii. **Thermotherapy prior to meristem-tip culture** — for certain plants (possessing viruses in the meristematic regions), heat treatment is first given and then the meristem-tips are isolated and cultured.

iii. **Culture medium —**MS medium with low concentrations of auxins and cytokinins is ideal.

A selected list of the plants from which viruses have been eliminated by meristem cultures is given in Table.

Table: A selected list of the plants with virus elimination by meristem cultures

Plant species	*Virus eliminated*
Solanum tuberosum (potato)	Leaf roll, potato viruses — A, X, Y, S
Nicotiana tabacum (tobacco)	Tobacco mosaic virus
Saccharum officinarum (sugar cane)	Mosaic virus
Allium sativum (garlic)	Mosaic virus
Anenas sativus (pineapple)	Mosaic virus
Brassica oleracea (cauliflower)	Cauliflower/mosaic virus turnip mosaic virus
Ipomoea batata (sweet potato)	Fealthery mottle virus
Ribes grassularia	Vein banding virus
Humulus lupulus	Hop latent virus
Armoracia rusticena	Turnip mosaic virus
Musa sp (Banana)	Cucumber mosaic virus
Hycinthus sp	Hycinth mosaic virus
Dahlia sp	Dahlia mosaic virus
Chrysanthemum sp	Virus B
Petunia sp	Tobacco mosaic virus
Iris sp	Iris mosaic virus
Cymbidium sp	Cymbidium mosaic virus
Fragaria sp	Pallidosis virus, yellow virus complex
Freesia sp	Freesia mosaic virus

3. Chemical treatment

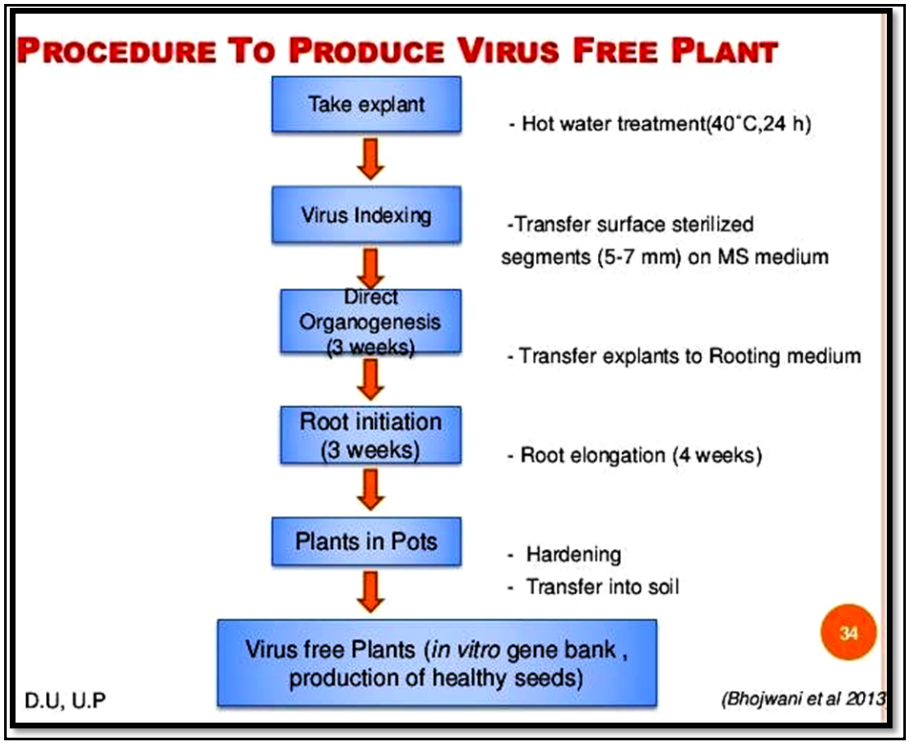
In the absence of effective therapeutic chemicals capable of eradicating virus from infected plants, there are reports of some attempts to suppress viruses in plant tissue and protoplast by the addition of some chemicals in the media. In some instances virus multiplication was suppressed by **the addition of cytokinins and other growth substances** while in others it was actually stimulated. Antimetabolites **malachite green, thiouraciol and acetylsalicylic acid** had little or a limited effect on virus elimination in meristem-tip regenerated plants, but the incorporation of nucleoside analog **rivavirin** (PVX,CMV, alfalfa mosic virus), **vidarbin, cyclohexamide** and **actinomycin-D** inhbit virus replication in plant protoplasts**.**

4. Cryotherapy

Cryotherapy is the prolonged exposure to a low temperature followed by shoot tip culture. It is a good method of virus elimination. Shoot tip cultures from *Chrysanthemum* plants treated with 5°C for 4 months yielded 67% plants free from *Chrysanthemum* stunt virus (CSV) and 22% plants free from *Chrysanthemum* chlorotic motile virus (CCMV). Cold treatment is preferable to heat treatment as it is less injurious to the plants and often more effective in virus elimination.

5. Electrotherapy

Electrotherapy assays were carried out either on infected in vivo or in vitro plants. Pulses of 15 V were applied for 5 min to 2- 3 cm long explants containing apical meristem. The meristems were then excised and placed on an MS culture medium. The efficiency of electrotherapy in producing virus- free regenerants from BSV-infected banana plants (cv. *W. Bungulan*) is 40-80 %.



Virus free assessment methods:

Tissue culture offers great promise for the production of quality planting material on account of disease free plants produced through meristem culture. But all the plants obtained through meristem culture with or without the therapeutic treatments are not virus free. Testing plants for the presence or absence of viruses is known as virus indexing. Every meristem tip or callus derived plant must be tested before using it as a mother plant to produce virus free stock. The three methods which are followed for virus indexing are sap transmission test, serology and EM examination.

Sap transmission test

It is done by taking the saps (filtered leaf extract) from test plants and which may be used to inoculate highly sensitive and healthy indicator plants. An Indicator plant for a virus is that plant species or variety which is highly susceptible to the virus and readily develops the symptoms. The inoculated indicator plants are maintained in a green house or aphid- proof cages separated from each other and from other plants. Sap transmission test is the most sensitive test among the three methods and can be easily performed on a commercial scale.

Serological test

It is a highly sensitive and precise technique for virus indexing employing antibodies specific to the concerned viruses. This test is performed by adding a drop of centrifuged sap from a test plant to a drop of antiserum taken from the blood of a rabbit. If the virus is present, the precipitation will take place due to the presence of specific antibodies in the blood. The ELISA (enzyme linked immunosorbent assay) is one of the serological methods used to identify viruses based on antibody reaction. ELISA is the most convenient, rapid and efficient test especially when a large number of samples are to be handled.

EM (Electron microscopy) examinations

These are particularly useful for identifying latent viruses (viruses those exhibit no visible symptoms). This method is not usually implemented as specialized equipment and trained personnel are required to carry out EM studies. Immunosorbent Electrom Microscopy (ISEM) described combines both serology and EM studies for detection of viruse.

Genetic assessment by RAPD and ISSR markers:

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generall determined by the technology that is used to reveal DNA-based polymorphism. Currently, the restriction fragment lengthpolymorphism (RFLP) assay has been the choice for many species to measure genetic diversity and construct a genetic linkage map. However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridisation, is, in general, time consuming and laborious. Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led to the development of several novel genetic assays based on selective amplification of DNA. This popularity of PCR is primarily due to its apparent simplicity and high probability of success.

Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes.

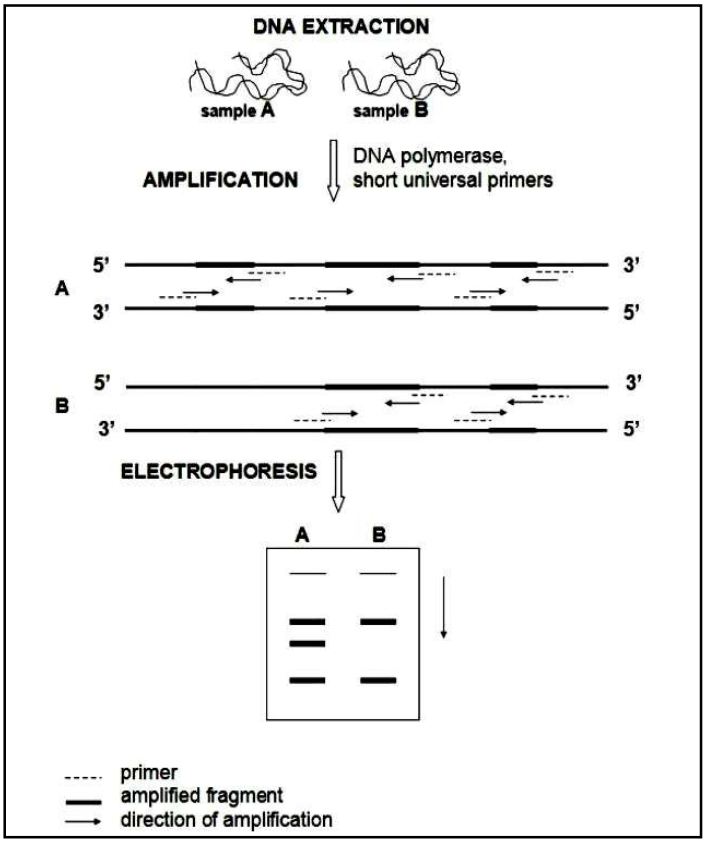
Randomly amplified polymorphic DNA markers (RAPD):

RAPD markers detect nucleotide sequence polymorphisms between individuals by employing a single, short (decamer), random oligonucleotide primers. DNA polymorphisms are detected due to thechanges of nucleotide at or between oligonucleotide primer binding sites in the genome. RAPD are dominant markers that can detect several loci in a single assay ranging from 0.5 to 5 kb. DNA fragments are fractionated by agarose gel electrophoresis and visualized with ethidium bromide (EtBr) that scores differences in the amplification pattern by their presence (+) and absence (-) at particular loci. Simplicity in methodology, no prior sequence information for primer designing, cost efficiency as well as no requirement radioactive probes has made RAPD technique a promising tool for identification of markers linked to agronomically important traits. However, inherent problems of reproducibility, sensitivity to experimental conditions, presence of artefactual bands (false positives) and inability to distinguish heterozygous from homozygous individuals makes them less preferential tool for genome wide studies.

RAPD primer	Sequence (5′-3′)	No. of amplification products
2	AGACGCGTAG	7
3	TGGACCCACA	3
4	GTGGCTTCTC	5
5	GCGCAGTATC	8
6	CCACCGTACT	6
7	TCCGGCTGTT	6
8	TGTCCCGTTG	7
11	CACACGAGAC	7
12	CGTACACCAG	5

RAPD involves following steps:-

1. The DNA of a selected species isisolated.
2. An excess of selecteddecaoligonucleotide added.
3. This mixture is kept in PCRequipment and is subjected torepeated cycles of DNA denaturation-renaturation- DNAreplication.
4. During this process, the decaoligonucleotidenwill pair with the homologous sequence present atdifferent locations in the DNA.
5. DNA replication extends the decaoligonucleotide and copy the sequence continuous with the sequence with which the selectedoligonucleotide has paired.
6. The repeated cycles of denaturation -renaturation-DNA replication will amplify this sequence of DNA.
7. Amplification will takes place only of those regions of the genome that has the sequence complementary to the decaoligonucleotide at their both ends.
8. After several cycles of amplification theDNA is subjected to gel electrophoresis.
9. The amplified DNA will form a distinct band. It is detected by ethidium bromide staining and visible fluorescence's under U.V. light.



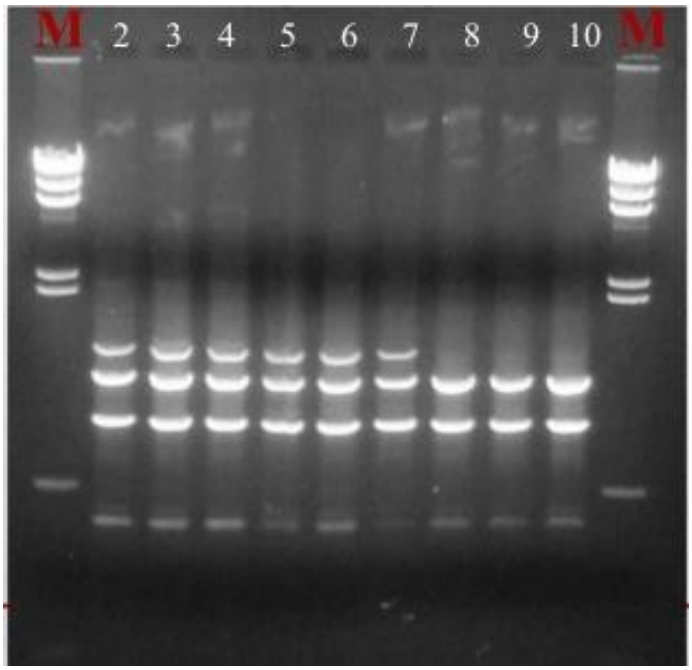


Fig.: Separated RAPD fragments

Advantages:

RAPD primers are readily available being universal. They provide moderately high genotyping throughput. This technique is simplePCR assay (no blotting and no radioactivity). It does not require special equipment. Only PCR is needed. The start-up cost is low. RAPD marker assays can be performed using very small DNA samples (5 to 25 ng per sample). RAPD primers are universal and can be commercially purchased. RAPD markers can beeasily shared between laboratories. Locus-specific, co-dominant PCR-based markers can be developed from RAPD markers. It provides more polymorphism than RFLPs.

Disadvantages:

The detection of polymorphism is limited. The maximum polymorphic information content for any bi- allelic marker is 0.5. This technique only detects dominant markers. The reproducibility of RAPD assays across laboratories is often low. The homology of fragments across genotypes cannot be ascertained without mapping. It is not applicable in marker assisted breeding programme.

Uses:

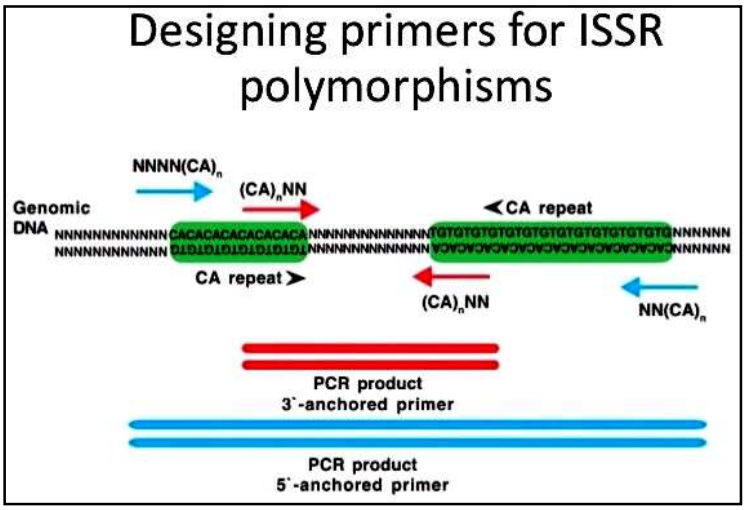
This technique can be used in various ways such as for varietal identification, DNA fingerprinting, gene tagging and construction of linkage maps. It can also be used to study phylogenetic relationship among species and sub-species and assessment of variability in breeding populations.

Inter simple sequence repeat (ISSR):

ISSR involves the amplification of DNA fragments flanked by inversely oriented SSR-microsatellites. The DNA polymorphism depends on the abundance and hypervariability of microsatellites in the genome. This technique uses microsatellites consisting of di-, tri-, tetra- or penta-nucleotides as primers complementary to microsatellite regions in the genome and amplify intersimple sequence repeats of different sizes. Depending on the usage ISSR primers can be either unanchored or anchored at 3' or 5' end with 1–4 degenerate bases extended into the flanking sequences. Primer extension with 1–4 degenerate nucleotide at 3' or 5' end

avoids internal priming and smear formation. The size of the amplified products varies between 200 and 2000 bp and can be separated using both agarose and polyacrylamide gel electrophoresis. The technique issimple, quick and need no sequence information for primer synthesis. ISSRs use longer semi-arbitrary SSR primers (15–30 mers) as compared to RAPD primers (10 mers), which allow the subsequent use of high annealing temperature leading to higher stringency and greater band reproducibility. However, dominant inheritance and homoplasy are the main limitations of ISSRs.

During the last twenty years, tissue culture and molecular biology have experienced a dynamic synergism. While tissue culture has provided model systems for molecular biology studies, molecular biology techniques have been applied to address limitations of tissue culture systems. Molecular marker systems have investigated genetic stability or somaclonal variation of plants after long term preservation or cryopreservation under in vitro conditions, and also of plants coming from long term culture conditions or following a large number of subcultures. Quality control of tissue cultured plants, molecular breeding in in vitro conditions and determining the genes involved in tissue culture responsiveness are other areas of usage of molecular markers.



RAPD and ISSR markers have been extensively applied to serve the purposes in the following areas:

- ***Determination of genetic stability or detection of somaclonal variation***

Somaclonal variation is the variation observed among plants regenerated from in vitro culture. These variations are heritable, i.e., transmitted through meiosis, and are usually irreversible. Many factors such as plant growth regulator balance (auxin and cytokinin concentration), culture duration (subculture number), macro and micro elements used in in vitro culture, and physiological stress induce somaclonal variation under in vitro conditionsThe source of explants and their pattern of regeneration are known to play major roles in determining the extent of genetic or somatic variation. Plants regenerated from adventitious buds around axillary buds, or from other well developed meristematic tissue, show the lowest tendency for genetic variation, whereas more changes are detected in plants derived from callus compared with those coming from embryogenic tissue.

- ***Genetic stability of plants after long term in vitro conservation and cryopreservation***

Germplasm preservation is essential to maintain biodiversity and avoid genetic erosion. In vitro culture may provide an alternative to standard methods for the conservation of many woody plants. Genetic conservation is based on the assumption that the material is conserved under conditions ensuring genetic stability. However, many factors associated with in vitro culture conservation procedures may result in somatic variation. It is essential to observe genetic stability during in vitro conservations. Currently, RAPD and ISSR markers are being increasingly used for monitoring genetic stability of germplasm during their long-term conservation.

- ***Genetic stability of plants after long subculture***

In some plants the variable number of proliferation cycles increases the occurrence of the somaclonal variation, mainly, due to chromosomal abnormalities.

- ***Molecular markers for quality control of in vitro plants***

The micropropagation industry is expanding and somaclonal variation and bacterial and viral contamination is becoming a problem for commercial propagators of a genotype. It is necessary that the plant material produced through tissue culture be certified before being

distributed to growers. Quality control of most micropropagated plants is being ensured using molecular markers (RAPD and ISSR) and DNA fingerprinting techniques. Polymorphism obtained in the progenies of tissue culture plants in comparison to the control mother explant source could be correlated with the apparent morphological changes. Although this is very time consuming, once done this will give an ideal marker system. These markers will further enhance the ability to understand the effect of different variables on the production of somaclonal variation in plants regenerated from tissue culture.

- ***Developing molecular markers linked to a trait in question***

The identification of gene or genomic regions that influence agriculturally related traits is very important in DNA marker-trait association study. This provides the basis for marker assisted selection (MAS) in plant breeding. Random molecular markers (RAPD and ISSR) are traditionally applied to establish a genetic linkage with a phenotypic trait. Having a tight linkage with the phenotypic trait RAPD and ISSR markers are the method of choice in many cases.

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6. Fermentation Technology: application of fermentation; batch, fed batch & continuous cultures of microbes; Bioreactors: Principles & their design; microbial strain improvement.

Fermentation:

Fermentation is a metabolic process that produces chemical changes in organic substrates through the action of enzymes. In biochemistry, it is narrowly defined as the extraction of energy from carbohydrates in the absence of oxygen. In the context of food production, it may more broadly refer to any process in which the activity of microorganisms brings about a desirable change to a foodstuff or beverage. The science of fermentation is known as **zymology**.

In microorganisms, fermentation is the primary means of producing ATP by the degradation of organic nutrients anaerobically. Humans have used fermentation to produce foodstuffs and beverages since the Neolithic age. For example, fermentation is used for preservation in a process that produces lactic acid found in such sour foods as pickled cucumbers, kimchi, and yogurt, as well as for producing alcoholic beverages such as wine and beer**.** Fermentation also occurs within the gastrointestinal tracts of all animals, including humans.

Louis Pasteur in the 19th century used the term fermentation in a narrow sense to describe the changes brought about by yeasts and other microorganisms growing in the absence of air (anaerobically); he also recognized that ethyl alcohol and carbon dioxide are not the only products of fermentation.

Types of Fermentation:

Two types of fermentation are common:

a) Alcoholic Fermentation:

In this type, the pyruvic acid is first decarboxylated to acetaldehyde in the presence of enzyme carboxylase. Thiamine pyrophosphate (TPP) is required as co-factor.

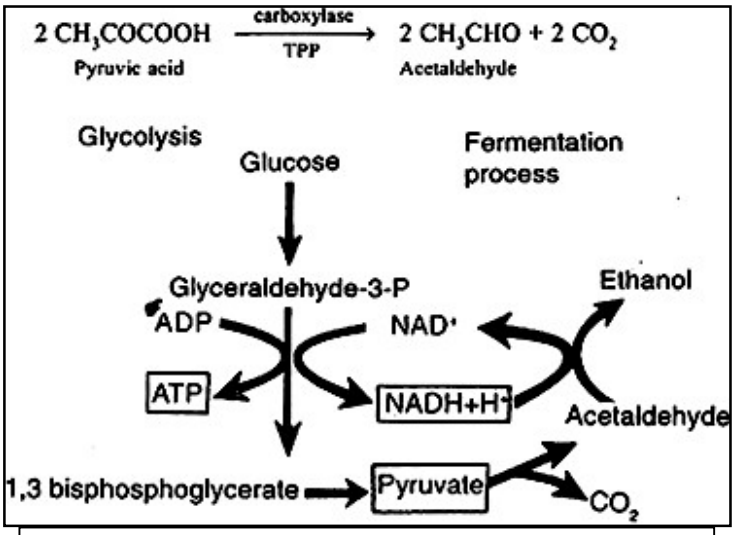
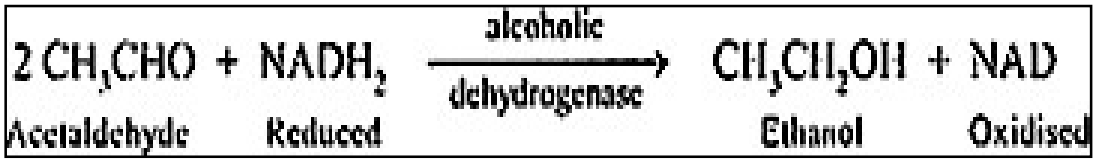
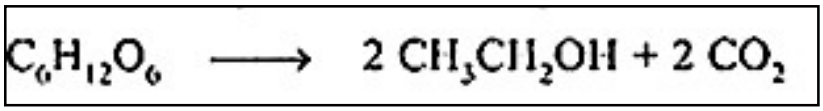


Fig.: Pathway of fermentation (anaerobic respiration) in yeast

Acetaldehyde is then reduced to ethanol (ethyl alcohol) by the enzyme alcohol dehydrogenase. Coenzyme $NADH_2$ (produced in glycolysis) is oxidised.

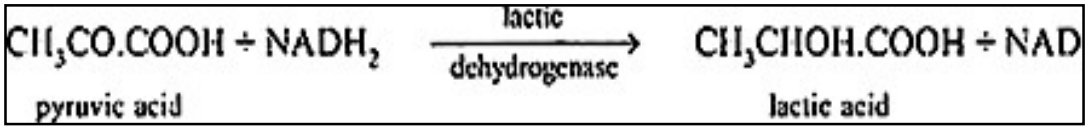


The overall equation for anaerobic respiration involving alcoholic fermentation is as follows –



b) Lactic acid Fermentation:

This type of fermentation occurs in lactic acid bacteria, certain fungi and muscles. Here, pyruvic acid is converted into lactic acid by the enzyme lactic dehydrogenase. Coenzyme NADH2, produced in glycolysis, is oxidised.



The overall equation for anaerobic respiration (lactic acid fermentation) is as follows –

There is net gain of only 2 ATP molecules (in glycolysis stage) during anaerobic respiration (orfermentation) and most of energy contained in glucose molecule is released as heat.

There are five major groups of commercially important fermentations –

(i) Those that produce microbial cells (or biomass) as the product.

(ii) Those that produce microbial enzymes.

(iii) Those that produce microbial metabolites.

(iv) Those that produce recombinant products.

(v) Those that modify a compound which is added to the fermentation the transformation process.

The component parts of a fermentation process are –

i. The formulation of media to be used in culturing the process organism during the development ofthe inoculum and in the production fermenter.
ii. The sterilization of the medium, fermenters and ancillary equipment.
iii. The production of an active, pure culture in sufficient quantity to inoculate the production vessel.
iv. The growth of the organism in the production fermenter under optimum conditions for productformation.
v. The extraction of the product and its purification.
vi. The disposal of effluents produced by the process.

Application of fermentation:

Many of the fermentive processes are of great importance in the household and industry. An example of the household is souring of milk or formation of curd. In the latter category are the alcoholic and acid fermentations.

The ripening of cheese, the retting of flax, sannhemp, ramie, and jute, the curing of tobacco and tea leaves, and the tanning of leather are some very important examples of commercial processes dependent upon fermentations brought about by different species of bacteria.

Alcoholic fermentation is used in brewing industry, where various types of beers, whisky and wines are produced, whereas CO_2 of alcoholic fermentation is used in baking industry for making bread and biscuits. Vinegar is produced by fermentation activity of acetic acid bacteria. Cleaning of raw hides is done by fermentive activity of bacteria.

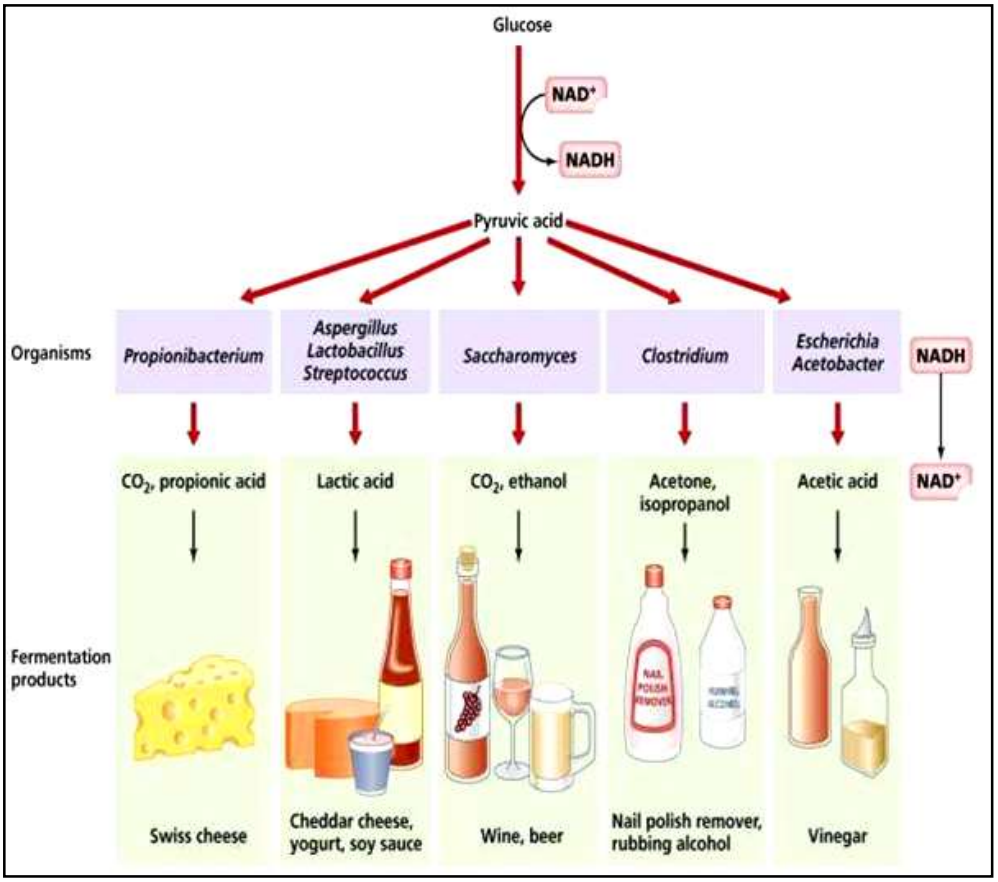
Products of Fermentation

Fermentation products include –

Food products – from milk (yogurt,kefir, fresh and ripened cheeses), fruits(wine, vinegar), vegetables (pickles,sauerkraut, soy sauce), meat(fermented sausages, salami)

Industrial chemicals – (solvents:acetone, butanol, ethanol, enzymes,amino acids)

Specialty chemicals – (vitamins,pharmaceuticals)



Antibiotics:

Antibiotic	**Produced by**	**Activity**	**Chemical nature**
Amphotericin B	*Sterptomyces nodosus*	antifungal	polyene
bacitracin	*Bacillus subtilis*	Gram +	peptide
Celphosporin C	*Cephalosporium acermonium*	Gram+	peptide
cycloheximide	*S.griseus*	antifugal	peptide

fungimycin	*S.coelicolor*	antifugal	polyene
gentamycin	*Micromonospora purpurea*	Gram+	aminoglyoside
gramicidin	*Bacillus brevis*	Gram+	peptide
sterptomycin	*S.grseus*	Gram+, mycobacteria	aminoglcoside
trichomycin	*S.hachijoensis*	antifugal	polyene

Enzymes:

Enzymes	**Source**	**Applications**	**Use in the form**
Alpha-amylase	*Bacillus licheniformis*	Hydrolysis of strach to dextrans	Extracelluler, soluble
Glucoamylase	*Aspergillus niger*	Dextran hydrolisis to glucose	Extracellular, soluble
Xylose isomerase	*Bacillus coagulans*	Pure glucose to equilibrium mixtureof glucose + fructose	Immobilized whole cell
Alkaline protease	*B.licheniformis*	detergent	Extracellular, soluble
Neutral protease	*B.subtilis*	Protien digestion in brewing sub	extracellular, soluble
Lipase	*A.niger*	Detergent, lipid	Extracellular, soluble

(i) Amino acid production

a) Lysine

b) Glutamic acid

(ii) Organic acids

c) Latic acid

d) Citric acid

e) Acidic acid

There are three different process of fermentation viz.:

1. Batch fermentation

2. Feb-batch fermentation and
3. Continuous culture

1. Batch fermentation:

This term is attributed to that type of fermentation wherein there is change in culture medium, number of microorganisms and the amount of the product produced (i.e. the metabolite or target protein). In batch fermentation six phases of the microbial growth are seen.

(a) Lag phase:

Immediately after inoculation, there is no increase in the numbers of the microbial cells for some timeand this period is called lag phase. This is in order that the organisms adjust to the new environment they are inoculated into.

(b) Acceleration phase:

The period when the cells just start increasing in numbers is known as acceleration phase.

(c) Log phase:

This is the time period when the cell numbers steadily increase.

(d) Deceleration phase:

The duration when the steady growth declines.

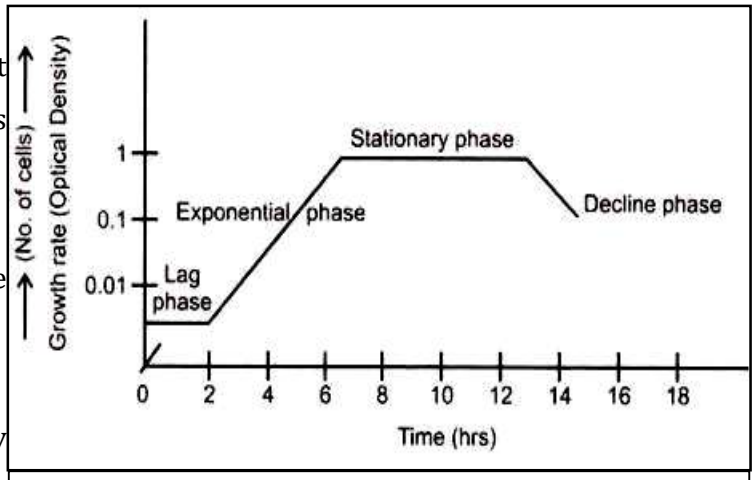


Fig.: Growth pattern of bacteria in batch culture

(e) Stationary phase:

The period where there is no change in the microbial cell number is the stationary phase. This phase is attained due to depletion of carbon source or accumulation of the end products.

(f) Death phase:

The period in which the cell numbers decrease steadily is the death phase. This is due to death of the cells because of cessation of metabolic activity and depletion of energy re- sources. Depending upon the product required the different phases of the cell growth are maintained. For microbial mass the log phase is preferred. For production of secondary metabolites i.e.

antibiotics, the stationary phase is preferred.

2. **Fed-batch culture:**

   - Fed-batch culture is also called as semi-closed system of cultivation.
   - In this technique, at first nutrient media is prepared and it is inoculated with culture organism and then incubated for particulate time.
   - During the course of incubation a particular nutrient is added at intervals without removing the used up media.so the volume of culture increases continuously.
   - Fed batch culture technique is applied in many types of fermentation process.
   - In fermentation some nutrient is very essential for the process but when these nutrients are provided in higher concentration in the culture they inhibit the growth of bacteria ultimately ceasing the fermentation. Therefore such nutrients are kept in lower concentration initially and it is added slowly and continuously during the course of fermentation.

Advantage:

Fed batch culture gives greater product yields than batch culture technique.

Disadvantage:

Chance of contamination of culture is higher in fed-batch than batch culture technique.

3. **Continuous culture technique:**

   - Continuous culture technique is also called as open system of cultivation.
   - In this technique fresh sterile medium is added continuously in the vessel and used up media with bacterial culture is removed continuously at the same rate. So the volume and bacterial density remain same in the cultivation vessel.
   - In this technique, bacteria grow continuously in their log phase. This type of growth is knownas steady state growth.
   - The cell density in continuous culture remains constant and it is achieved by maintaining constant dilution and flow rate.

Types of approach to continuous culture:

1. **Chemostat:**
    - ✓ It is the most common type of approach which controls the population density and growth of culture.
    - ✓ Two elements are used in chemostat, the dilution rate and concentration of limiting nutrient.
    - ✓ In continuous culture, endproducts do not accumulates and nutrients are not completely depleted, therefore bacteria never reach in stationary phase because fresh nutrients are supplied continuously and end products are removed continuously.
    - ✓ In chemostat, the liquid media contain some nutrient in growth limiting concentration and the concentration of limiting nutrient determines the rate of bacterial growth.
    - ✓ During steady state chemostat, concentration of limiting nutrient remains constantbecause the rate of addition of nutrient equals the rate at which it is used by organism plus flow through outlet.
    - ✓ To check whether there is constant cell density or not, concentration of that essential nutrient in the vessel is checked.
    - ✓ If the concentration of that nutrient is altered then it indicates bacterial density is changing. Therefore in this case flow rate is adjusted to maintain constant cell density.

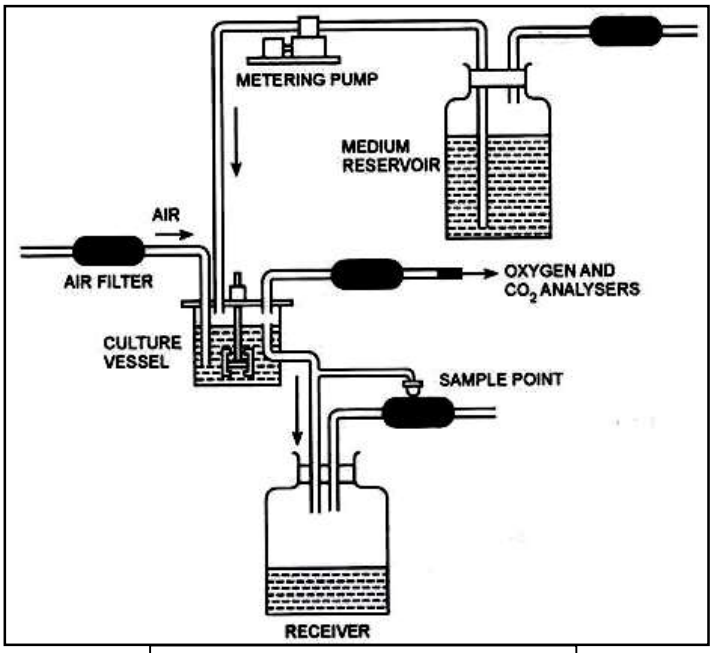


Fig.: The Chemostat

2. **Turbidostat:**
    - ✓ In turbidostat, a photoelectric device is used to monitor the cell density in the cultivation vessel.
    - ✓ The optical sensing device measures the turbidity (absorbance) of the culture in the vessel.
    - ✓ If concentration is altered, it is noticed by the photoelectric device and the flow rate is adjusted to maintain constant cell density in the culture.

Advantages of continuous fermentation:

- The size of the bioreactor and other equipment used in continuous fermentation are relativelysmaller compared to batch fermentation for the production of the same quantity of product.
- The yield of the product is more consistent since the physiological state of the cells is uniform.

- The ‘down time’ between two successive fermentations for cleaning and preparing the bioreactorfor reuse is avoided in continuous fermentation.
- Continuous fermentation can be run in a cost-effective manner.

Disadvantages of continuous fermentation:

Despite many advantages of continuous fermentation (described above), it is not very widely used inindustries. Some of the drawbacks are listed –

- Continuous fermentation may run continuously for a period of 500 to 1,000 hours. Maintenance ofsterile conditions for such a long period is difficult.
- The recombinant cells with plasmid constructs cannot function continuously and therefore theproduct yield decreases.
- It is not easy to maintain the same quality of the culture medium for all the additions. Nutrient variations will alter the growth and physiology of the cells, and consequently the product yield.

Bioreactors:

A bioreactor (fermentor) is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the waste biomass of cultured microorganisms along-with their products.

A fermentor is used for commercial production in fermentation industries and is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value. Fermentors are extensively used for food processing, fermentation, waste treatment, etc

Design of bioreactor:

All bioreactors deal with heterogeneous systems dealing with two or more phases, e.g., liquid, gas, solid. Therefore, optimal conditions for fermentation necessitate efficient transfer of mass, heat and momentum from one phase to the other. Chemical engineering principles are employed for design andoperation of bioreactors.

A bioreactor should provide for the following:

(i) Agitation (for mixing of cells and medium),

(ii) Aeration (aerobic bioreactor); for O_2 supply,

(iii) Regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, liquid level etc.,

(iv) Sterilization and maintenance of sterility, and

(v) Withdrawal of cells/medium (for continuous bioreactor).

Modern fermentors are usually integrated with computers for efficient process monitoring, data acquisition, etc.

Generally, 20-25% of fermentor volume is left unfilled with medium as "head space" to allow for splashing, foaming and aeration. The fermentor design varies greatly depending on the type and the fermentation for which it is used. Bioreactors are so designed that they provide the best possible growth and biosynthesis for industrially important cultures and allow ease of manipulation for all operations.

Size of Fermentors:

The size of fermentors ranges from 1-2 litre laboratory fementors to 5,00,000 litre or, occasionally, even more, fermentors of upto 1.2 million litres have been used. The size of the fermentor used depends on the process and how it is operated. A summary of fermentor or size of fermentor (litres)

Construction of Fermentors:

Industrial fermentors can be divided into two major classes, anaerobic and aerobic. Anaerobic fermentors require little special equipment except for removal of heat generated during the fermentation process, whereas aerobic fermentors require much more elaborate equipment to ensure that mixing and adequate aeration are achieved.

Since most industrial fermentation processes are aerobic, the construction of a typical aerobicfermentor is the following –

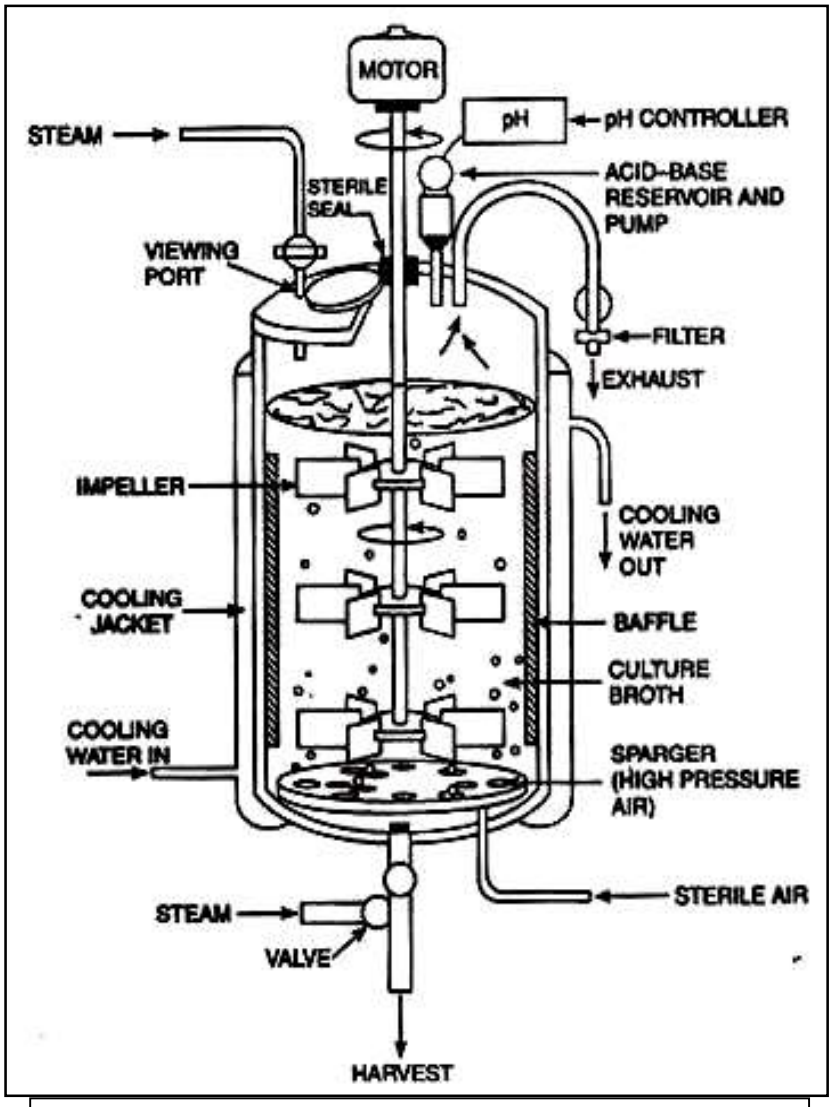


Fig.: An Industrial aerobic fermentor (Internal View)

1.Cooling Jacket:

Large-scale industrial fermentors are almost always constructed of stainless steel. A fermentor is a large cylinder closed at the top and the bottom and various pipes and valves are fitted into it. The fermentor is fitted externally with a cooling jacket through which steam (for sterilization) or cooling water (for cooling) is run.

Cooling jacket is necessary because sterilization of the nutrient medium and removal of the heat generated are obligatory for successful completion of the fermentation in the fermentor. For very large fermentors, insufficient heat transfer takes place through the jacket and therefore, internal coils are provided through which either steam or cooling water is run.

2. Aeration System:

Aeration system is one of the most critical part of a fermentor. In a fermentor with a high microbial population density, there is a tremendous oxygen demand by the culture, but oxygen being poorly soluble in water hardly transfers rapidly throughout the growth medium. It is necessary, therefore, that elaborate precautions are taken using a good aeration system to ensure proper aeration an oxygen availability throughout the culture. However, two separate aeration devices are used to ensure proper aeration in fermentor. These devices are sparger and impeller. The sparger is typically just a series of holes in a metal ring or a nozzle through which filter- sterilized air (or oxygen-enriched air) passes into the fermentor under high pressure. The air enters the fermentor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium. The impeller (also called agitator) is an agitating device necessary for stirring of the fermenter.The stirring accomplishes two things –

(i) It mixes the gas bubbles through the liquid culture medium and

(ii) It mixes the microbial cells through the liquid culture medium. In this way, the stirring ensuresuniform access of microbial cells to the nutrients.

The size and position of the impeller in the fermentor depends upon the size of the fermentor. In tall fermentors, more than one impeller is needed if adequate aeration and agitation is to be obtained. Ideally, the impeller should be 1/3 of the fermentors diameter fitted above the base of the fermentor. The number of impeller may vary from size to size to the fermentor.

3. Baffles:

The baffles are normally incorporated into fermentors of all sizes to prevent a vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the fermentors diameter and attached radially to the walls.

4. Controlling Devices for Environmental Factors:

In any microbial fermentation, it is necessary not only to measure growth and product formation but also to control the process by altering environmental parameters as the process

proceeds. For this purpose, various devices are used in a fermentor. Environmental factors that are frequently controlled includes temperature, oxygen concentration, pH, cells mass, levels of key nutrients, and product concentration.

Use of Computer in Fermentor:

Computer technology has produced a remarkable impact in fermentation work in recent years and the computers are used to model fermentation processes in industrial fermentors. Integration of computers into fermentation systems is based on the computers capacity for process monitoring, data acquisition,data storage, and error-detection.

Some typical, on-line data analysis functions include the acquisition measurements, verification of data, filtering, unit conversion, calculations of indirect measurements, differential integration calculations of estimated variables, data reduction, tabulation of results, graphical presentation of results, process stimulation and storage of data.

Types of Fermentor:

The fermentor (bioreactor) types used extensively in industries are the stirred tank fermentor, airlift fermentor, and bubble column fermentor.

1. **Stirred Tank Fermentor:**

Stirred tank fermentors consists of a cylindrical vessel with a motor driven central saft that supports one or more impellers.

2. **Airlift Fermentor:**

In airlift fermentor the liquid culture volume of the vessel is divided into two interconnected zones by means of a baffle or draft tube. Only one of the two zones is sparged with air or other gas and this sparged zone is known as the riser.

The other zone that receives no gas is called down- comer. The bulk density of the gas-liquid dispersion in the gas-sparged riser tends to be lower than the bulk density in the down-comer, consequently the dispersion flows up in the riser zone and down-flow occurs in the down- comer.

Airlift fermentors are highly energy-efficient and are often used in large-scale manufacture of biopharmaceutical proteins obtained from fragile animal cells. Heat and mass transfer

capabilities of airlift reactors are at least as good as those of other systems, and airlift reactors are more effective in suspending solids than are bubble column fermentors.

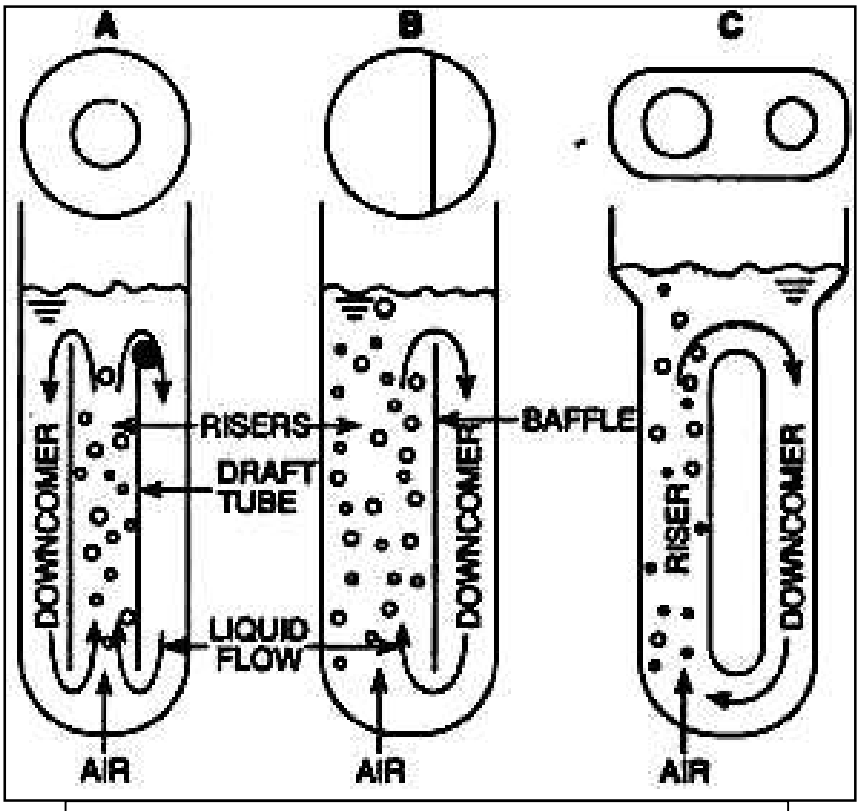


Fig.: Airlift fermentor. (A) Draft-tube Internal loop configuration, (B) a split cylinder device, and (c) an external loop device.

All performance characteristics of airlift -fermentor are related ultimately to the gas injection rate and the resulting rate of liquid circulation. Usually, the rate of liquid circulation increases with the square root of the height of the airlift device. Because the liquid circulation is driven by the gas hold-up difference between the riser and the down- comer, circulation is enhanced if there is little or no gas in the down-comer. All the gas in the down- comer comes from being entrained in with the liquid as it flows into the down-comer from the riser near the top of the reactor.

3. Bubble Column Fermentor:

A bubble column fermentor is usually cylindrical with an aspect (height- to- diameter) ratio of 4-6. Gas is sparged at the base of the column through perforated pipes, perforated plates, or

sintered glass or metal micro-porous spargers. O_2transfer, mixing and other performance factors are influenced mainly by the gas flow rate and the rheological properties of the fluid. Internal devices such as horizontal perforated plates, vertical baffles and corrugated sheet packing's may be placed in the vessel to improve mass transfer and modify the basic design. The column diameter does not affect its behaviour so long as the diameter exceeds 0.1 m. One exception is the axial mixing performance. For a given gas flow rate, the mixing improves with increasing vessel diameter. Mass and heat transfer and the prevailing shear rate increase as gas flow rate is increased.

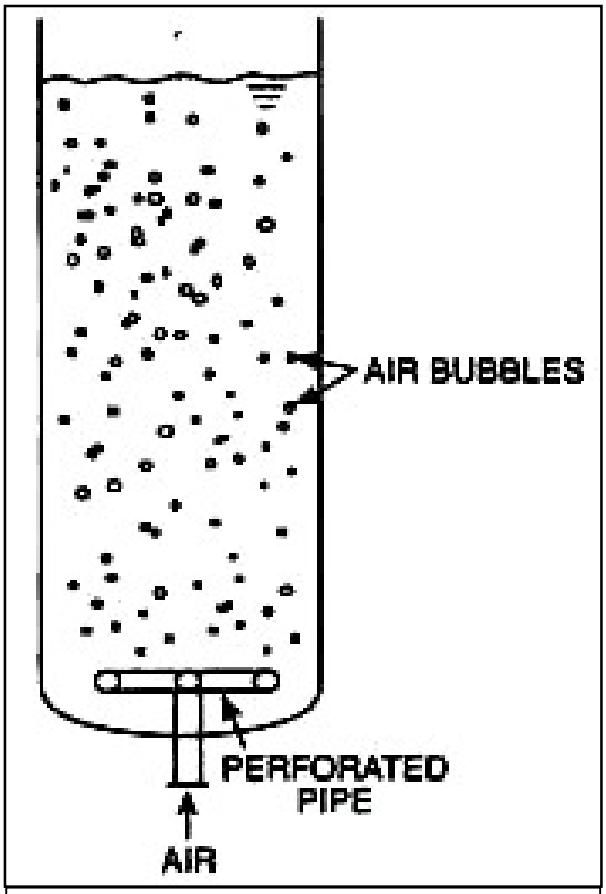


Fig.: A bubble column ferementor.

Microbial strain improvement:

Strain – A Strain is a group of species with one/ more characteristics that distinguish it from other sub groups of the same species of the strain. Each strain is identified by a name, number

or letter. Example:- *E.coli* Strain K12.

Strain Improvement- The Science and Technology of manipulating and improving microbial strains in order to enhance their metabolic capacities is known as Strain Improvement

Ideal Characteristics of Strain –

- Rapid growth
- Genetic stability
- Non-toxicity to humans
- Ability to use cheaper substrates
- Elimination of the production of compounds that may interfere with downstream processing
- To improve the use of carbon and nitrogen sources.
- Reduction of cultivation cost
- Shorter fermentation time.

Purpose of Strain Improvement –

- Increase the productivities
- Regulating the activity of the enzymes
- Introducing new genetic properties into the organism by Recombinant DNA technology /Genetic engineering.

1. Methods of Strain Improvement:

A mutant requiring oleic acid for neomycin formation by *Streptomyces fradiae* showed a decrease in the intracellular level of neomycin precursors in the mutant. On the other hand supersensitive mutants of β-lactam antibiotics are another example.

Recent approaches towards strain improvement are given below –

(i) Role of Plasmid:

Plasmid genes are involved in antibiotic production in *Streptomyces* sp. Although, plasmids are involved in genetic characteristics on curing experiments. Involvement of plasmids in biosynthesis of aureothricin and kasugamycin in Str. kasuaensis was demonstrated more than decades ago by Okanishi (1970). The genetic study using Str. venezuelae ISP 5230 a

chloramphenicol (CM) producer contains most of the structural genes for the CM biosynthetic steps treated between met and ilu on the chromosome and the plasmid played role in increasing CM production. A linear plasmid like DNA (pSLA2) of 11.2 × 106 dalton molecular weight from Streptomyces sp. produced antibiotics.

(ii) Protoplast Fusion:

Protoplast fusion is one of the useful techniques for obtaining hybrids or recombinants of different microorganism strains. Various studies have been carried out by using protoplast fusion in *Streptomyces*, *Saccharomyces* and fungi.

Protoplast formation in Sterptomyces was first reported by Okanishi and his team in the year 1966. Further, they have worked on formation stabilization and regeneration of protoplast of *Str. griseus* and *Str. venezuelae*. Fusion of yeast protoplasts has been reported with *Sacchromyces cerevisiae*. Technique for protoplast fusion in *Brevibacterium flavum*, has been used for strain improvement.

(iii) Mutation:

Screening after major subjection of a parent strain to physical or chemical mutagen greatly increased the probability of finding improved strain.

a) Major mutations:

It involves the selection of mutants with a pronounced change in a biochemical character of practical interest. Such variants are commonly used in genetic studies and are generally low mutants'.

They are isolated routinely from population surviving after prolonged exposure to a mutagen, for example, selection of non-pigmented *Penicillium chrysogenum* strains with high penicillin production. The initial strain of *Sterptomyces griseus* (a streptomycin producing organism) synthesized the small amount of streptomycin but its variant was isolated which produced greater amount of streptomycin.

For further improvement it is also necessary to study the biosynthetic pathways which contribute to the identification of precursors as in case of a modified tetracycline synthesized by a mutant strain of *Str. aureofacies*.

The molecule got changed at the C-5 position and was almost devoid of antibiotic activity. Another mutant strain S-604 synthesized 6-dimethyl tetracycline, a new antibiotic, not

elaborated by the parentstrains, proved to have several advantages. Today it is one of leading commercial forms of tetracycline.

b) Minor mutations:

It plays a dominant role in strains improvement. By definition such mutation affects only the amount of product synthesized. Such variants are usually phenotypically similar to the parent, with rapid and abundant mycelial and conidial development.

A 10 to 15% increase in conidial population exposed to moderate doses of a mutagen, obtained after repeated isolation of minor (positive) variants and using each succeeding strain for further mutation and selection Such increases have also been obtained by repeated selection without the introduction ofmutagen In this case, the population to be tested must be large and assay for the desired product also must be accurate and specific.

This technique fetched importance in improving P. chrysogenum. For example, Wisconsin series werethe famous Q-176 culture with significantly improved antibiotic titres, and strains BL3-D10, which does not produce the characteristic and trouble some chrysogenin pigment. All further mutant selections over the next decade were derived from Q-176.

2. Mutation Concept for Strain Development:

Strains selected as obvious variants after exposure to mutagen are usually inferior in their capacity for accumulation of antibiotic. Improvements are extremely few and their selection and evaluation is extremely important.

Mutagen dose is important. Mutants sought for major mutation rates are best isolated from populations surviving prolonged doses of mutagen, whereas variants for increased productivity are generally isolated from population surviving intermediates dose level.

Strains with enhanced altered morphology, etc. may be inherently better producers but may require considerable fermentation development. Step wise selection implies small increment in productivity, and the probability of getting hyper producing strains decreases. Variant strains may require special propogation and preservation procedures and actual production gains depend also on stability and reliability of performance. Though, strains may prove better in their productivity at laboratory scale, there is no guarantee that enhanced productivity will occur in production fermenters. The long term pilot plant studies are often

necessary before any enhanced strains potential can be realized in actual production.

3. Isolation of Mutant Classes and Their Use in Microbial Processes:

(i) Localized Mutagenesis and Computation:

Localized mutagenesis affecting the small selected regions of the chromosomes, offers a promising new approach. Mutation programmes can be directed to maximize mutations in any marked area on the chromosome, specially the areas known to affect the formation of end products. Isolation of strains in unknown loci linked to the revertant site can be done by a heterokaryon method or by the use of temperature sensitive mutants.

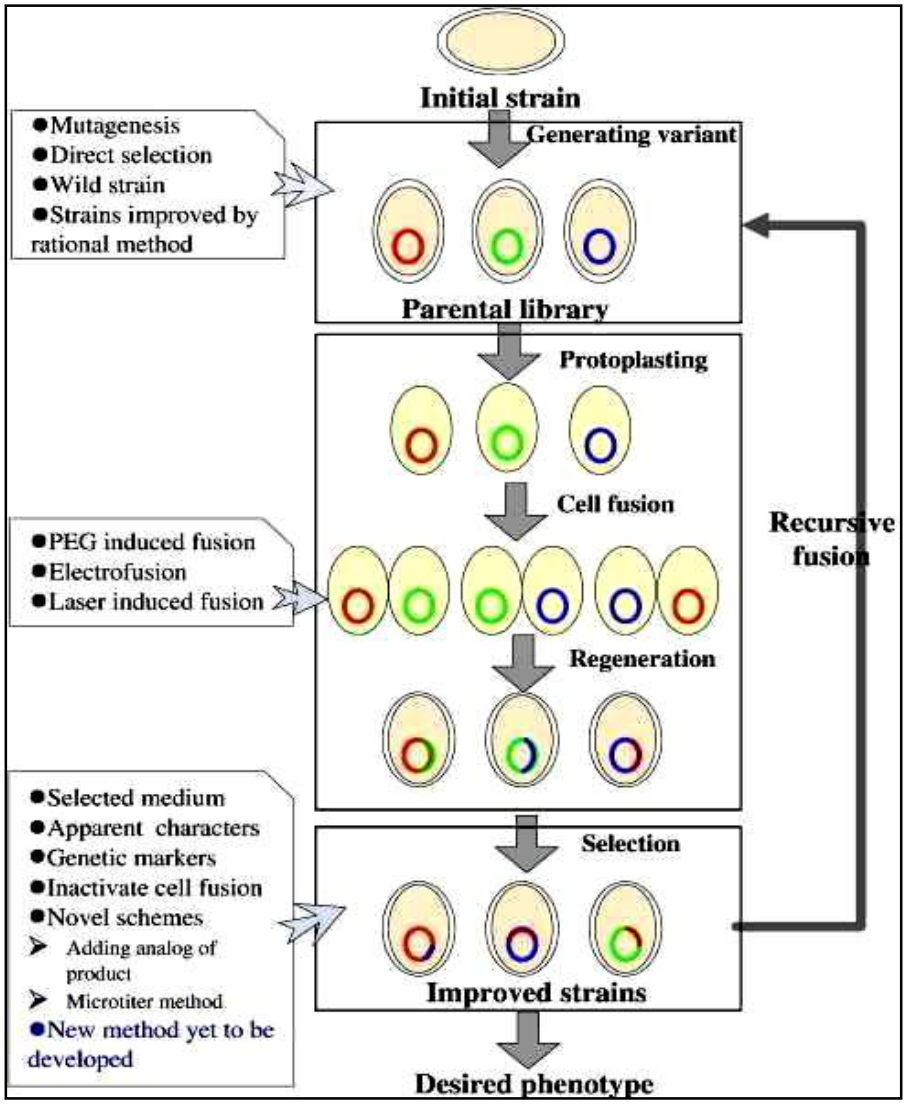
(ii) Sexual and Parasexual Processes:

In fungi, the vegetative mycelium is haploid and can be propogated almost indefinitely by serial transfer of hyphal fragments and can also be propogated by asexual spores/conidia.

Two strains of opposite mating types (A or B) are required to initiate the sexual cycle and allow to mate by mixing the conidia of mating type A with mycelia on appropriate media. After a period of nuclear division and migration, fusion between A and B nuclei takes place. Each fused nucleus (diploid) undergoes meiosis to form four haploids, which divide mitotically into eight nuclei contained in ascus.

Few of the industrially important fungi form heterokaryon in which rare diploid nuclei result from the fusion of two haploid nuclei. This process is called Parasexuality. Although, recombination in fewer fragments in the parasexual cycle compared to the meiotic process it can occur by mitotic crossing over or by other mechanism.

The importance of mitotic crossing over or recombination is that it makes possible genetic analysis and controlled breeding in organisms with no sexual cycle. Stram improvement through parasexual cycle has been reported in *P. chrysogenum* and in one study; a homozygous diploid representing parent was an efficient producer of penicillin V.



Recombinant proteins:

These are the proteins produced by the transferred gene / transgene; they themselves are of commercial value. Ex: Insulin, Interferons etc. are produced in Bacteria

Metabolic Engineering:

When metabolic activities of an organism are modified by introducing into it transgenes, which affect enzymatic, transport and/or regulatory function of its cells its known as

Metabolic Engineering. Ex: Over production of the amino acid Isoleucine in *Corynebacterium glutamicum* & Ethanol by *E.coli*.

Product Modification include the new enzymes which modifies the product of existing biosynthetic pathway e.g. Conversion of Cephalosporin C into 7-amino cephalosporanic acid by D-amino acidoxidase (in *A. chrysogenum*).

Completely new metabolite formation include in which all the genes of a new pathway are transferred e.g. *E. coli*, transfer of 2 genes for polyhydroxybutyrate synthesisfrom Alcaligenes eutrophus.

Enhance growth include enhanced substrate utilization. e.g. *E.coli*, glutamate dehydrogenase into *M.methylotrophus* carbon conversion increased from 4% to 7%.

Genome Shuffling:

It is a novel technique for strain improvement that allows for recombination between multiple parents at each generation and several rounds of recursive genome fusion were carried out resulting in the final improved strain involving genetic trait from m ultiple initial strains.

7. Immobilization of microbial enzymes & whole cells and their applications in industries.

Immobilization of enzymes:

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain theirstructural conformation necessary for catalysis.

There are several advantages of immobilized enzymes –

- ✓ Stable and more efficient in function.
- ✓ Can be reused again and again.
- ✓ Products are enzyme-free.
- ✓ Ideal for multi-enzyme reaction systems.
- ✓ Control of enzyme function is easy.
- ✓ Suitable for industrial and medical use.
- ✓ Minimize effluent disposal problems.

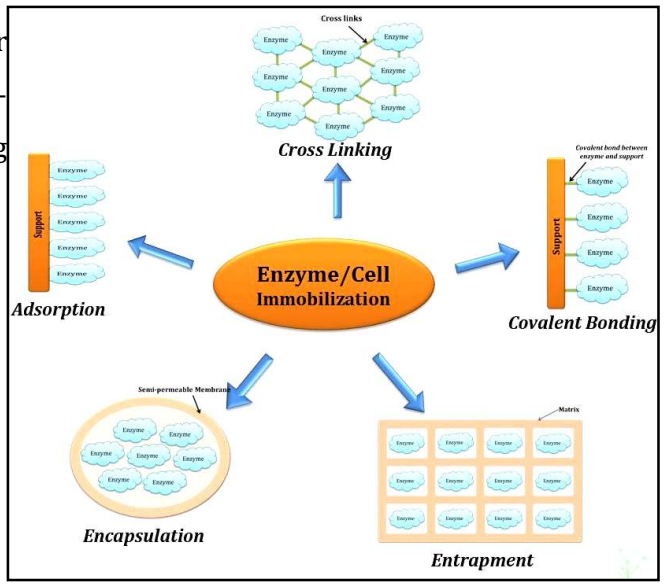
There are however, **certain disadvantages** also associated with immobilization.

- The possibility of loss of biological activity of an enzyme during immobilization or while it is inuse.
- Immobilization is an expensive affair often requiring sophisticated equipment.

Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

Methods of Immobilization:

The commonly employed techniques for immobilization of enzymes are—adsorption, entrapment, covalent binding and cross-linking.



Adsorption:

Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex). Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds. Therefore, the adsorbed enzymes can beeasily removed by minor changes in pH, ionic strength ortemperature. This is a disadvantage for industrial use of enzymes.

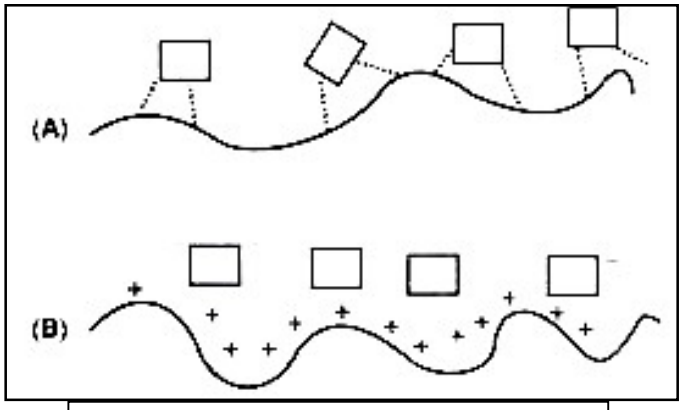


Fig.: Immobilization of enzymes by adsorption (A) By van der Waals forces (B) By hydrogen bonding

Entrapment:

Enzymes can be immobilized by physical entrapmentinside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.

Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways, viz –

1. Enzyme inclusion in gels: This is an entrapment of enzymes inside the gels.

2. Enzyme inclusion in fibres: The enzymes are trapped in a fibre format of the matrix.

3. Enzyme inclusion in microcapsules: In this case, the enzymes are trapped inside a microcapsule matrix. The hydrophobic and hydrophilic forms of the matrix polymerise to form a microcapsule containing enzyme molecules inside. The major limitation for entrapment of enzymes is their leakage from the matrix. Most workers prefer to use the technique of entrapment for immobilization of whole cells. Entrapped cells are in use for industrial production of amino acids (L-isoleucine, L-aspartic acid), L- malic acid and hydroquinone.

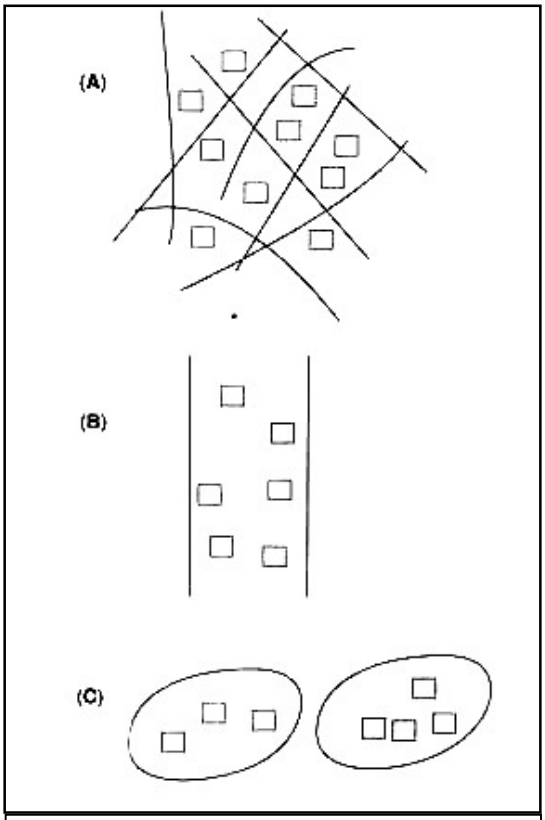


Fig.: Immobilization of enzymes by entrapment (A) Inclusion in gels (B) Inclusion in fibres (C) Inclusion in microcapsules

Microencapsulation:

Microencapsulation is a type of entrapment. It refers to the process of spherical particle

formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation, viz –

1. Building of special membrane reactors.
2. Formation of emulsions.
3. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Covalent Binding:

Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemicalgroups of enzymes and the chemical groups of the support. This technique is widely used. However, covalent binding is often associated with loss ofsome enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme.

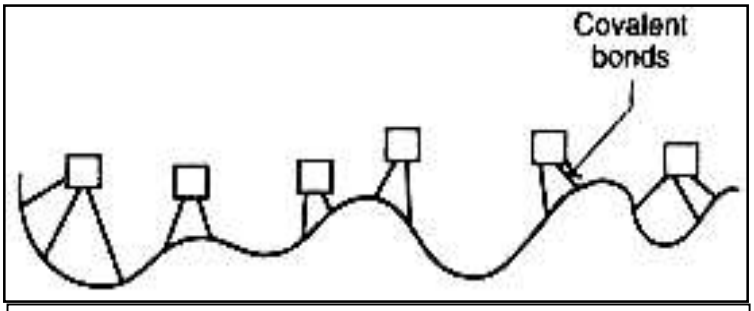


Fig.: A general representation of immobilization of enzymes by covalent binding

The following are the common methods of covalent binding –

1. Cyanogen bromide activation: The inert support materials (cellulose, sepharose, sephadex) containing glycol groups are activated by CNBr, which then bind to enzymes and immobilize them.

2. Diazotation: Some of the support materials (amino benzyl cellulose, amino derivatives of polystyrene, aminosilanized porous glass) are subjected to diazotation on treatment with $NaNO_2$ and HCI. They,in turn, bind covalently to tyrosyl or histidyl groups of enzymes.

3. Peptide bond formation: Enzyme immobilization can also beachieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and the carboxyl (or amino) groups of enzymes. The support material is first chemically treated to form active functional groups.

4. Activation by bi- or poly- functional reagents: Some of the reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support (e.g. aminoethylcellulose, albumin, amino alkylated porous glass).

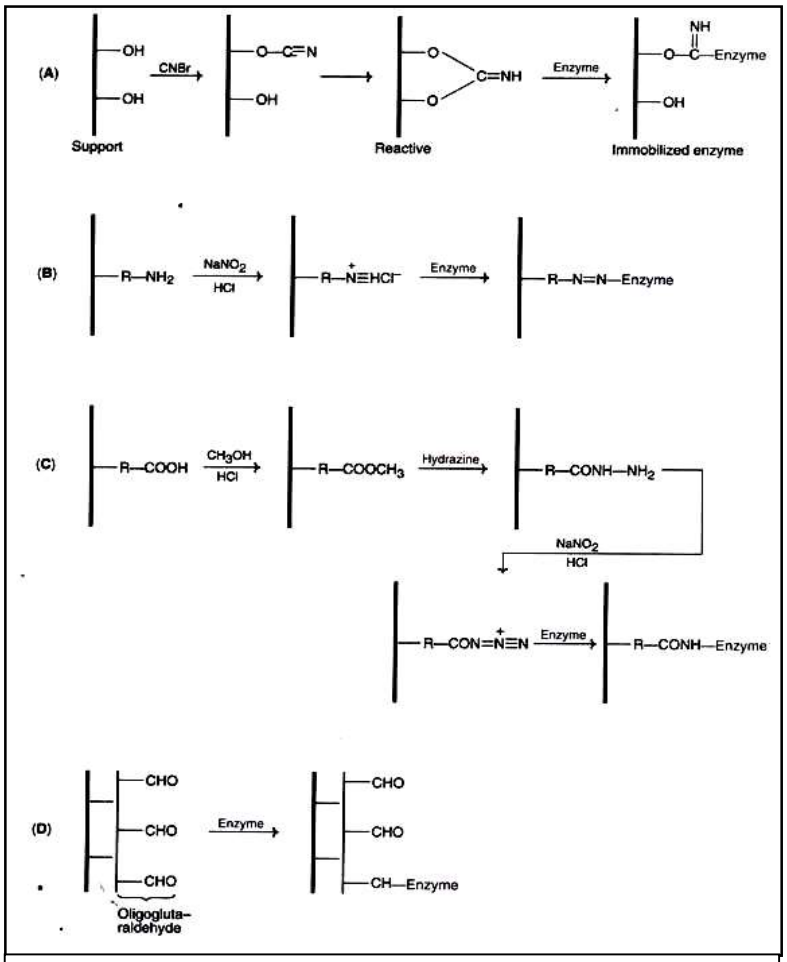


Fig.: Immobilization of enzymes by covalent binding (A) Cyanogen bromide activation (B) Diazotation (C) Peptide bond formation (D) Activation by

Cross-Linking:

The absence of a solid support is a characteristic feature of immobilization of enzymes by cross- linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules. There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene di-isocyanate and toluene di-isothiocyanate. Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross-linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the polyfunctional reagent.

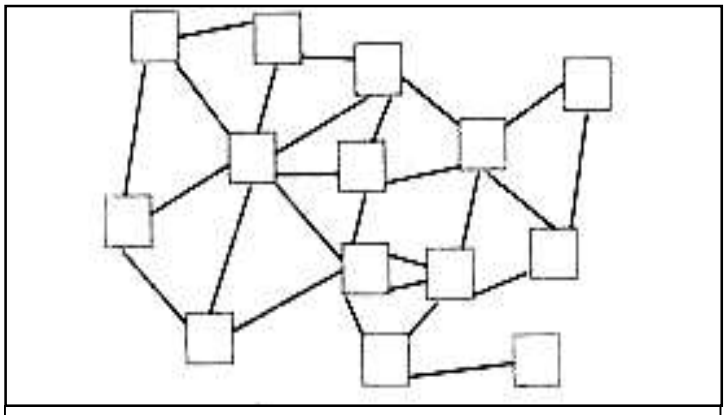


Fig.: Immobilization of enzymes by cross linking.

Choice of Immobilization Technique:

The selection of a particular method for immobilization of enzymes is based on a trial and error approach to choose the ideal one. Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.

Immobilization of L-amino acid acylase:

L-Amino acid acylase was the first enzyme to be immobilized by a group of Japanese workers (Chibata and Tosa, 1969). More than 40 different immobilization methods were attempted by this group. Only three of them were found be useful. They were covalentbinding to iodoacetyl cellulose, ionic binding to DEAE-Sephadex and entrapment within polyacrylamide.

Stabilization of Soluble Enzymes:

Some of the enzymes cannot be immobilized and they have to be used in soluble form e.g. enzymes used in liquid detergents, some diagnostic reagents and food additives. Such enzymes can be stabilized by using certain additives or by chemical modifications. The stabilized enzymes have longer half-lives, although they cannot be recycled. Some important methods of enzyme stabilization are briefly described.

Solvent Stabilization:

Certain solvents at low concentrations stabilize the enzymes, while at high concentrations the enzymes get denatured e.g. acetone (5%) and ethanol (5%) can stabilize benzyl alcohol dehydrogenase.

Substrate Stabilization:

The active site of an enzyme can be stabilized by adding substrates e.g. starch stabilizes a-amylase; glucose stabilizes glucose isomerase.

Stabilization by Polymers:

Enzymes can be stabilized, particularly against increased temperature, by addition of polymers such as gelatin, albumin and polyethylene glycol.

Stabilization by Salts:

Stability of metalloenzymes can be achieved by adding salts such as Ca, Fe, Mn, Cu and Zn
e.g. proteases can be stabilized by adding calcium.

Stabilization by Chemical Modifications:

Enzymes can be stabilized by suitable chemical modifications without loss of biological activity. There are several types of chemical modifications.

a. Addition of poly-amino side chains e.g. polytyrosine, polyglycine.
b. Acylation of enzymes by adding groups such as acetyl, propionyl and succinyl

Stabilization by Rebuilding:

Theoretically, the stability of the enzymes is due to hydrophobic interactions in the core of the enzyme. It is therefore, proposed that enzymes can be stabilized by enhancing hydrophobic interactions. For this purpose, the enzyme is first unfold and then rebuilt in one of the following ways –

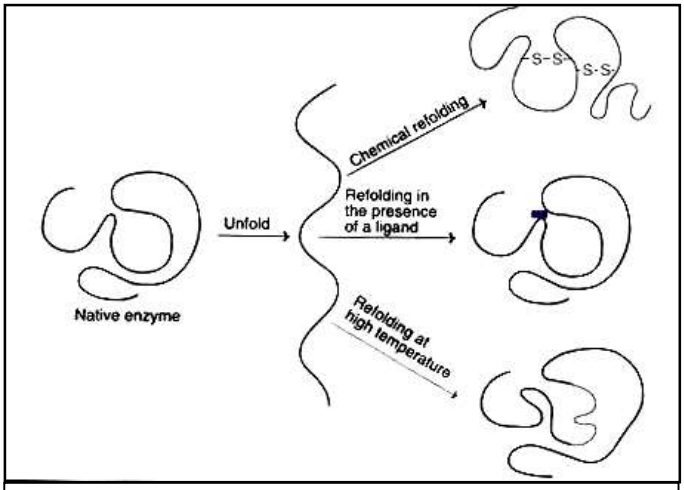


Fig.: Stabilization of an enzyme by refolding.

1. The enzyme can be chemically treated (e.g. urea and a disulfide) and then refolded.
2. The refolding can be done in the presence of low molecular weight ligands.
3. For certain enzymes, refolding at higher temperatures (around 50°C) stabilize them.

Stabilization by Site-Directed Mutagenesis:

Site-directed mutagenesis has been successfully used to produce more stable and functionally more efficient enzymes e.g. subtilisin E.

Immobilization of Cells:

Immobilized individual enzymes can be successfully used for single-step reactions. They are, however, not suitable for multi-enzyme reactions and for the reactions requiring cofactors. The whole cells or cellular organelles can be immobilized to serve as multi-enzyme systems,

In addition, immobilized cells rather than enzymes are sometimes preferred even for single reactions, due to cost factor in isolating enzymes. For the enzymes which depend on the special arrangement of the membrane, cell immobilization is preferred.

Immobilized cells have been traditionally used for the treatment of sewage. The techniques employed for immobilization of cells are almost the same as that used for immobilization of enzymes with appropriate modifications. Entrapment and surface attachment techniques are commonly used. Gels, and to some extent membranes, are also employed.

Immobilized Viable Cells:

The viability of the cells can be preserved by mild immobilization. Such immobilized cells are particularly useful for fermentations. Sometimes mammalian cell cultures are made to function as immobilized viable cells.

Immobilized Non-viable Cells:

In many instances, immobilized non-viable cells are preferred over the enzymes or even the viable cells. This is mainly because of the costly isolation and purification processes. The best example is the immobilization of cells containing glucose isomerase for the industrial production of high fructose syrup. Other important examples of microbial biocatalysts and their applications are given in Table.

Table: Selected examples of immobilized cells (to bring out one or two enzyme reactions) in industrial applications.

Immobilized microorganism (microbial biocatalyst)	*Application(s)*
Escherichia coli	For the synthesis of L-aspartic acid from fumaric acid and NH_3
Escherichia coli	For the production of L-tryptophan from indole and serine
Pseudomonas sp	Production of L-serine from glycine and methanol
Saccharomyces cerevisiae	Hydrolysis of sucrose
Saccharomyces sp	Large scale production of alcohol
Zymomonas mobilis	Synthesis of sorbitol and gluconic acid from glucose and fructose
Anthrobacter simplex	Synthesis of prednisolone from hydrocortisone
Pseudomonas chlororaphis	Production of acrylamide from acrylonitrile
Humicola sp	For the conversion of rifamycin B to rifamycin S
Bacteria and yeasts (several sp)	In biosensors

Limitations of Immobilizing Eukaryotic Cells:

Prokaryotic cells (particularly bacterial) are mainly used for immobilization. It is also possible to immobilize eukaryotic plant and animal cells. Due to the presence of cellular organelles, the metabolism of eukaryotic cells is slow. Thus, for the industrial production of biochemical, prokaryotic cells are preferred. However, for the production of complex proteins (e.g. immunoglobulin's) and for the proteins that undergo post- translational modifications, eukaryotic cells may be used.

Effect of Immobilization on Enzyme Properties:

Enzyme immobilization is frequently associated with alterations in enzyme properties, particularly thekinetic properties of enzymes. Some of them are listed below –

1. There is a substantial decrease in the enzyme specificity. This may be due to conformational changes that occur when the enzyme gets immobilized.
2. The kinetic constants Km and Vmax of an immobilized enzyme differ from that of the native enzyme. This is because the conformational change of the enzyme will affect the affinity between enzyme and substrate.

Immobilized Enzyme Reactors:

The immobilized enzymes cells are utilized in theindustrial processes in the form of enzyme reactors. They are broadly of two types — batch reactors and continuous reactors. Other types are stirred tank reactors and plug flow type reactors.

1. Batch Reactors:

In batch reactors, the immobilized enzymes and substrates are placed, and the reaction is allowed to take place under constant stirring. As the reaction is completed, the product is separated from the enzyme (usually by denaturation).

Soluble enzymes are commonly used in batch reactors. It is rather difficult to separate the soluble enzymes from the products; hence there is a limitationof their reuse. However, special techniques have been developed for recovery of soluble enzymes, although this may result in loss of enzyme activity.

2. Continuous Reactors:

In continuous enzyme reactors, the substrate is added continuously while the product is removed simultaneously. Immobilized enzymes can also be used for continuous operation. Continuous reactors have certain advantages over batch reactors. These include control over the product formation, convenient operation of the system and easy automation of the entire process. There are mainly two types of continuous reactors-continuous stirred tank reactor (CSTR) and plug reactor (PR). CSTR is ideal for good product formation.

3. Stirred tank reactors:

The simplest form of batch reactor is the stirred tank reactor. It is composed of a reactor fitted with a stirrerthat allows good mixing, and appropriate temperature and pH control. However, there may occur loss of some enzyme activity. A modification of stirred tank reactor is basket reactor. In this system, the enzyme is retained over the impeller blades. Both stirred tank reactor and basket reactor have a well-mixed flow pattern.

4. Plug flow type reactors:

These reactors are alternatives to flow pattern type of reactors. The flow rate of fluids controlled by a plug system. The plug flow type reactors may be in the form of packed bed or fluidized bed. These reactors are particularly useful when there occurs inadequate product formation in flow type reactors. Further, plug flow reactors are also useful for obtaining

kinetic data on the reaction systems.

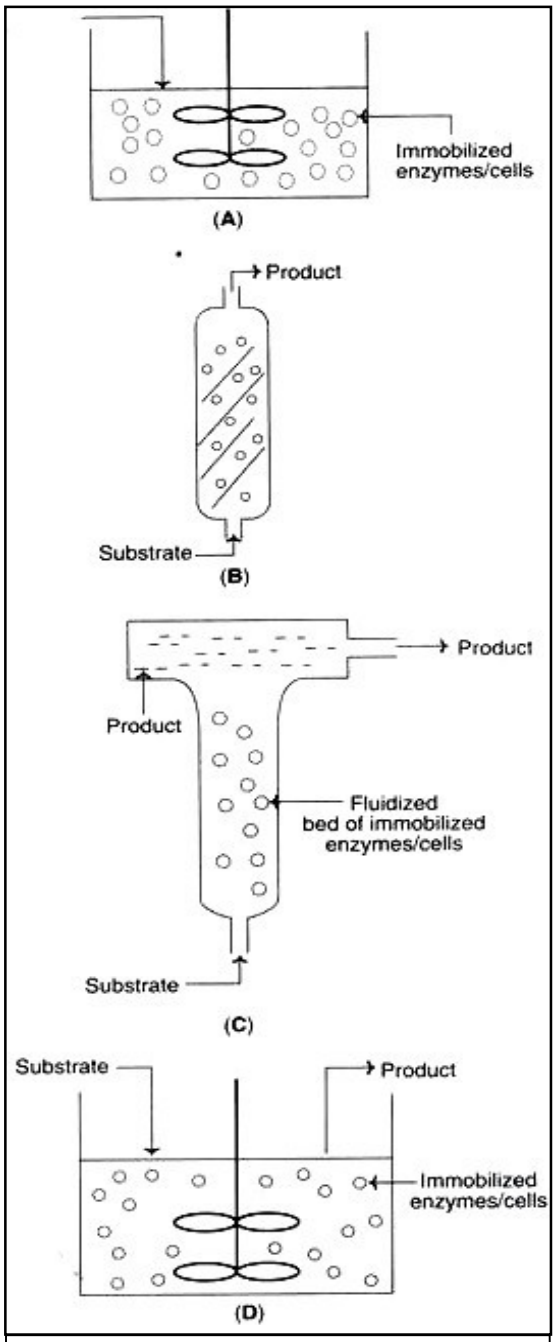


Fig.: Immobilized enzyme (cell) reactors (A) Batch stirred tank reactor, (B) Packed bed reactor, (C) Fluidized bed reactor, (D) Continuous stirred tank reactor.

Membrane Reactors:

Several membranes with a variety of chemical compositions can be used. The commonly used membrane materials include polysulfone, polyamide and cellulose acetate. The biocatalysts (enzymes or cells) are normally retained on the membranes of the reactor. The substrate is introduced into reactor while the product passes out. Good mixing in the reactor can be achieved by using stirrer. In a continuous membrane reactor, the biocatalysts are held over membrane layers on to which substrate molecules are passed.

In a recycle model membrane reactor, the contents (i.e. the solution containing enzymes, cofactors, and substrates along with freshly released product are recycled by using a pump. The product passes out which can be recovered.

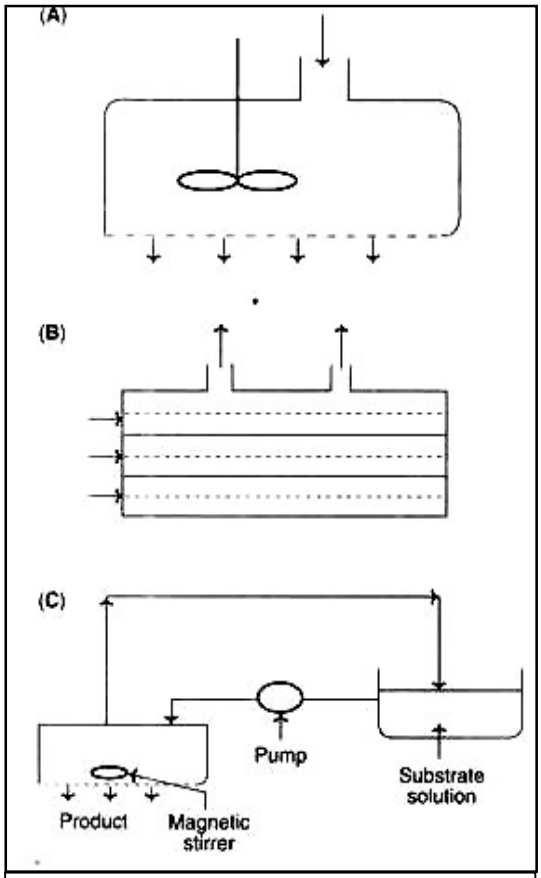


Fig.: Membrane reactors (A) Batch membrane reactor, (B) Continuous membrane reactor, (C) Recycle membrane reactor.

Applications of Immobilized Enzymes and Cells:

Immobilized enzymes and cells are very widely used for industrial, analytical and therapeutic purpose, besides their involvement in food production and exploring the knowledge of biochemistry, microbiology and other allied specialties. A brief account of the industrial applications of immobilized cells is given in Table.

Manufacture of Commercial Products:

A selected list of important immobilized enzymes and their industrial applications is given in Table.

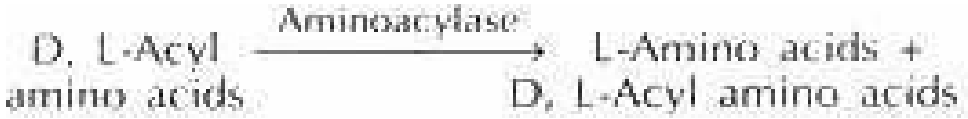
Table: A selected list of important immobilized enzymes and their industrial applications

Immobilized enzyme	*Application(s)*
Aminoacylase	Production of L-amino acids from D, L-acyl amino acids
Glucose isomerase	Production of high fructose syrup from glucose (or starch)
Amylase	Production of glucose from starch
Invertase	Splitting of sucrose to glucose and fructose
β-Galactosidase	Splitting of lactose to glucose and galactose
Penicillin acylase	Commercial production of semi-synthetic penicillins
Aspartase	Production of aspartic acid from fumaric acid
Fumarase	Synthesis of malic acid from fumaric acid
Histidine ammonia lyase	Production of urocanic acid from histidine
Ribonuclease	Synthesis of nucleotides from RNA
Nitrilase	Production of acrylamide from acrylonitrile

Some details on the manufacture of L-amino acids and high fructose syrup are given here under.

Production of L-Amino Acids:

L-Amino acids (and not D-amino acids) are very important for use in food and feed supplements and medical purposes. The chemical methods employed for their production result in a racemic mixture of D- and L-amino acids. They can be acylated to form D, L-acyl amino acids. The immobilized enzyme aminoacylase (frequently immobilized on DEAE sephadex) can selectively hydrolyse D, L-acylamino acids to produce L-amino acids



The free L-amino acids can separated from the un-hydrolysed D-acyl amino acids. The latter can be recemized to D, L-acyl amino acids and recycled through the enzyme reactor containing immobilized aminoacylase. Huge quantities of L-methionine, L-phenylalanine L-tryptophan and L-valine are produced worldwide by this approach.

Production of High Fructose Syrup:

Fructose is the sweetest among the monosaccharide's, and has twice the sweetening strength of sucrose. Glucose is about 75% as sweet as sucrose. Therefore, glucose (the most abundant monosaccharide) cannot be a good substitute for sucrose for sweetening. Thus, there is a great demand for fructose which is very sweet, but has the same calorific value as that of glucose or sucrose.

High fructose syrup (HFS) contains approximately equivalent amounts of glucose and fructose. HFS is almost similar to sucrose from nutritional point of view. HFS is a good substitute for sugar in the preparation of soft drinks, processed foods and baking

High fructose syrup can be produced from glucose by employing an immobilized enzyme glucose isomerase. The starch containing raw materials (wheat, potato, corn) are subjected to hydrolysis to produce glucose. Glucose isomerase then isomerizes glucose to fructose. The product formed is HFS containing about 50% fructose. (Note: Some authors use the term high fructose corn syrup i.e. HFCS in place of HFS).

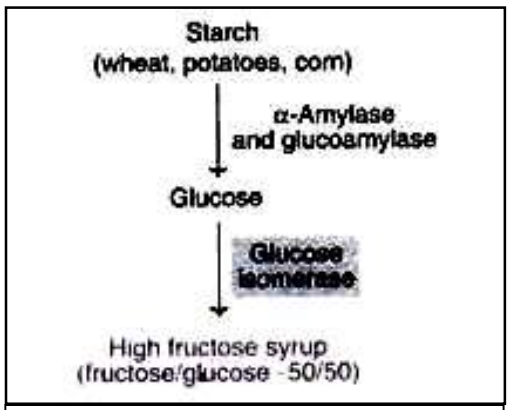


Fig.: Production of high fructose syrup from starch (glucose isomerase) is the immobilized enzyme.

Glucose isomerase: This is an intracellular enzyme produced by a number of microorganisms. The species of Arthrobacter, Bacillus and Streptomyces are the preferred sources. Being an intracellular enzyme, the isolation of glucose isomerase without loss of biological activity requires special and costly techniques. Many a times, whole cells or partly broken cells are immobilized and used.

Immobilized Enzymes and Cells- Analytical Applications:

In Biochemical Analysis –

Immobilized enzymes (or cells) can be used for the development of precise and specific analytical techniques for the estimation of several biochemical compounds. The principle of analytical assay primarily involves the action of the immobilized enzyme on the substrate.

A decrease in the substrate concentration or an increase in the product level or an alteration in the cofactor concentration can be used for the assay. A selected list of examples of immobilized enzymes used in the assay of some substances is given in Table.

Table: Selected examples of immobilized enzymes used in analytical biochemistry

Immobilized enzyme	*Substance assayed*
Glucose oxidase	Glucose
Urease	Urea
Cholesterol oxidase	Cholesterol
Lactate dehydrogenase	Lactate
Alcohol oxidase	Alcohol
Hexokinase	ATP
Galactose oxidase	Galactose
Penicillinase	Penicillin
Ascorbic acid oxidase	Ascorbic acid
L-Amino acid oxidase	L-Amino acids
Cephalosporinase	Cephalosporin
Monoamine oxidase	Monoamine

Two types of detector systems are commonly employed. Thermistors are heat measuring devices which can record the heat generated in an enzyme catalysed reaction. Electrode devices are used for measuring potential differences in the reaction system. In the Figure, an

enzyme thermistor and an enzyme electrode, along with a specific urease electrode are depicted.

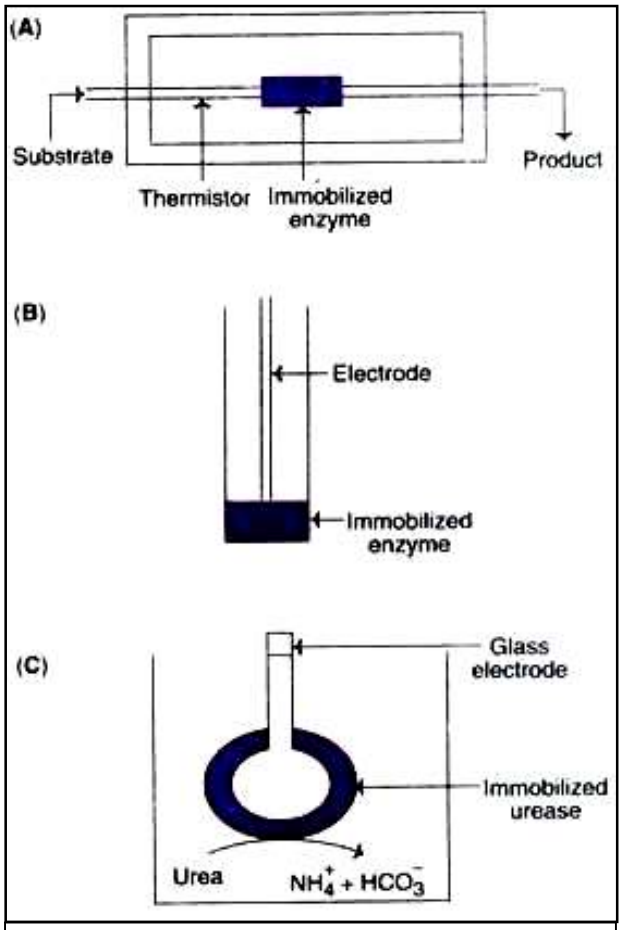


Fig.: Immobilized enzymes or cells in analytical biochemistry (A) Enzyme thermistor, (B) Enzyme electrode, (C) Urease electrode.

In Affinity Chromatography and Purification:

Immobilized enzymes can be used in affinity chromatography. Based on the property of affinity, it ispossible to purify several compounds e.g. antigens, antibodies, cofactors.

8. Microbes as Food & in food processing, single cell protein.

There are many useful application of microbes in the food industry. They influence the quality, availability and quantity of food. Microorganisms are used to change one substance to another which is used as food, such as milk to yoghurt and cheese, sugar to wine and bread.

Fermented Dairy Products:

Fermented milk is produced by inoculating pasteurised milk with specific culture of microorganisms. The different fermented dairy products include yoghurt and cheese.

Bacteria is used in Yoghurt Making:

Yoghurt is a dairy product which is produced by the bacterial fermentation of milk. Most commonly, cow's milk is used, though it can be made from any kind of milk. It can be prepared from a variety of milk including whole, skimmed, dried, evaporated or semi- skimmed milk.

The steps involved in yoghurt making are illustrated in Fig.

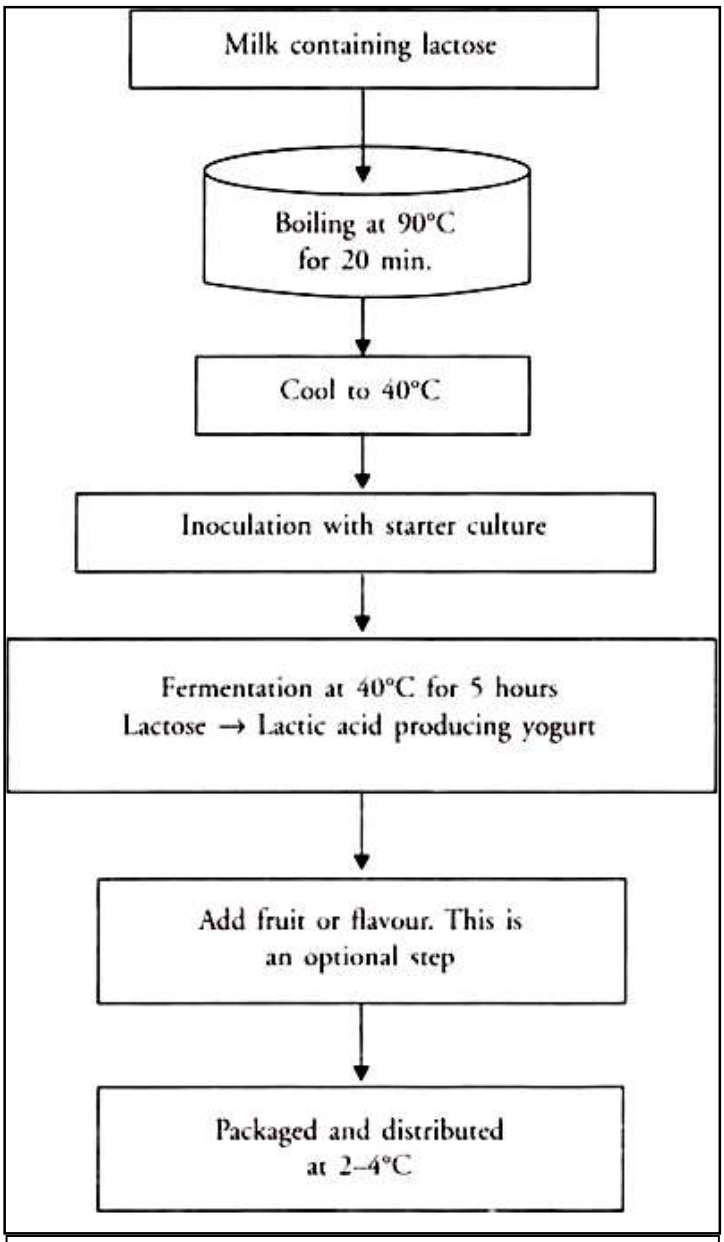


Fig.: Manufacture of yogurt

The milk sugar, i.e. lactose is fermented into lactic acid by the friendly bacteria, Streptococcus salivarius, S. thermophilus and Lactobacillus bulgaricus. These bacteria are collectively known as lactic acid bacteria or LAB. The bacteria feed on the lactose and release lactic acid as a waste product.The acid cause the curdling of the milk protein, casein into a solid mass called

curd. The gel like texture and taste of yoghurt is due to the fermentation of lactose to lactic acid. The increased acidity (pH = 4-5) also prevents the proliferation of other potentially pathogenic bacteria.

Both unpasteurised and pasteurized milk may be used for yoghurt making. The use of unpasteurised milk maintains the healthy balance of bacteria and enzymes of milk in its unprocessed state under very carefully controlled temperature and environmental conditions. To ensure complete fermentationtwo or more different bacteria may be used together.

Yoghurt is often sold sweetened and flavoured, or with fruit added at the bottom. The flavour varies indifferent countries.

a. Lassi is yoghurt-based beverage in India and is consumed either salty or sweet. Salty lassi is usually flavoured with ground- roasted cumin and black pepper powder, while the sweet variety is served with lemon, mango or other fruit juice.
b. A lassi-like, salty drink called ayran is popular in Turkey and Bulgaria and is prepared by mixing yoghurt with water and salt.

In India, Bulgaria and Turkey yogurt is prepared at home using a small amount of plain active culture yogurt as the starter culture. The milk is boiled to kill undesirable microbes. It is cooled to about 40°C. A tablespoon of starter culture is added and mixed thoroughly. It is left undisturbed for about 6 hours.

Bacteria and Fungi are used in Cheese Making:

Cheese is prepared by inoculating milk with a starter culture containing specific micro-organisms. Cheese is a solid food made from the milk of various animals, most commonly cows. Milk from goat, sheep, reindeer and water buffalo may also be used. There are several types of cheese.

Fermentation of milk leads to lactic acid production, which sours the milk. This leads to coagulation of milk protein, casein. The solid part of the milk produced by coagulation is known as curd and the liquid is known as whey.

The curds can be separated and pressed into desired shape and whey is used as food source for yeasts,which in turn can be processed as cattle feed and is rich in protein and vitamins. The cheese can be matured or ripened by the addition of bacteria or fungi or both. The

bacteria added reduce the pH,alters texture and develops a flavour.

Coagulation can be controlled using rennet tablets, which contains the enzyme rennin. Rennin is an enzyme present in the stomach of Calves but now is also available in genetically engineered bacteria. Coagulation can also be done using acids such as vinegar or lemon juice.

Depending on the nature of the organism added, cheese is of the following types:

a. Cheddar cheese is prepared by the addition of bacteria to enhance its flavour and texture.
b. The use of mould fungi produces Roquefort cheese and blue cheese.
c. A combination of both bacteria and fungi produces camembert cheese.
d. Swiss cheese is prepared by the addition of Propionibacterium sharmanii. The big holes in thecheese are because of the production of large amounts of CO_2.

The natural colour of cheese ranges from off-white to yellow. Herbs and spices may also be added to the cheese. Other factors that contribute to a different flavours and styles of cheese are different levels of milk fat, variations in length of aging, different processing treatments and different breeds of cows, sheep or other mammals.

Cheese production steps:

Step 1. Coagulum Formation:

Milk coagulation occurs due to two distinct activities.

(i) Inoculation with bacterial cultures, e.g., *Streptococcus lactis* or *S. cremoris* for incubation at 31°C, or *S. thermophilic* combined- with *Lactobacillus lactis*. *L. bulguricus* or *L. helveticus* (for incubation at 50°C), results in lactose degradation to produce lactic acid, which lowers the pH to about 4.6.

(ii) Incubation with rennet cleaves K-casein into para-K-casein and caseino macropeptide. This cleavage occurs at a specific peptide bond between phenylalanine at position 105 and methionine at position 106 (-phe 105-met 106-), and leads to coagulation of α- and β-caseins and the K-casein hydrolysis products.

Traditionally, rennet obtained from the fourth stomach of unweaned calves has been used. But at present, rennet from microbial sources is used extensively, and is responsible for about 70% of US and 30% of the worldwide cheese productions. But the rennet obtained from

Mucor miehei is relatively more thermostable and hence remains active during ripening, which often produces bitter off-flavours.

Therefore, it is treated with oxidising agents like H_2O_2, peracids, etc., which converts the methionine residues to their sulphoxides. This reduces the temperature tolerance of the enzyme by 10°C and makes M. miehei rennet more comparable to calf rennet. Attempts to clone calf chymosin gene in *E. coli* and *Saccharomyces cerevisiae* have been successful, but active renin is secreted only by the yeast cells.

Step 2. Separation of Curd:

The coagulum is heated to 37°C and cooled. This eliminates the remaining rennet activity and separates, to some extent the watery fluid called whey. The curd is separated from whey, salted, and mixed with proteases and/or lipases; alternatively, bricks of cheese may be inoculated with specific strains if fungi, e.g., *Penicillium roquefortii*, *P. camembertii*, etc. The bricks are pressed to remove excess moisture to enable proper ripening.

Step 3. Ripening:

Ripening procedures will vary with the type of cheese to be produced. The cheese bricks are inoculated with specific strains of fungi for the development of appropriate flavours through protease and lipase activities. Alternatively, proteases and lipases may be used for this purpose. Proteases from *Bacillus amyloliquefaciens* are used to enhance flavour in cheddar cheese.

Proteases hydrolyse proteins to produce peptides of variable sizes. Peptides having terminal acidic amino acid residues produce meaty, appetising flavours. But hydrophobic amino acid residues located non-terminally produce bitter flavours: the, flavours are the strongest in medium-sized peptides, absent in longer peptides, and decrease with a decrease in the peptide size.

Therefore, the kind and the degree of flavour in cheese can be controlled by regulating protein hydrolysis. The stronger flavours of Italian cheeses are produced by a modest lipid hydrolysis, which increases the amount of-free butyric acid. Lipolysis is brought about by lipase from *M. miehei* or *Aspergillus niger*; the lipase is added to the milk at 30 U/l before addition of rennet.

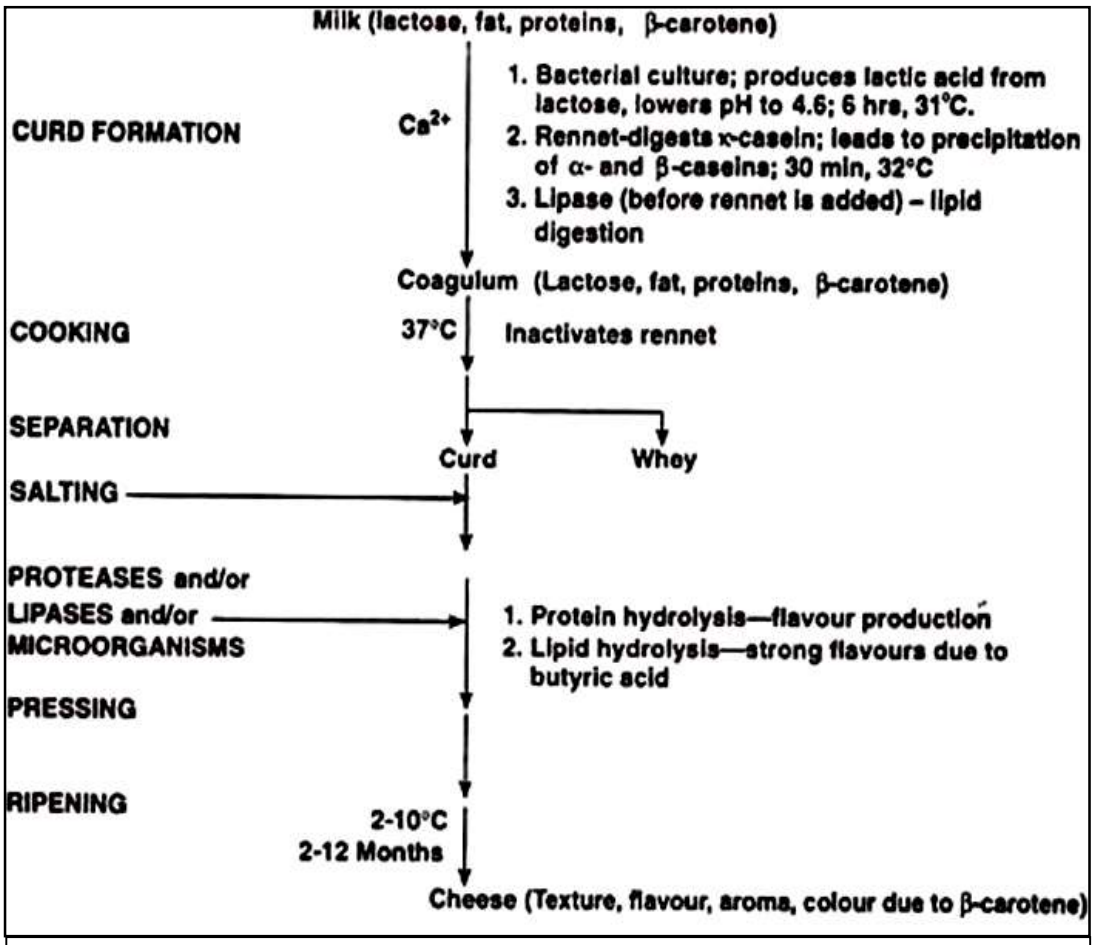


Fig.: A schematic representation of the various steps in cheese production.

Other Fermented Foods:

Some important food produced in whole or in part by microbial fermentation are pickles, sausages, etc. Different microorganisms are added to specific stages of food production to produce the desired effect. Moulds are used for the fermentation of rice to produce a variety of oriental foods.

Yeast is used for Making Bread:

Yeast is a fungus that feeds saprotrophically. The enzymes secreted by the yeast cell, digest food that contains sugar and minerals. Yeast is used to make bread. When yeast is added to raising flour and water, carbon dioxide is produced which gets trapped in the dough prepared

from the flour.

The dough rises and bread is made. The flour is usually made from wheat and contains starch. Starch is the energy source for the yeast. The flour also contains a protein called gluten, which forms sticky stretchy threads as the yeast works on the sugar. The threads trap the carbon dioxide and make the dough rise well. Some commercial uses of yeast are shown in Table.

Table: Important commercial products of yeast

Types of yeast	Product	Uses
Saccharomyces cerevisiae	Beer, Wine, Bread, Baker's yeast	Baking industry and brewing industry
Saccharomyces rouxii	Soy sauce	Food condiment
S. cerevisiae	Ethanol	Fuel, solvent
Eremothecium ashbyi	Riboflavin	Vitamin supplement

Baker's Yeast:

Yeast is used as leavening agent in baking since earlier times. The most commonly used species is Saccharomyces cerevisiae because of its ability to ferment sugar in the dough vigorously and to grow rapidly. The carbon dioxide used during the fermentation is responsible for the leavening or the rising of the dough. The procedure of mass production of Baker's yeast is elaborate under controlled conditions of pH, temperature conditions.

Microorganisms as Food – Single Cell Protein:

Algae, yeasts and bacteria can be grown in large quantities to yield a cell crop which is rich in protein known as single cell protein. The protein may be used for human consumption or as animal feed. It may be a useful source of minerals, vitamins, fat and carbohydrates. The composition of the different SCP depends upon the organism and the substrate on which it grows.

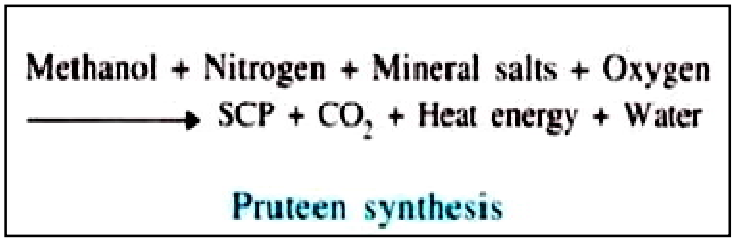
Advantages of using microorganisms as a food source are –

a. They grow very fast and do not need much space as conventional crops.
b. They grow on a wide range of cheap, waste products of agriculture and industry

such as petroleum products, methanol, ethanol, sugar, molasses, waste from paper mills etc. The secondary advantage is that they help in recycling the materials and thereby clean up the wastes.

c. They are high yielding. In a growth medium of 1000 lb of yeast in one day, many tonnes of protein is produced. This is about 10-15 times greater than soyabean and about 25-50 times greater than corn.
d. The protein content of the cells is very high. Yeast cells have a protein content as high as 40- 50%; for algae the range is 20- 40%.
e. The proteins of the microorganism contain all the essential amino acids.
f. Some microorganisms, particularly yeasts, have high vitamin content.
g. Factors, such as climate do not affect them, since they do not occupy large areas of land. Pruteen was the first major SCP to be produced. It was produced by a bacterium, Methylophilus methylotrophus. Methanol was used as a source of energy and the temperature was maintained at 30-40°C and pH at 6.7.

Pruteen was rich in essential amino acids and has high vitamin content. It is twice as nutritious as soyabean meal and was used as an animal feed.



Disadvantages of using SCP:

a. The high nucleic acid content causes intestinal disturbances. It can also lead to an increase in the uric acid in the blood that will eventually lead to gout. Additional processing can be done to reduce the nucleic acid content, but this would increase the cost.
b. Bacterial cells have small size and low density, which makes harvesting from the fermented medium difficult and costly.
c. The taste is not acceptable for many persons. Individual taste and customs make

microorganism unattractive as a food to some individuals.

Chocolate Making:

Chocolate is prepared with the help of microbes. Chocolate comes from the seeds of cacao trees. These seeds are found in a white fleshy pod. To remove the seeds out of the pod, the pod is allowed toferment with naturally occurring microbes that include yeasts and bacteria such as *Lactobacilli* and *Acetobacter*.

Single cell protein:

Single-cell protein (SCP) refers to edible unicellular microorganisms. The biomass or protein extract from pure or mixed cultures of algae, yeasts, fungi or bacteria may be used as an ingredient or a substitute for protein-rich foods, and is suitable for human consumption or as animal feeds.

Whereas industrial agriculture is marked by a high water footprint, high land use, biodiversity destruction, general environmental degradation and contributes to climate change by emission of a third of all greenhouse gases, production of SCP does not necessarily exhibit any of these serious drawbacks. As of today, SCP is commonly grown on agricultural waste products, and as such inherits the ecological footprint and water footprint of industrial agriculture. However, SCP may also be produced entirely independent of agricultural waste products through autotrophic growth. Thanks to the high diversity of microbial metabolism, autotrophic SCP provides several different modes of growth, versatile options of nutrients recycling, and a substantially increased efficiency compared to crops.

SCP production in India:

- National Botanical Research Institute (NBRI).
- Central Food Technological Research Institute (CFTRI).
- In CFTRI, SCP is produced from algae cultured on sewage.

Raw materials:

- Production of SCP requires micro-organisms that serve as the protein source and the substratethat is biomass on which they grow.

- There is a variety of both the sources that can be used for the production of SCP.
- The biomass used can be plant biomass or organic biomass.
- The micro-organisms used belong to the group of Algae, Fungi and Bacteria.

Micro organisms:

Micro-organisms used are fungi, yeast, algae & bacteria. The following table shows average differentcompositions of main groups of micro–organisms (% dry wt.).

COMPOSITON	FUNGI	ALGAE	YEAST	BACTERIA
PROTEIN	30- 40 %	40- 60 %	45- 55 %	50- 65 %
FAT	9-14 %	8-10 %	5-10 %	3-7 %
NUCLEIC ACID	7-10 %	3-8 %	6-12 %	8-12 %

A list of the micro-organisms used for SCP production is as follows –

Fungi:

- *Aspergillus fumigatus*
- *Aspergillus niger*
- *Rhizopus cyclopium*

Yeast:

- *Saccharomyces cerevisae*
- *Candida tropicalis*
- *Candida utilis*

Algae:

- *Spirulina sps.*
- *Chlorella pyrenoidosa*
- *Chondrus crispus*

Bacteria:

- *Pseudomonas fluroescens*
- *Lactobacillus*
- *Bacillus megaterium*

SCP production:

1) Selection of suitable strain
2) Fermentation
3) Harvesting
4) Post harvest treatment
5) SCP processing for food

1) Selection of strain:

- It a very critical step as the quality of protein depends totally on the microbe that is used forthe production.
- Thus careful selection of the strain should be done.
- Care should be taken that the selected strain should not produce any toxic or undesirableeffects in the consumer.

2) Fermentation:

- It can be carried out in the fermentor which is equipped with aerator, thermostat, pH, etc. or in the trenches or ponds.
- Microbes are cultured in fed- batch culture.
- Engineers have developed deep liftfermentor & air lift fermentor

3) Harvesting:

- When the colonies of microbes are fullydeveloped, they are then harvested.
- The bulk of cells are removed from thefermentor by decantation.

4) Post harvest treatment:

- After harvesting, the cells are subjected toa variety of processes.
- Post harvesting treatments includes steps like separation by centrifugation, washing, drying,etc.

5) Processing for food:

It includes –

a) Liberation of cell proteins by destruction of indigestible cell wall.

i. **Mechanical methods**: Crushing, crumbling, grinding, pressure homogenization, etc.

ii. **Chemical methods:** Enzymes & salts are used to digest or disrupt the cell wall. Salts like NaCl, sodium dodecyl sulfate, etc. whereas nuclease enzymes are used.

iii. **Physical methods:** Freeze- thaw, osmotic shock, heating & drying.

b) Reduction of nucleic acid content

- Chemical & enzymatic treatments are preferred.
- Chemicals which are used includes acidified alcohol, salts, acids & alkalies.
- Use of such chemicals leads to formation of lygino-alanine which causes hypersensitivity skinreactions.
- Enzymes which are used include ribonuclease & nuclease enzymes.
- These enzymes can be used exogenously or can be induced endogenously.

Nutritional and Safety Evaluations of SCP:

I. The SCP chemical composition must be characterised in terms of protein, amino acid, nucleic acid, lipid, vitamin, etc. contents.

II. Analysis of substrate residues and toxic substances, e.g., heavy metals, mycotoxins, polycyclic hydrocarbons, etc. must be done.

III. Physical properties like density, particle size, texture, colour, storage, etc. properties should be determined.

IV. Microbiological description, e.g., species, strain, should be provided, and information on contamination be also given.

V. The nutritional value should be evaluated on the target species, and other species should also be included. The products for human use will, of necessity, be evaluated over a longer period using a multistaged process.

VI. Possible toxic or carcinogenic compounds must be assayed for. These compounds may have been present in the substrate, may be synthesized by the organism or produced during the processing of SCP.

Advantages of SCP:

The SCP processes and products offer several advantages as listed below –

- ✓ The SCP is rich in high quality protein and is rather poor in fats, which is rather desirable.
- ✓ They can be produced all the year round and are not dependent of the climate (except thealgal processes).
- ✓ The microbes are very fast growing and produce large quantities of SCP from relatively verysmall area of land.
- ✓ They use low cost substrates and, in some cases, such substrates which are being wasted andcausing pollution to the environment.
- ✓ When the substrate used for SCP process is a source of pollution, SCP production helpsreduce pollution.
- ✓ Strains having high biomass yields and a desirable amino acid composition can be easilyselected or produced by genetic engineering.

9. Biofertilizers and Biopesticides in agriculture.

Bio-fertilizers:

Bio-fertilizers are microorganisms which bring about nutrient enrichment of soil by enhancing the availability of nutrients to crops. The micro-organisms which act as bio-fertilizers are bacteria, cyanobacteria (blue green algae) and mycorrhizal fungi. Bacteria and cynobacteria have the property of nitrogen fixation while mycorrhizal fungi preferentially withdraw minerals from organic matter for the plant with which they are associated.

Nitrogen fixation is the process of conversion of molecular or dinitrogen into nitrogen compounds. Insoluble forms of soil phosphorus are converted into soluble forms by certain micro-organisms. This makes the phosphorus available to the plants. Phosphate is also

solubilised by some bacteria and by some fungi that form association with plant roots.

The various bio-fertilizers are as follows –

1. Free Living Nitrogen Fixing Bacteria:

They live freely in the soil and perform nitrogen fixation. Some of them are saprotrophic, living on organic remains, e.g., *Azotobacter*, *Bacillus polymyxa*, *Clostridium*, *Beijerinckia*. They are further distinguished into aerobic and anaerobic forms The property of nitrogen fixation is also found in photoautotrophic bacteria, e.g., *Rhodopseudomonas*, *Rhodospirillum*, *Chromatium*. Inoculation of soil with these bacteria helps in increasing yield and saving of nitrogen fertilizers. For example, *Azotobacter* occurring in fields of Cotton, Maize, Jowar and Rice, not only increases yield but also saves nitrogen fertilizer to the tune of 10-25 kg/ha. Its inoculation is available under the trade name of azotobactrin.

2. Free Living Nitrogen Fixing Cyanobacteria:

A number of free living cyanobacteria or blue-green algae have the property of nitrogen fixation, e.g., *Anabaena, Nostoc, Aulosira, Totypothrix, Cylindrospermum, Stigonema*. Cyanobacteria are photosynthetic. Therefore, they add organic matter as well as extra nitrogen to the soil.

3. Loose Association of Nitrogen Fixing Bacteria:

Certain nitrogen fixing bacteria like *Azospirillum* live around the roots of higher plants without developing any intimate relationship. It is often called rhizosphere association. The bacteria obtain some plant exudate and use the same as part of their food requirement. The bacteria fix nitrogen and exude a part of the fixed nitrogen for use by the plant. The phenomenon is termed as associative mutualism (= associative symbiosis).

4. Symbiotic Nitrogen Fixing Bacteria:

They form a mutually beneficial association with the plants. The bacteria obtain food and shelter fromplants. In return, they give a part of their fixed nitrogen to the plants. The most important of the symbiotic nitrogen fixing bacteria is *Rhizobium* (pl. *Rhizobia*). It forms nodules on the roots of legume plants. There are many species of *Rhizobium* which form

association with different legume roots, e.g., *R. leguminosarum, R. lupini, R. trifolii, R. meliloti, R. phaseoli.*

These bacteria, also called rhizobia, live freely in the soil but cannot fix nitrogen except for a strain of Cowpea *Rhizobium* (Me Comb et al, 1975). They develop the ability to fix nitrogen only when they are present inside the root nodules. In the nodule cells, bacteria (bacteroids) lie in groups surrounded by membrane of the host which is lined by a pink-red pigment called leghaemoglobin. Presently cultures of *Rhizobium* specific for different crops are raised in the laboratory.

Frankia, a nitrogen fixing mycelial bacterium (Actinomycetes), is associated symbiotically with the root nodules of several non-legume plants like *Casuarina, Alnus* (Alder) *Myrica, Rubus* etc. Leaves ofa few plants (e.g., *Ardisia*) develop special internal cavities for providing space to symbiotic nitrogenfixing bacteria, *Xanthomonas* and *Mycobacterium*. Such leaves are a constant source of nitrogen fertilizer to the soil.

5. Symbiotic Nitrogen Fixing Cyanobacteria:

Nitrogen fixing cyanobacteria (blue-green algae) form symbiotic association with several plants, e.g., cycad roots, lichens, liverworts, *Azolla* (fern). Out of these, *Azolla-Anabaena* association is of great importance to agriculture.

Azolla pinnata is a small free floating fresh water fern which multiplies rapidly, doubling every 5-7 days. The fern can coexist with rice plants because it does not interfere with their growth. In some South-East Asian countries, especially China, the rice fields are regularly provided with *Azolla*.

6. Microphos Biofertilizers:

They release phosphate from bound and insoluble states, e.g., *Bacillus polymyxa, Pseudomonas striata, Aspergillus* sp.

7. Mycorrhiza:

It is a mutually beneficial or symbiotic association of a fungus with the root of a higher plant. The most common fungal partners of mycorrhiza are *Glomus* species. Mycorrhizal roots show

a sparse or dense wooly growth of fungal hyphae on their surface. Root cap and root hairs are absent. The shape is irregular, tuberous, nodulated or coralloid. The fungus remains restricted to the cortex of the root. The vascular strand and growing point are not affected. Mycorrhiza often remains in the upper layers of the soil where organic matter is abundant.

Depending upon the residence of the fungus, mycorrhizae are of two types— ectomycorrhiza and endomycorrhiza.

(i) Ectomycorrhiza (= Ectotrophic Mycorrhiza):

The fungus forms a mantle on the surface of the root. Internally, it does not lie in the cortex, i.e. does not penetrate into the cortical cells. The root cells secrete sugars and other food ingredients into the intercellular spaces for feeding the fungal hyphae.

(ii) Endomycorrhiza (= Endotrophic Mycorrhiza):

Fewer fungal hyphae lie on the surface. The remaining live in the cortex of the root, mostly in the intercellular spaces with some hyphal tips passing inside the cortical cells, e.g., grasses, crop plants, orchids and some woody plants. In seedling stage of orchids, the fungal hyphae also provide nourishment by forming nutrients rich cells called pelotons. Intracellular growth occurs in order to obtain nourishment because unlike ectomycorrhiza, the cortical cells do not secrete sugars in the intercellular spaces.

Importance of Bio-fertilizers:

- They increase the yield of plants by 15-35%.
- Bio-fertilizers are effective even under semi-arid conditions.
- Farmers can prepare the inoculum themselves.
- They improve soil texture.
- Bio-fertilizers do not allow pathogens to flourish.
- They produce vitamins and growth promoting bio-chemicals.
- They are non-polluting.

Biopesticide:

Biopesticide is a formulation made from naturally occurring substances that controls pests by non-toxic mechanisms and in ecofriendly manner. Biopesticides may be derived from animals

(e.g. nematodes), plants (*Chrysanthemum*, *Azadirachta*) and micro-organisms (e.g. *Bacillus thuringiensis*, *Trichoderma*, nucleopolyhedrosis virus), and include living organisms (natural enemies) etc. However, biopesticides are generally less toxic to the user and are non-target organisms, making themdesirable and sustainable tools for disease management.

Advatages of biopesticides:

- ✓ Inherently less harmful and less environmental load,
- ✓ Designed to affect only one specific pest or, in some cases, a few target organisms,
- ✓ Often effective in very small quantities and often decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problems.
- ✓ When used as a component of Integrated Pest Management (IPM) programs, biopesticides can contribute greatly.

Types of biopesticides:

1) Microbial pesticides
2) Plant-incorporated-protectants (PIPs)
3) Biochemical pesticides
4) Botanical pesticides
5) Biotic agents (parasitoids and predators)

1) Microbial Pesticides:

Microbial pesticides are composed of microscopic living organisms (viruses, bacteria, fungi, protozoa, or nematodes) or toxin produced by these organisms. Applied as conventional insecticidal sprays, dusts, or granules. Their greatest strength is their specificity as most are essentially nontoxic and non pathogenic to animals and humans. Microbial pesticides includes insecticides, fungicides, herbicides and growth regulators of microbial origin.

Some of the important microbial pesticides are as follows –

a) Bacillus thuringiensis

- ❖ Discovered in Japan in early 20th century and first become a commercial product in France in1938.

- ❖ Control lepidopterous pests like American bollworm in cotton and stem borers in rice.
- ❖ When ingested by pest larvae, Bt releases toxins which damage the mid gut of the pest,eventually killing it.
- ❖ Main sources for the production of Bt preparations are the strains of the subspecies kurstaki,galeriae and dendrolimus

***b) Agrobacterium radiobacter* (Agrocin)**

- ❖ *Agrobacterium radiobacter* is used to treat roots during transplanting, that checks crowngall.
- ❖ Crown gall is a disease in peaches, grapevine, roses and various plants caused by soilborne pathogen *Agrobacterium tumefaciensm*.
- ❖ The effective strains of *A. radiobacter* posses two important features –
    - They are able to colonize host roots to a higher population density.
    - They produce an antibiotic, agrocin that is toxic to *A. tumefaciens*.

***c) Pseudomonas fluorescens* (Phenazine)**

- ❖ This bacterium is used to control damping off caused by *Pythium* sp., *Phytophthora sp., Rhizoctonia solani*,*Gaeumannomyces graminis*.
- ❖ It has ability to grow quickly in the rhizosphere

d) Trichoderma

- ➢ *Trichoderma* is a fungicide effective against soil born diseases such as root rot.
- ➢ This is also used against *Necteia galligena* that causes silver leaf disease of fruit trees byentering through pruning wounds.

e) Metarhizium anisopliae

- ➢ It infects spittlebugs, rhinoceros beetles.

f) Beauveria bassiana

- ➢ Controls Colorado potato beetle.

g) Verticillium lecanii

- ➢ Controls aphids and whiteflies.

h) Nomuraea riley

- ➢ Controls soybean caterpillars.

i) Baculoviruses (Bvs)

- Control lepidopterous and hymenopterous pests.
- Contains rod shaped, circular double stranded super coiled DNA.

2) Plant-incorporated-protectants (PIPs):

- Pesticidal substances that plant produce from the genetic material that has been added to theplant.
- As the pest feed on such plants theywill eventually die.

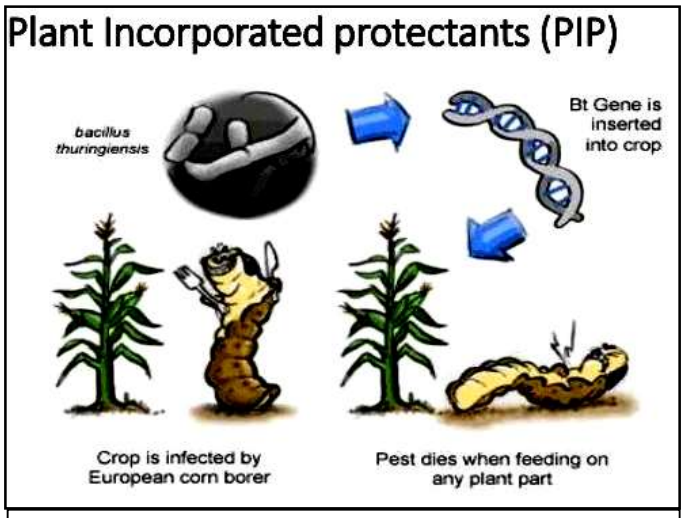


Fig.: Showing the mode of action of cry protein in crystal produced by genetically modified plant

3) Botanical pesticides:

- These are naturally occurring plant material that may be crudepreparation of the plant parts ground to produce a dust or powder that can be used in full strength or dilute form in a carrier such as clay, talc or diatomaceous earth.
- "Azadirachtin" affects the reproductive and digestive proceesof pest.
- Several plant based insecticides as nicotinoids, natural pyrethrins, rotenoids, neem products etc are used.

4) Biochemical pesticides:

- They are naturally occurring substance to control pest by non-toxic mechanisms.
- Biochemical pesticides include substances as insect sex pheromones that interfere

withmating that attract insect pest to traps.

- The synthetic attractants are used in one of four ways –
    - As a lure in traps used to monitor pest populations;
    - As a lure in traps designed to "trap out" a pest population;
    - As a broadcast signal intended to disrupt insect mating
    - As an attractant in a bait containing an insecticide

5) Biotic agents/Natural enemies:

- **Predators:**
    - They consume several to many prey over the course of their development, they are free living and they are usually as big as or bigger than their prey. lady beetles, rove beetles, many ground beetles, lacewings, true bugs such as *Podisus* and *Orius*, syrphid fly larvae, mantids, spiders, and mites suchas *Phytoseiulus* and *Amblyseius*.
- **Parasitoids:**
    - Parasitoids are almost the same size as their hosts, and their development always kills the hostinsect.
    - An adult parasitoid deposits one or more eggs into or onto the body of a host insect orsomewhere in the host's habitat.
    - The larva that hatches from each egg feeds internally or externally on the host's tissues andbody fluids, consuming it slowly.
    - Later in development, the host dies and the parasitoid pupates inside or outside of the host'sbody.
    - Example: *Bathyplectes, Trichogramma, Encarsia, Muscidifurax* etc.

Application of Biopesticides:

- ✓ Biopesticides are usually applied in a similar manner to chemical pesticides but in environmental friendly way.
- ✓ For effective control, microbial agents require appropriate formulation.
- ✓ Biopesticides used to control internal seed borne fungal pathogens.

10. Environmental Biotechnology: Treatment of waste & waste water; bioremediation.

Environmental Biotechnology:

The most important topics at the threshold of the 21st century are the environment and biotechnology. Environmental biotechnology can be defined as the marriage of environmental issues with the advances in biotechnology.

It is concerned with the application of biotechnology as an emerging technology in the context of environmental protection, since rapid industrialization, urbanization and other developments have resulted in a threatened clean environment and depleted natural resources.

It is not a new area of interest, because some of the issues of concern are familiar examples of "old" technologies, such as: composting, wastewater treatment, etc.

In its early stage, environmental biotechnology has evolved from chemical engineering, but later, other disciplines (biochemistry, environmental engineering, environmental microbiology, molecular biology, ecology) also contribute to environmental biotechnology development.

The development of multiple human activities in the sector of industry, transport, agriculture, domestic space, etc. has amplified the pollution of air, water and soil.

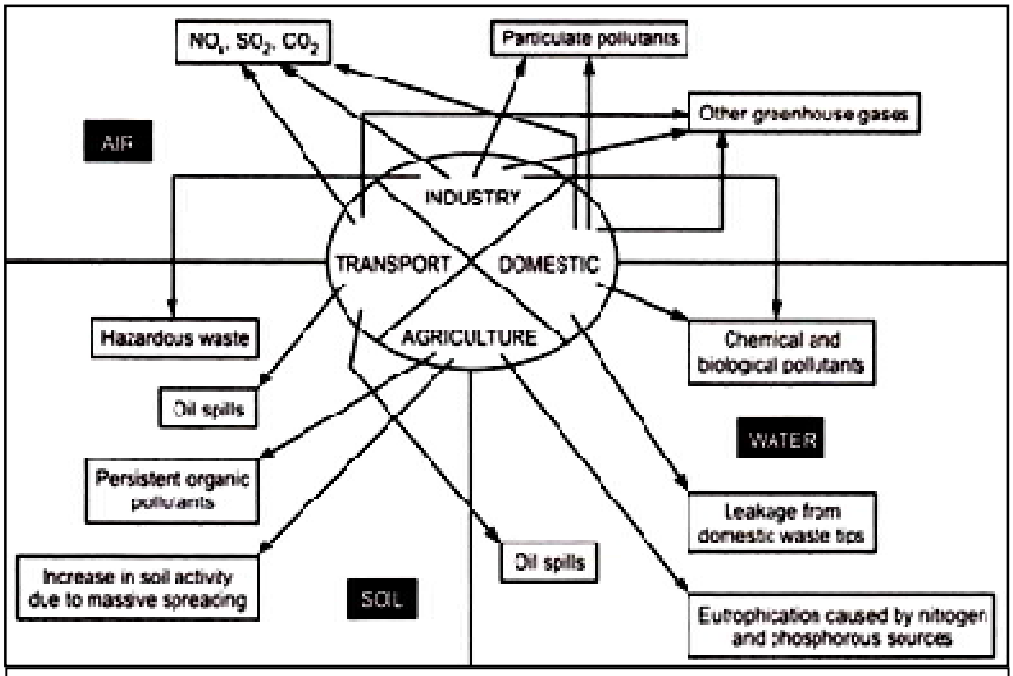


Fig.: The spider of environmental pollution due to anthropogenic activities.

Studies and researches demonstrated that some of these pollutants can be readily degraded or removedby means of biotechnological solutions.

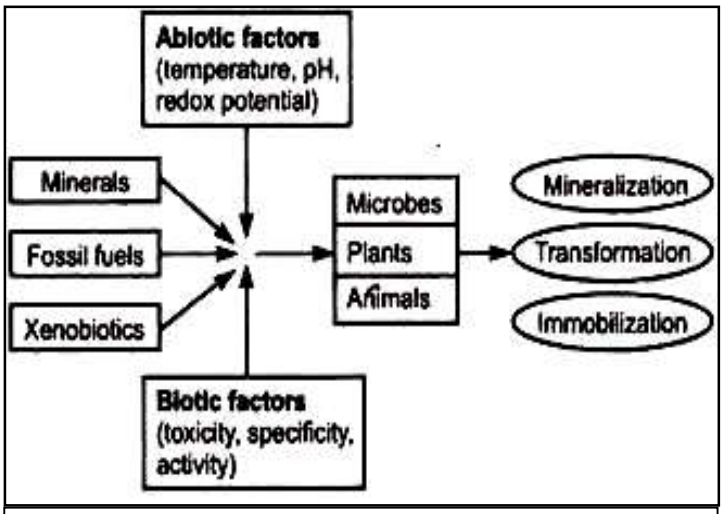


Fig.: Sources of environmental pollutants and factors that influence their removal from the environment.

Three key points are considered for environmental biotechnology. These are as follows –

1. To detect the pollution and any other environmental changes by means of biosensors and biomonitoring.
2. To prevent the unfavorable environmental changes in the manufacturing process by substitution oftraditional processes.
3. To control and remediate the emission of pollutants into the environment.

By considering all these issues, biotechnology may be regarded as a driving force for integrated environmental protection by environmental bioremediation, waste minimization, environmental bio monitoring, bio maintenance.

Wastewater treatment:

Wastewater treatment is a process used to remove contaminants from wastewater or sewage and convert it into an effluent that can be returned to the water cycle with minimum impact on the environment, or directly reused. The latter is called water reclamation because treated wastewater can then be used for other purposes. The treatment process takes place in a wastewater treatment plant (WWTP), often referred to as a Water Resource Recovery Facility (WRRF) or a Sewage Treatment Plant (STP). Pollutants in municipal wastewater (households and small industries) are removed or broken down.

The treatment of wastewater is part of the overarching field of sanitation. Sanitation also includes the management of human waste and solid waste as well as stormwater (drainage) management. By-products from wastewater treatment plants, such as screenings, grit and sewage sludge may also be treated in a wastewater treatment plant.

The four processes are as follows –

1. **Preliminary Treatment:**

As already stated, preliminary treatment involves the removal of floating materials (leaves, papers, rags) and settleable inorganic solids (sand, grit), besides oily substances (fats, oils, greases). The three major types of equipment-screeners, grit chambers, and skimming tanks, employed in preliminary screening are briefly described.

Screeners:

A screener is a device with openings (usually uniform in size) to remove the floating materials

and suspended particles. The process of screening can be carried out by passing sewage through different types of screeners (with different pore sizes).

The screeners are classified as coarse, medium or fine, depending on the size of the openings. The coarse screen has larger openings (75-150 mm). The openings for medium and fine screens respectively are 20-50 mm and less than 20 mm. Different types of screens-fixed bar screen (coarse ormedium) disc type fine screen, drum type fine screen are in use.

A shredder or comminatory is a special screen that can cut and retain the floating and suspended materials.

Grit Chambers:

The heavy inorganic materials (specific gravity 2.4-2.7) like sand, ash and others can be removed by using grit chambers. This technique is based on the process of sedimentation due to gravitational forces. Grit chambers may be kept either before or after the screens. A diagrammatic representation ofa typical grit chamber is depicted in.

Skimming Tanks:

Several greasy and oily materials (fats, oils, waxes, soaps etc.) from the domestic or industrial outlets find their entry into the sewage. They can be removed by using a skimming tank which is fitted with baffle walls that divide the tank. The skimming tank is divided into three compartments that are interconnected.

2. Primary Treatment:

Primary treatment is aimed at the removal of fine suspended organic solids that cannot be removed in the preliminary treatment. Primary treatment basically involves the process of sedimentation or settling. In the normal process of sewage treatment, sedimentation is usually carried out twice-once before the secondary treatment, referred to as primary sedimentation, and then after the secondary treatment is complete, a process known as secondary sedimentation. It is sometimes necessary to use chemical coagulants to facilitate or aid sedimentation, and this process is referred to as chemical precipitation or coagulation-aided sedimentation.

Principle of Sedimentation:

The solid particle of the sewage tend to settle down due to gravity. However, most of the solid

particles of organic compounds remain in a suspended state in a flowing sewage. If the flow of the sewage is stopped and if it is stored in a tank referred to as sedimentation tank, the solid particles can settle down at the bottom. The process of sedimentation is influenced by several factors. These include the size, shape and specific gravity of particles, besides viscosity and flow velocity of sewage.

Types of Settling:

There are four major types of settling—discrete settling, flocculent settling, hindered or zone settling and compression. This categorization is mainly based on the tendency of the particles to interact and form solids.

The particles which do not change their size, shape and weight are referred to as discrete particles or granular particles. The use of grit in sewage may be considered as an example of discrete settling.

Flocculent settling:

The flocculent particles can change their size, shape and weight, and thus lose their identity. These particles actually coalesce during settling. Settling of bioflocs, and chemical . floes in secondary sedimentation tanks are good examples of flocculent settling.

Hindered or zone settling:

The particles as such, tend to remain in a fixed position with respect to each other. When flocculated, the whole mass of particles settle as a unit or a zone. In the hindered settling the concentration of particles increases from top to the bottom and this results in the thickening of the sludge. Zone settlingis employed in conjunction with biological treatment facilities.

Compression:

Settlement of particles in the lower layers can occur by compression of the weight of the particles onthe upper layers. This process facilitates sludge thickening at the bottom.

Chemical-aided Sedimentation:

It is not always possible to remove the colloidal wastes in sewage by plain sedimentation. However, addition of certain chemicals aids sedimentation, a process referred to as chemical precipitation or chemical-aided sedimentation. By this technique, about 60-80% of the suspended particles can be removed. Chemical precipitation involves three stages—

coagulation, flocculation and sedimentation. Coagulation is mainly a chemical process wherein the charged particles are destabilized (by the addition of chemical agents). On the other hand, flocculation involves the physical phenomena of aggregating the destabilized particles to finally form settleable solids (i.e. sedimentation). The chemicals used in chemical-aided sedimentation are of two types-coagulants and coagulant aids.

Coagulants:

These are the chemicals (normally positively charged) which form insoluble and gelatinous precipitates with colloidal particles (negatively charged ones present in sewage). The most commonly used coagulants in sewage treatment are alum (alluminium sulfate) iron salts (ferric sulfate, ferrous sulfate, ferric chloride), lime and soda ash (sodium carbonate), sodium silicate and sodium aluminate.

3. Secondary or Biological Treatment:

Biological treatment of sewage is required for the removal of dissolved and fine colloidal organic matter. This process involves the use of microorganisms (bacteria, algae, fungi, protozoa, rotifers, nematodes) that decompose the unstable organic matter to stable inorganic forms.

The biological treatment processes of sewage are broadly classified as aerobic, anaerobic and pond processes. Depending on the nature of the use of the microorganisms, the biological processes are categorized as suspended growth systems and attached growth systems.

Aerobic Suspended-Growth Treatment Processes:

The most important suspended-growth biological treatment systems used for the removal of organic matter are listed:

i. Activated sludge process
ii. Aerated lagoons
iii. Sequencing batch reactor
iv. Aerobic digestion.

Among these, activated sludge process is the most widely used for the secondary treatment of sewage.

i. Activated Sludge Process:

The activated sludge process, first developed in England in 1914, continues to be the most commonly used modern process for the biological treatment of sewage. In this method, the sewage containing organic matter with the microorganisms is aerated (by a mechanical aerator) in an aeration tank. The reactor contents are referred to as mixed liquor. Under aerobic conditions, the microorganisms metabolize the soluble and suspended organic matter. The generalized metabolic reaction is as follows.

A part of the organic matter is utilized for the synthesis of new bacterial cells while the remaining getsoxidized to CO_2 and H_2O. The newly formed microorganisms are agglomerated to form floes, technically referred to as sludge.

The separated sludge which is not in contact with organic matter becomes activated. It is separated from the settling tank, and returned to the aeration tank, and recycled. The activated sludge recycled in aeration tank serves as a seed or inoculum. The excess and waste sludge can be removed.

For efficient operation of activated sludge process, it is necessary to maintain a constant supply of O_2 which can be done by mechanical aeration or through the use of rotating paddles. Growth of protozoa in a sludge is an indication of its healthy condition. The disposal of a waste sludge is a problem. It may be used as a fertilizer in crop lands or as landfills, after drying.

Conventional activated sludge process: In the normal treatment of sewage, the activated sludge is proceeded by primary sedimentation tank. The conventional activated sludge system consists of a separation tank, settling or sedimentation tank and sludge removal line (Fig. 57.4). The sewage after the primary treatment is introduced at the head of the tank. It is desirable to supply O2 uniformly throughout the tank.

Modified activated sludge processes: For increasing the performance of the activated sludge system, several modifications have been done in the recent years. Most of them are directed to bring out efficient aeration. Aeration can be done by step aeration, tapered aeration, and high rate aeration by complete mixing and extended aeration.

ii. Aerated Lagoons:

Aerated lagoons, also called as aerated ponds, are the facultative stabilization ponds wherein surface aerators are installed to overcome the bad adours (due to overload of organic

materials). The microbiological treatment of aerated ponds is comparable to the activated sludge process. The major difference is the large surface area in aerated ponds and this is more susceptible for temperature effects. It is possible to carry out continuous nitrification in aerated lagoons. This however, depends on the design and operating conditions of the pond (particularly the temperature).

iii. Sequencing Batch Reactor:

Sequencing batch reactor (SBR) is a modification of activated sludge treatment system. The processes namely aeration and sedimentation are carried out in both the systems. The major difference is that while in the conventional activated sludge system, aeration and sedimentation occur simultaneously in separate tanks, these two processes are carried out sequentially in the same tank in SBR. Thus, the sequencing batch reactor may be regarded as fill- and-draw activated *sludge process.*

iv. Aerobic Digestion:

The organic sludge's produced from various treatment processes (activated sludge treatment, trickling filter-sludge) are subjected to aerobic digestion in special reactors referred to as aerobic digesters.

Aerobic Attached — Growth Treatment Processes:

Aerobic attached-growth treatment processes are commonly used to remove the organic matter found in the sewage. These processes are also useful for the nitrification (conversion of ammonia to nitrate). The commonly used attached-growth processes are listed:

i. Trickling filters
ii. Roughing filters
iii. Rotating biological contractors
iv. Packed bed reactors.

Among these, trickling filter is most widely used.

Anaerobic Digestion:

Anaerobic digestion is mostly useful for the stabilization of concentrated sludge's that are produced on the treatment of industrial sewage. The process of anaerobic digestion is carried out in an air tight reactor. Sludge is introduced continuously or intermittently. In the high-rate digestion system, the contents of the digester are heated and mixed completely. And it takes

about 15 days for the process to be complete.

Biodegradation of organic matter of sludge (or sewage):

The biological degradation of organic matter of sludge occurs in three stages— hydrolysis, acidogenesis and methanogenesis.

Hydrolysis:

In the enzyme-catalysed reactions, high molecular weight compounds (proteins, polysaccharides, lipids and nucleic acids) are degraded to low molecular weight compounds (amino acids, monosaccharide's, fatty acids, purines and pyrimidine's). The latter serve as substrates for energy supply and microbial growth.

Acidogenesis:

The low molecular weight compounds are converted to acidic products (propionate, butyrate, andlactate).

Methanogenesis:

This is the third and final stage and involves the production of methane and carbon dioxide, from the intermediates formed in acidogenesis. Methane gas is highly insoluble and its departure from the digester represents the stabilization of sewage or sludge. Microorganisms to degrade organic matter of sludge (or sewage) A consortium of anaerobic microorganisms work together for degradation of sludge (or sewage) organic matter. They may be categorized into two types, viz –

Acid-forming bacteria: These are also known as acidogens or non-methanogenic bacteria. They bring out the hydrolysis of macromolecules (e.g. carbohydrate) to simple substrates (e.g. monosaccharide's), and the latter to acids e.g. *Clostridium* sp, *Corynebacterium* sp, *Lactobacillus* sp, *Actinomyces* sp, *Staphylococcus* sp, *Peptococcus anaerobus*, *Escherichia coli*.

Methanogenic bacteria: These bacteria also referred to as methanogens or methane formers are responsible for the conversionof acetic acid and hydrogen to methane and carbon dioxide. The most important methanogens belong to the genera *Methanobacterium*, *Methanobacillus*, *Methanococcus* and *Methanosarcina*.

Anaerobic contact process is carried out in a specially designed reactors. The treatment process consists of mixing of sewage with recycled sludge solids and then digestion under anaerobic conditions. After the digestion is complete, the supernatant effluent is discharged

and the settled sludge is recycled. Anaerobic contact process is successfully used for efficient industrial wastes with high BOD e.g. meat packing wastes.

Anaerobic Attached — Growth Treatment Processes:

There are mainly two treatment processes under the anaerobic attached—growth treatment system— anaerobic filter process and expanded bed process.

Anaerobic Filter Process:

Anaerobic filter consists of a column filled with solid media for the treatment of organic matter in sewage. In this process system, waste water (sewage) flows upwards through the column containing anaerobic bacteria. Due to the presence of solid media, the bacteria are retained in the column. This makes the treatment process more efficient.

Expanded-Bed Process:

The sewage can be treated by pumping it through a bed of inert materials (sand or coal expanded aggregates) on which the bacteria have grown and formed a film. The effluent that comes out can be recycled to maintain the flow rate.

Pond Treatment Processes:

Pond treatment processes for the treatment of sewage (containing biodegradable wastes) are carried out by specially designed and constructed ponds. These ponds, referred to as stabilization ponds, arelarge, shallow earthen basins. The treatment process is a natural one involving the combined use of bacteria and algae. The stabilization ponds are classified as aerobic, anaerobic and facultative ponds.

Aerobic Ponds: The aerobic ponds, as the name indicates, maintain complete aerobic conditions. These ponds usually have a depth of about 0.5 to 1.5 feet (150 to 450 mm) and allow the penetration of light throughout the liquid depth. A second type of aerobic ponds with a depth of 5 feet (1.5 m) are also in use.

In all these ponds, oxygen is maintained through continuous atmospheric diffusion (by surface aerators or pumps), besides the production by algae grown in the pond. The aerobic stabilization ponds contain bacteria and algae in suspension. They are particularly useful for the treatment of soluble wastes.

The algae and bacteria exhibit a symbiotic and cyclic relationship in the aerobic ponds. The

algae can carry out photosynthesis and release oxygen to maintain aerobic conditions in the pond. The bacteria degrade the organic matter to produce CO2 and other nutrients to be utilized by algae (Fig. 57.11). Some higher organisms like protozoa and rotifers present in the pond are responsible for the polishing of the effluent.

4. Tertiary Treatment:

Tertiary treatment or advanced treatment is sometimes needed for the removal of suspended and dissolved substances, after the conventional primary and secondary treatments. In general, the effluent of the sewage obtained after secondary treatment can be conveniently disposed without causing any nuisance.

However, tertiary treatment is needed under the following circumstances:

i. When the quality of the effluent to be discharged does not meet the standard requirements(particularly in the developed countries).
ii. When there is a necessary to reuse the sewage/ waste water (reclamation of water is quiteexpensive, but is required in certain situations of water shortage).
iii. For the removal of nitrogen and phosphorus compounds.

Tertiary treatment process broadly involves the removal of suspended and dissolved solids, nitrogen, phosphorus and pathogenic organisms. In the conventional hierarchy of sewage treatment, the unit operations are carried out in the order of preliminary, primary, secondary and finally tertiary treatment.

However, sometimes advanced (tertiary) treatment process may be directly carried out bypassing theother unit operations. This mainly depends on the composition of waste water and the requirements.

There are four major processes under the tertiary treatment:

I. Solids removal
II. Biological nitrogen removal
III. Biological phosphorus removal
IV. Disinfection.

I. Solids Removal:

The techniques for the removal of suspended and dissolved solids in waste water treatment

are comparable with those employed for the processing of potable (drinking) water.

The effluents obtained from secondary treatment may contain suspended solids in the size 0.1 to 100μm. The concentration of these solids is variable, and is usually 20-40 mg/l. The removal of suspended solids is carried out by granular medium (sand) filtration and micro screening. Sometimes, diatomaceous earth filters and coagulation-cum sedimentation techniques are also used.

Removal of dissolved solids: The dissolved solids can be removed mainly by two techniques—adsorption and ion-exchange.

Adsorption by activated carbon: Activated carbon is highly porous and provides large surface area for the adsorption of dissolved solids in the advanced treatment. The compounds that can be removed by adsorption include organic materials (herbicides, pesticides, tannins, lignin's, colour and odour producing substances), inorganic materials (toxic trace metals) and several other pollutants.

Ion-exchange for dissolved solids removal: As the name indicates, ion-exchange involves the displacement of one ion by another. The exchange occurs between the ions of insoluble exchange material (ion-exchange materials) and the ions of different species in solution (i.e. waste water for advanced treatment).

The ion-exchange process is carried out by employing two types of ion-exchange materials—cation exchangers and anion exchangers. The synthetic resins with strong acidic (H^+) and basic (OH^-) functional groups serve as ion exchangers. The cation exchangers (with H^+ or Na^+) can replace the positively charged ions (Ca^{2+}, Mg^{2+}) in water by hydrogen ions. This is what is done for removing the hardness of water.

II. Biological Nitrogen Removal:

Decomposition products of proteins and the urea present in sewage are the major constituents of biological nitrogen. Although, nitrogen is a nutrient, its excess concentration causes eutrophication, and thus its removal is required. Biological nitrogen removal (BNR) is carried out by the methods based on the following principles.

Assimilation of nitrogen: Since nitrogen is a nutrient, the microorganisms in the sewage can assimilate ammonia nitrogen, and grow. As some of these cells die, a portion of this ammonia

nitrogen will be returned to the sewage.

Nitrification: Ammonia nitrogen first gets oxidized to nitrite (NO–2) by the bacteria *Nitrosomonas* sp. This is followed by further oxidation nitrite (NO–2) to nitrate (NO–3) by *Nitrobacter* sp.

The bacteria involved in nitrification are auxotrophs. The nitrification process is accomplished by aerobic suspended growth and aerobic attached-growth systems. In the general practice, nitrification is carried out along with the BOD removal in the secondary treatment with suitable modifications. Trickling filters, rotating biological contactors and packed towers can be used for nitrification process.

Denitrification: The removal of nitrogen in the form of nitrate by converting to nitrogen gas is referred to as de-nitrification. This process occurs under anaerobic conditions and is brought out by certain genera of bacteria-*Aerobacter, Bacillus, Brevibacterium, Lactobacillus, Micrococcus, Pseudomonas and Spirillum.*

These bacteria are heterotrophs and require no oxygen, but the presence of organic carbon is essential. The presence of even minute quantities of O2 suppresses de-nitrification. The heterotrophic bacteria can reduce nitrate in the following stages, to finally nitrogen gas.

III. Biological Phosphorus Removal:

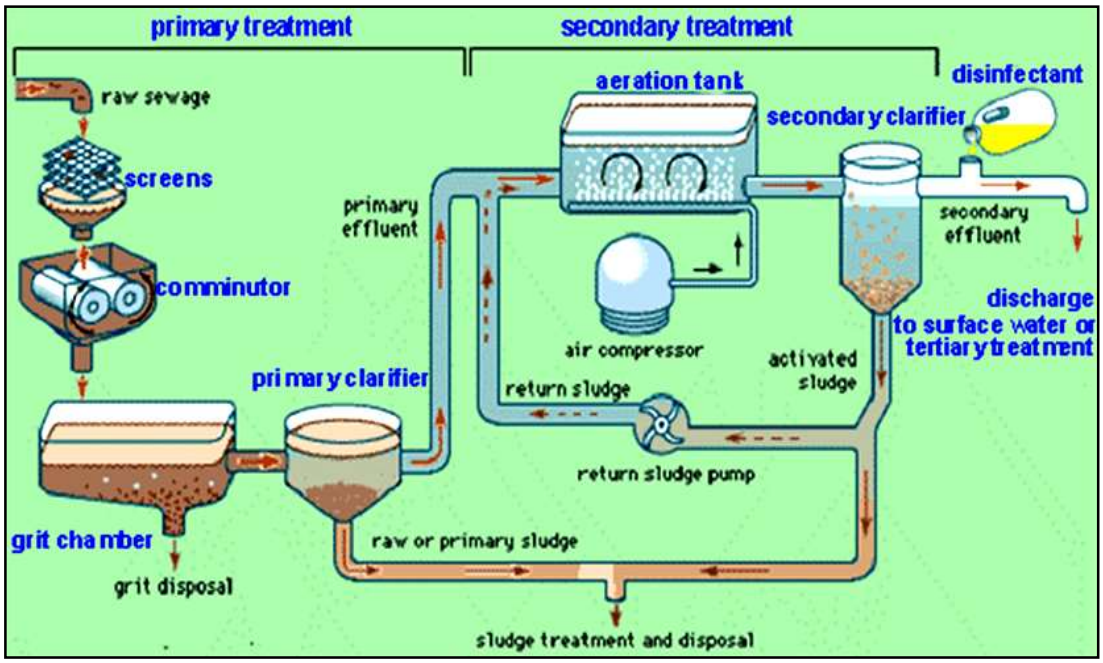
Phosphorus in the sewage is mostly present in the form of orthophosphate (PO43-), polyphosphate (P2O7) and organic bound phosphorus. In fact, phosphorus is an essential nutrient for microorganisms. Thus, during the normal secondary treatment process, 10-30% of the sewage phosphorus is utilized by the microorganisms for growth and energy purposes. Phosphorus removal from waste water is required to control eutrophication and to maintain water quality.

IV. The process of phosphorus removal:.

The phosphorus enriched supernatant that comes out of the phosphorus stripper is treated with lime to precipitate the phosphorus. The resultant liquid supernatant can be returned to the aeration tank for further treatment.

V. Disinfection:

Disinfection broadly refers to the selective destruction or inactivation of disease-causing (pathogenic) organisms. In the process of disinfection, all the organisms are not destroyed. This is in contrast to sterilization which involves the destruction of all the organisms. There are several water borne diseases (typhoid, cholera, dysentery) caused by bacteria, viruses and other pathogens. The very purpose of disinfection is to control these diseases.



Agents for disinfection:

Disinfection is accomplished by using chemical and physical agents, besides mechanical and radiationmeans.

Chemical agents:

Chlorine and its compounds are most commonly used. The other chemicals— bromine, iodine, ozone, alcohols, phenols, heavy metals, hydrogen peroxide, alkalies and acids are sometimes employed. After chlorine (regarded as most universal disinfectant), bromine and iodine are in use. In recent years, ozone as a disinfectant is gaining importance, since it is very effective.

Physical agents:

Heat and light can be effectively used as disinfectants. Sunlight (particularly ultra-violet rays) is in fact a good disinfectant.

Mechanical means:

The pathogenic organisms can also be removed by mechanical means, during the course of waste water treatment. The processes involving screens (coarse and fine), grit chambers and sedimentation can partly remove the disease- causing organisms.

Radiation means:

The gamma rays emitted from radioisotopes can serve as effective disinfectants. Characteristics of an ideal disinfectant:

An ideal disinfectant should possess the following characteristics –

1. Toxic to pathogens at low concentration
2. Soluble and stable in water
3. Non-toxic to man and higher organisms
4. Cheap and easily available.

Disinfection with chlorine:

Chlorine is a very widely used disinfectant, as it satisfies the criteria of an ideal disinfectant. The mostcommonly used chlorine compounds are — chlorine gas (Cl_2), calcium hypochloride [$Ca(COCI_2)$], sodium hypochlorite (NaOCI) and chlorine dioxide (CIO_2).

The disinfection efficiency of chlorine depends on the number of microorganisms in the water being treated, pH and temperature.

Sewage/Waste Water Treatment:

A conventional sewage treatment plant has the requisite operating units arranged one after another fortreatment and final disposal of sewage.

Bioremediation:

Bioremediation is a treatment technology that uses biodegradation of organic contaminants through stimulation of indigenous microbial populations by providing certain amendments, such as adding oxygen, limiting nutrients, or adding exotic micro•bial species.

It uses naturally occurring or exter•nally-applied microorganisms to degrade and trans•form hazardousorganic constituents into com•pound of reduced toxicity and/or availability.

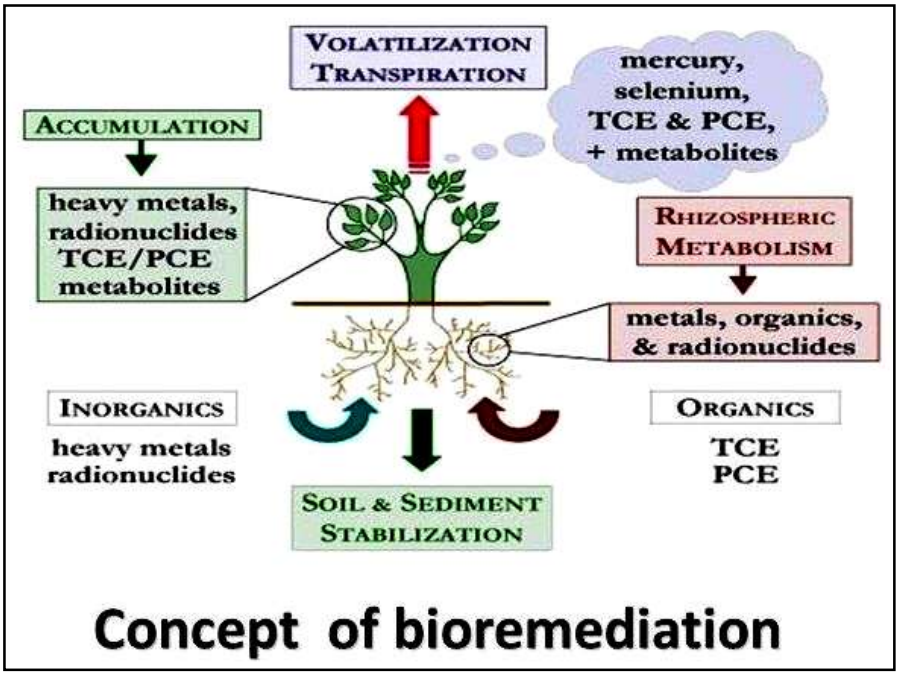
Xenobiotics broadly refer to the unnatural, foreign and synthetic chemicals such as pesticide, herbicide & other organic compounds.

Types of Bioremediation:

There are far more than 9 types of bioremediation, but the following are the most common ways in which it is used.

1. **Phytoremediation** – use of plants to remove contaminants. The plants are able to draw the contaminants into their structures and hold on to them, effectively removing them from soil or water.
2. **Bioventing** – blowing air through soil to increase oxygen rates in the waste. This is an effective way to neutralize certain oxygen sensitive metals or chemicals.
3. **Bioleaching** – removing metals from soil using living organisms. Certain types of organisms are draw to heavy metals and other contaminants and absorb them. One new approach was discovered when fish bones were found to attract and hold heavy metals such as lead and cadmium.
4. **Landfarming** – turning contaminated soil for aeration and sifting to remove contaminants, or deliberately depleting a soil of nitrogen to remove nitrogen based organisms.
5. **Bioreactor** – the use of specially designed containers to hold the waste while bioremediation occurs.
6. **Composting** – containing waste so a natural decay and remediation process occurs.
7. **Bioaugmentation –** adding microbes and organisms to strengthen the same in waste to allow them to take over and decontaminate the area.

8. **Rhizofiltration** – the use of plants to remove metals in water.
9. **Biostimulation –** the use of microbes designed to remove contamination applied in a medium to the waste.



There are two classes of bioremediation used. Don't confuse the class type with the actual types of bioremediation available, the classes describe the general application of the organisms. The two classes are –

***In-situ* –** In situ refers to when contaminated waste is treated right at its point of origin. For example, there may be soil that is contaminated. Rather than remove the soil from its point of origin, it is treated right where it is. The benefit to in situ treatment is that it prevents the spread of contamination during the displacement and transport of the contaminated material.

***Ex-situ* –** Ex situ refers to treatment that occurs after the contaminated waste has been removed to a treatment area. To use soil as the example again, the soil may be removed and transported to an area where the bioremediation may be applied. The main advantage to this is it helps to contain and controlthe bioremediation products, as well as making the area that was contaminated available for use.

Advantages of bioremediation:

- ✓ Bioremediation is a natural process and is therefore perceived by the public.
- ✓ Bioremediation is useful for the complete destruction of a wide variety of contaminants.
- ✓ Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible.
- ✓ Bioremediation can often be carried out on site, often without causing a major disruption ofnormal activities.
- ✓ Bioremediation can prove less expensive than other technologies that are used for cleanup ofhazardous waste.

Disadvantages of bioremediation:

- Bioremediation is limited to those compounds that are biodegradable. Not all compounds aresusceptible to rapid and complete degradation.
- There are some concerns that the products of biodegradation may be more persistent or toxicthan the parent compound.
- Biological processes are often highly specific. microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants.
- It is difficult to extrapolate (deduce) from bench and pilot-scale studies to fullscale fieldoperations.
- Bioremediation often takes longer than other treatment options.

11. Let's sum up

- Plants are multicellular and mostly photosynthetic organisms which found essentially everywhere, both in water and on land. The aquatic plants include red, brown and green algae and the land plants include mosses, ferns, gymnosperms and angiosperms.
- The microorganisms have always played an essential role in the biosphere with fermented foods and beverages, plant and animal diseases and nutrient cycling foremost.
- Mushrooms are the fruit bodies of edible fungi, commonly belonging to Basidiomycotina (*Agaricus campestris, A. brunnescens, Pleurotus sajor-caju, Volvariella volvacea* etc.) and rarely to Ascomycotina (*Morchella conica, M. esculenta*).
- Most remediation activity still makes use of conventional methods such as excavation and reburial, capping, and soil washing and burning. However, newly emerging biological cleanup methods, such as phytoremediation, are often simpler in design and cheaper to implement.
- Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant. This chapter will concern itself with environmental or abiotic factors that produce stress in plants, although biotic factors such as weeds, pathogens, and insect predation can alsoproduce stress.
- Genetically modified crops are defined as crops whose genomes have been altered in ways that do not occur naturally. Genetically engineered crops have genes added or removed using genetic engineering techniques, originally including gene guns, electroporation, microinjection and *Agrobacterium*.
- Plants are a vital component of biodiversity and healthy ecosystems. They provide a range of ecosystem services, from production of oxygen and removal of atmospheric carbon dioxide emissions, creation and stabilization of soil, protection of watersheds and provision of natural resources including food, fibre, fuel, shelter

and medicine.

- Conservation of biodiversity is protection, upliftment and scientific management of biodiversity so as to maintain it at its threshold level and derive sustainable benefits for the present and future generation.

12. Suggested Readings

1. Chawla, H.S. An Introduction to Plant Biotechnology (2nd ed.), 2002,Oxford & IBH
2. Singh, B.D. Biotechnology Latest ed., Kalyani Publishers.
3. Bhojwani, S.S. & Razdan, M.I. Plant Tissue Culture: Theory and Practise, Elsevier
4. Gupta, P.K. Biotechnology & Genomes, latest Ed., Rastogi Publications
5. Borer, A., Sentos, F.R. & Bowen, D.B. Understanding Biotechnology, 2003, Pearson Education
6. Ingacimuthu, S. Plant Biotechnology, 1997, Oxford & IBH
7. Walker, J.M. & Rapley, R. Molecular Biology & Biotechnology, 2000, Royal Society of Chemistry
8. Collin, H.A. and Edwards, S. Plant Cell Culture, 1998, Bios Scientific Publishers54
9. Dixon, R.A. & Gonzales, R. A. Plant Cell Culture: A Practical Approach, 1994, Oxford University Press
10. Dubey, R.C. Biotechnology, Latest Ed., S.Chand & Company Pvt. Ltd.
11. Rajdan, M.K. An Introduction to Plant Tissue Culture, Latest Ed., Oxford & IBH
12. Jha, T.B. & Ghosh, B. Plant Tissue Culture, 2003, Universities Press
13. Kar, D.K. & Halder, S. Plant Breeding, Biometry & Biotechnology, 2010, New Central Book Agency
14. 16. Slatter, A., Scott, N. & Fowler, N. Plant Biotechnology, 2003, Oxford University Press
15. Dey, K.K. Plant Tissue Culture, 1992, New Central Book Agency

16. http://www.biologydiscussion.com/
17. https://en.wikipedia.org/

13. Assignments

1. What is Glyphosate? What are the different mechanisms of glyphosate function?
2. How bt toxin helps to production of insecticide resistance plant.
3. What conditions do plant cells need to multiply *in vitro*?
4. Define embryoids.
5. Briefly describe the stages of somatic embryogenesis.
6. Mention the role of SERK and LEC genes during somatic embryogenesis.
7. What are main features doubled haploids? State its application in crop improvement?
8. How would you induce haploids?
9. Why plants produce secondary metabolites? What are the types of secondary metabolites?
10. Explain the different ways for the production of in vitro secondary metabolites.
11. What is meant by cellular totipotency?
12. Differentiate between somatic and zygotic embryogenesis.
13. What is a bioreactor? How is it used to scale up multiplication of commercially importantplants?
14. How is the antisense RNA technology used for production of transgenic plant varieties?
15. What is its significance in micropropagation?
16. What are transgenic crops? What are the advantages of such crops? Outline the method ofobtaining such crop for a specific attribute.

17. Write notes on Biopesticides.
18. What is bioremediation? Enlist different techniques of bioremediation.

All the materials are self writing and collected from e-book, journals and websites.