CHAPTER-9

BIOTECHNOLOGY: PRINCIPLES AND PROCESSES

Topic-1

Principles of Biotechnology and Tools of Recombinant DNA Technology

Concepts Covered • *Biotechnology, Principles of Biotechnology, Tools of Recombinant DNA technology.*

Revision Notes

- **Introduction**
  - **Biotechnology** deals with the techniques of using live organisms or their enzymes for products and processes useful to humans.
  - The term biotechnology was given by Karl Ereky (1919).
  - The **European Federation of Biotechnology (EFB)** defines Biotechnology as 'the integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'.
- **Biotechnology deals with:**
  - Microbe-mediated processes (making curd, bread, wine, etc.)
  - *In vitro* fertilisation ('test-tube' baby programme).
  - Synthesis and using of a gene.
  - Preparation of a DNA vaccine.
  - Correcting a defective gene.

Principles of Biotechnology

- **The two core techniques of modern biotechnology are:**
  - **(a) Genetic engineering:** The technique in which the genetic material (DNA and RNA) is chemically altered and introduced into host organisms to change the phenotype is known as genetic engineering.
  - **(b) Maintenance of sterile ambience:** It is necessary for chemical engineering processes to grow only the desired microbe / eukaryotic cell in large quantities for the manufacture of antibiotics, vaccines, enzymes, etc.
- Traditional hybridisation techniques lead to the inclusion and multiplication of undesirable genes along with desired genes.
- Genetic engineering helps to isolate and introduce only desirable genes into the target organism.
- A piece of DNA is not only able to multiply itself in the progeny cells of the organism, but, when it gets integrated into the recipient **genome**, it multiplies and inherits along with the host DNA.
- First recombinant DNA was emerged from the possibility of linking a gene of antibiotic resistance with a native plasmid of *Salmonella typhimurium*. The plasmid is an autonomously replicating circular extra-chromosomal DNA.
- Stanley Cohen and Herbert Boyer (1972) constructed the first recombinant DNA. They isolated the antibiotic resistance gene by cutting out a piece of DNA from a **plasmid**.

Steps in Genetically Modifying an Organism

- **There are three basic steps in genetically modifying an organism:**
  - **(a)** Identification of DNA with desirable genes.
  - **(b)** Introduction of the identified DNA into the host.
  - **(c)** Maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

Tools of Recombinant DNA technology

1. **Restriction Enzymes ('molecular scissors')**
   - The restriction enzymes are called molecular scissors and are responsible for cutting DNA.
   - In 1963, two enzymes responsible for restricting the growth of bacteriophage in *E. coli* were isolated. One of these added methyl groups to DNA. The other (restriction endonuclease) cut DNA.
   - The first restriction endonuclease is Hind II. Isolated by Smith, Wilcox and Kelley (1968) from *Haemophilus influenzae* bacterium. It always cuts DNA molecules at a particular point by recognizing a specific sequence of six base pairs. This is known as the recognition sequence for Hind II.
   - Today more than 900 restriction enzymes have been isolated from over 230 strains of bacteria.

Naming of the restriction enzymes

- First letter indicates genus and the second two letters indicate species of the **prokaryotic cell** from which they were isolated e.g., EcoRI comes from *E. coli* RY 13, where R = the strain, Roman numbers = the order in which the enzymes were isolated from that strain of bacteria.
- Restriction enzymes belong to a class of enzymes called **nucleases**.
- The nucleases include **exonucleases** and **endonucleases**.

Key Words

Genome : It is an entire set of DNA instructions found in a cell.

Plasmid : It is a small, circular, double stranded ANA molecule; It naturally exist in bacterial cells and in some eukaryotes.

Prokaryotic cell : Single-celled, lack true nucleus (i.e., nucleus is not bounded by a true nuclear membrane).

Palindrome : A DNA or RNA sequence that reads exactly the same in both directions.

(i) Exonucleases

- They remove nucleotides from the ends of the DNA.

(ii) Endonucleases

- They cut at specific positions within the DNA.
- Each restriction endonuclease can bind to a specific recognition sequence of the DNA and cut each of the two strands at specific points in their sugar-phosphate backbones.
- Each restriction endonuclease recognizes a specific palindromic nucleotide sequence in the DNA.
- The palindrome in DNA is a sequence of base pairs that read the same on the two strands in the 5' → 3' direction and in 3' → 5' direction. e.g.,

  5' —— GAATTC —— 3'

  3' —— CTTAAG —— 5'
- Restriction enzymes cut the strand a little away from the centre of the **palindrome** sites but between the same two bases on the opposite strands. This leaves single-stranded overhanging stretches at the ends. They are called sticky ends.

- They form H-bonds with their complementary cut counterparts. This stickiness facilitates the action of the enzyme **DNA ligase**.
- When cut by the same restriction enzyme, the resultant DNA fragments have the same kind of sticky-ends and these are joined together by the enzyme DNA ligases.

Separation and isolation of DNA fragments:

- DNA fragments formed by restriction endonuclease can be separated by a technique called **Gel electrophoresis**.
- DNA fragments are negatively charged. So, they can be separated by moving them towards the anode under the influence of an electric field through a medium / matrix such as **agarose** (which is a natural polymer of D-galactose and 3, 6 anhydro L-galactose and is extracted from sea weeds).
- The DNA fragments separate (resolve) according to their size through the sieving effect provided by the agarose gel.
- The smaller sized fragments move farther.
- The separated DNA fragments can be visualized after staining the DNA with **ethidium bromide** followed by exposure to UV radiation. Bright orange coloured DNA bands can be seen.
- The separated DNA bands are cut out from agarose gel and extracted from the gel piece. This step is called elution.
- These purified DNA fragments are used in constructing recombinant DNA by joining them with cloning vectors.

2. Cloning Vectors

- These are the DNA molecules that can carry a foreign DNA segment and replicate inside the host cells e.g., **plasmids** (circular extra-chromosomal DNA of bacteria) and **bacteriophages**.
- Bacteriophages (high number per cell) have very high copy numbers of their genome within the bacterial cells.
- Some plasmids have only 1-2 copies per cell. Others may have 15-100 copies per cell.
- When the cloning vectors are multiplied in the host the linked piece of DNA is also multiplied to the numbers equal to the copy number of the vectors.

Key Fact

Humans shares about 98% of their genes with chimpanzees, 92% with mice, 18% with (*E. coli*) bacteria.

Features of cloning vector:

(a) Origin of replication (*ori*)

- This is a DNA sequence from where **replication** starts. A piece of DNA linked to *ori* site can replicate within the host cells. This also controls the copy number of the linked DNA. So, to get many copies of the target DNA, it should be cloned in a vector whose origin support high copy number.

(b) Selectable marker (marker gene)

- It helps to select the transformants and eliminate the non-transformants.
- **Transformation** is a procedure in which a piece of DNA is introduced in a host bacterium.
- Selectable markers of *E. coli* include the genes encoding resistance to antibiotics like ampicillin, chloramphenicol, tetracycline or kanamycin, etc.
- The normal *E. coli* cells do not carry resistance against any of these antibiotics.

(c) Cloning sites

- To link the alien DNA, the vector needs very few recognition sites for restriction enzymes.
- Presence of more than one recognition site generates several fragments, which complicates the gene cloning.
- The ligation of alien DNA is carried out at a restriction site present in one of the two **antibiotic resistance genes**. e.g., ligation of a foreign DNA at the BamHI site of the tetracycline resistance gene in the **vector pBR322**.
- The recombinant plasmids lose tetracycline resistance due to insertion of foreign DNA. But, they can be selected out from non-recombinant ones by plating the transformants on **ampicillin** containing medium.
- Then, these transformants are transferred to a **tetracycline** medium.
- The recombinants grow in ampicillin medium but not on tetracycline medium. But, non-recombinants will grow on the medium containing both the antibiotics.

- In this case, one antibiotic resistance gene helps to select the transformants, whereas the other antibiotic resistance gene gets inactivated due to the insertion of alien DNA and helps in the selection of recombinants.
- Selection of recombinants due to the inactivation of antibiotics requires simultaneous plating on two plates having different antibiotics.
- Therefore, alternative selectable markers have developed to differentiate recombinants from non-recombinants based on their ability to produce colour in the presence of a chromogenic substrate.
- A recombinant DNA is inserted within the coding sequence of an enzyme, β-galactosidase. So, the enzyme is inactivated. It is called **insertional inactivation**. Such colonies do not produce any colour. These are identified as recombinant colonies.
- If the plasmid in bacteria do not have any insert it gives blue coloured colonies in presence of chromogenic substrate.

(d) Vectors for cloning genes in plants and animals

- Genetic tools of some pathogens can be transformed into useful vectors for delivering genes to plants and animals. e.g., *Agrobacterium tumefaciens* (a pathogen of many dicot plants) can deliver a piece of DNA (T-DNA) to transform normal plant cells into a tumor.
- These tumor cells produce the chemicals required by the pathogen.
- The **tumor-inducing (Ti) plasmid** of *A. tumefaciens* is modified into a cloning vector which is not pathogenic to the plants but can use the mechanisms to deliver genes of interest into plants.
- Retroviruses in animals can transform normal cells into **cancerous** cells. So, they are used to deliver desirable genes into animal cells.

3. Competent Host (For Transformation with Recombinant DNA)

- Competent cells are capable of uptaking DNA from the surrounding. For the process of transformation, bacterial cells are made competent, so that DNA can enter the cells.
- DNA is a hydrophilic molecule. So it cannot pass through cell membranes.
- To avoid this problem, bacterial cells are treated with a specific concentration of a divalent cations (e.g., calcium), so as to increase the pore size in the cell wall.
- So, DNA enters the bacterium through pores in the cell wall. Such cells are incubated with recombinant DNA on ice.
- They are then placed briefly at 42°C (heat shock) and then put them back on ice. This enables the bacteria to take up the recombinant DNA.

Other methods to introduce alien DNA into host cells:

(a) Micro-injection: In this, recombinant DNA is directly injected into the nucleus of an animal cell.

(b) Biolistics (gene gun) method: In this, cells are bombarded with high-velocity micro-particles of gold or tungsten coated with DNA. This method is suitable for plants.

(c) 'Disarmed pathogen' vectors: These vectors, when infect the cell, transfer the recombinant DNA into the host.

IMPORTANT DIAGRAMS.

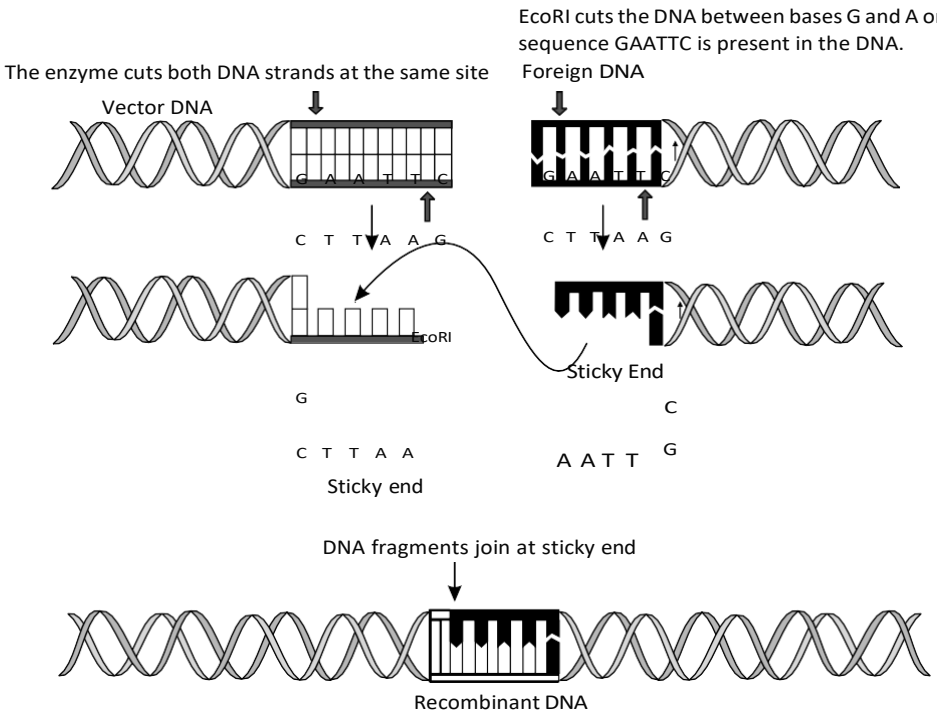

Fig 9.1: Steps in formation of recombinant DNA by action of restriction endonuclease enzyme- EcoRI.

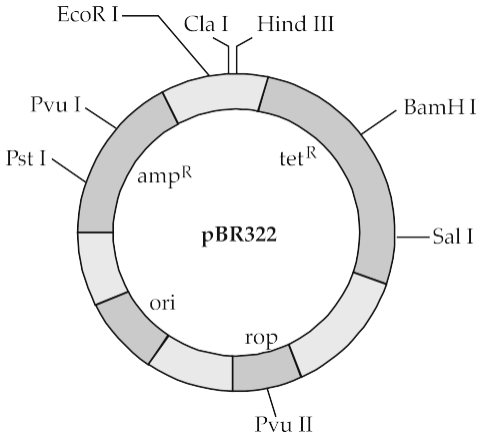

Fig 9.2: Cloning vector pBR322

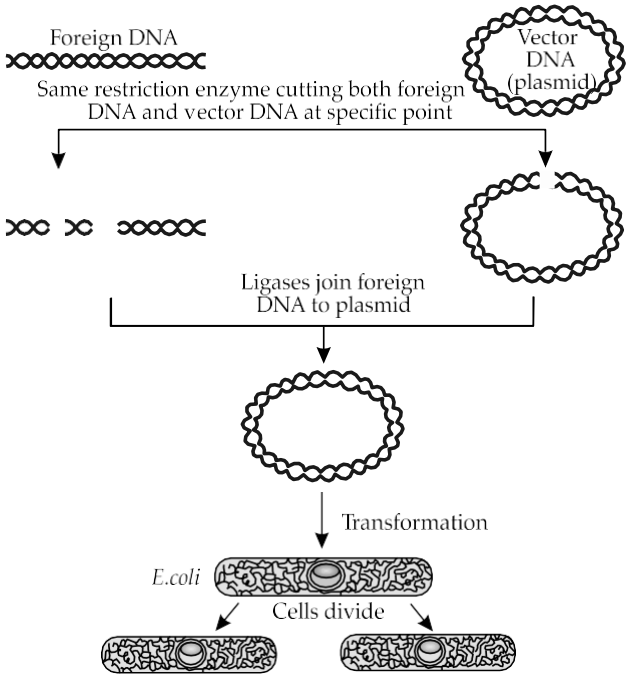

Fig 9.3: Diagrammatic representation of recombinant-DNA technology

Mnemonics

1. Concept: Important tools in **B**iotechnology.
Mnemonics: HELina **G**yi **G**aate **G**ate: **S**peed of 40 **H**ilte-dulte **G**un **G**unaate by **R**oad **H**ighway
Interpretations: Tools : Characters
Heat Shock : Sudden temperature raised to 40°C
Electroporation : High voltage pulse
Liposomes : Genes to cells in vivo
Gene gun : Gold particles
Genetic marker : R plasmid
Gene synthesis : Hormone somatostatin

2. Concept: Animal Clones
Mnemonic: No **D**ue **N**ames : **P**ut **S**ome **A**lmonds
Interpretation: Clone name : Species
Noori : Pashmina Goat
Dolly : Sheep
Noah : Asian Gaur

Example 1

Q. Describe the formation of recombinant DNA by the action of EcoRI.

Sol. The formation of recombinant DNA by the action of EcoRI are as follows:

(a) Each restriction endonuclease recognizes a specific palindromic nucleotide sequences in the DNA.

(b) It cuts the strand of DNA a little away from the centre of the palindrome sites, but between the same two bases on the opposite strands. This leaves single stranded portions at the ends.

(c) There are overhanging stretches called sticky ends on each strand.

(d) These are named so because they form hydrogen bonds with their complementary cut counterparts.

(e) This stickiness of the ends facilitates the action of the enzyme DNA ligase.

Topic-2 Process of Recombinant DNA Technology

Concepts Covered ● *Processes of rDNA technology* ● *Bioreactors*

Revision Notes

- **Isolation of the Genetic Material (DNA)**
  - To get pure DNA (free from other macro-molecules), the bacterial cells / plant or animal tissue are treated with enzymes such as **lysozyme** (bacteria), **cellulase** (plant cells), **chitinase** (fungus), etc.
  - The cell is broken to release DNA along with other macromolecules (RNA, proteins, polysaccharides and lipids).
  - Genes (DNA) are interwined with proteins such as **histones**.
  - RNA is removed by treating with **ribonuclease**.
  - Proteins are removed by treatment with **protease**.
  - Other molecules are removed by appropriate treatments.
  - When chilled ethanol is added, purified DNA precipitates out as a collection of fine threads in the suspension.
- **Cutting of DNA at Specific Locations**
  - Restriction enzyme digestions are performed by incubating purified DNA with the restriction enzyme, at the optimal conditions.
- **Isolation of derived DNA fragments.**
  - **Agarose gel electrophoresis** is employed to check the progression of a restriction enzyme digestion. As DNA is negatively charged, it moves towards the anode. The process is repeated with the vector DNA also.
  - After cutting the source DNA and the vector DNA, the cutout gene (DNA segment) of interest from the source DNA and the cut vector are mixed and ligase is added.
  - This creates recombinant DNA.
- **Amplification of Gene of Interest Using PCR**
  - **Polymerase Chain Reaction (PCR)** is the synthesis of multiple copies of the gene of interest *in vitro* using two sets of **primers** and the enzyme **DNA polymerase**.
  - The technique was developed by Kary Mullis in 1985 and for this, he was awarded the Nobel Prize in 1993.
  - DNA polymerase enzyme joins the nucleotides
  - Most commonly enzyme is *Taq* polymerase

Key Fact

Polymerase Chain Reaction (PCR) can be used to identify diseases. This technology was used during SARS epidemic, 2003.

- **Insertion of Recombinant DNA into the Host Cell / organism**
  - There are several methods of introducing the ligated DNA into recipient cells.
  - Recipient cells take up DNA present in its surrounding.

- If a recombinant DNA bearing **ampicillin resistant gene** (a selectable marker gene) is transferred into ***E. coli*** cells, the host cells become ampicillin-resistant cells.
- If the transformed cells are spread on agar plates containing ampicillin, only transformants will grow, non transformed recipient cells will die.

Key Word

Histone: A type of protein found in chromosomes. Histones bind to DNA, help give shape to chromosomes and help control the activity of genes.

- **Obtaining the Foreign Gene Product**
  - The ultimate aim of recombinant DNA technology is to produce a desirable protein.
  - The foreign gene gets expressed under appropriate conditions.
  - If a protein-encoding gene is expressed in a heterologous host, it is called a **recombinant protein**.
  - The cells with foreign genes may be grown on a small scale in the laboratory.
  - The cultures may be used to extract the desired protein and purified using different separation techniques.
  - The cells can also be multiplied on large scale in a continuous culture system.
  - Here, the used medium is drained out from one side while the fresh medium is added from the other side.
  - It maintains the cells more physiologically active and so produces a larger biomass leading to higher yields of the desired protein.
- **Downstream Processing:** All the processes to which the product is subjected to before being marketed as a final and finished product are called as downstream processing.
  - It includes a series of processes such as separation and purification of products after the biosynthetic stage.
  - The product is formulated with suitable preservatives.
  - Such formulation undergoes through clinical trials as in the case of drugs.
  - Strict quality control testing for each product is also required.
  - The downstream processing and quality control testing vary from product to product.
- **Bioreactors**
  - To produce large quantities of products, the bioreactors are used where large volumes (100-1000 litres) of culture can be processed.
  - Bioreactors are the vessels in which raw materials are biologically converted into specific products, enzymes etc., using microbial plant, animal or human cells.
  - A bioreactor provides the optimal growth conditions (temperature, pH, substrate, salts, vitamins, oxygen) for achieving the desired product.
  - There are two types of bioreactors namely,
    (a) Simple stirred-tank bioreactor
    (b) Sparged stirred-tank bioreactor
  - The most commonly used bioreactors are of stirring type.
- **Stirred-tank Reactor**
  - It is usually cylindrical or with a curved base to facilitate the proper mixing of the reacting contents.
  - The stirrer facilitates even mixing and oxygen availability throughout the bioreactor.
  - Alternatively, air can be bubbled through the reactor.
  - The bioreactor has
    (a) An agitator system.
    (b) An oxygen delivery system.
    (c) A foam control system.
    (d) A temperature control system.
    (e) pH control system.
    (f) Sampling ports (for periodic withdrawal of the culture).
    (g) The contents are mixed by stirrer. This makes the oxygen available throughout the bioreactor.

IMPORTANT DIAGRAMS:

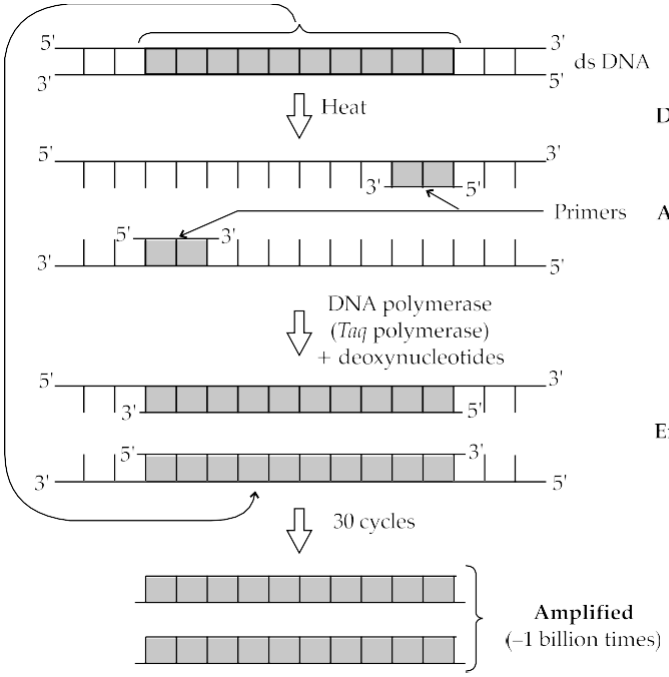

Fig. 9.4: Polymerase Chain reaction

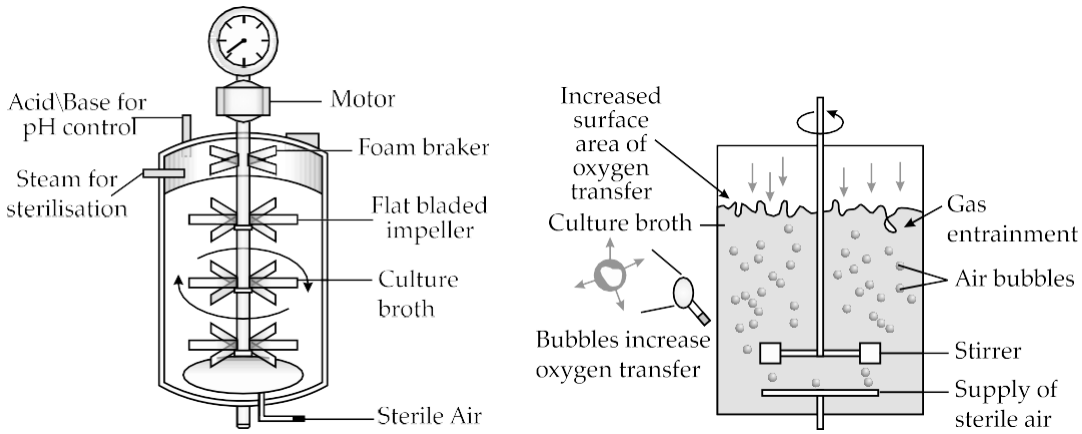

Fig. 9.5: Bioreactors- (a) Simple stirred-tank Bioreactor, (b) Sparged stirred-tank bioreactor

Example 2

Q. Write the steps you would suggest to be undertaken to obtain a foreign-gene-product.

Sol. The steps to be undertaken to obtain a foreign-gene-product are as follows:

- Insertion of a piece of desired DNA into cloning vector to get recombinant DNA.
- Transfer of recombinant DNA into a host cell. (Plant or animal or bacterial cell).
- The alien DNA will get multiplied.
- After the cloning of gene of interest, optimised conditions are provided to the culture to induce the expression of the target gene.
- Extraction of the desired product.
- Purification of desired products by using different separation technique.