CHAPTER - 11
BIOTECHNOLOGY: PRINCIPLES AND PROCESSES

1. **Biotechnology :** Biotechnology deals with microorganisms, plant or animal cells or their enzymes to produce products and processes useful to humans. The term 'Biotechnology' was given by Karl Ereky (1919).
   According to **European Federation of Biotechnology (EFB),** ' biotechnology is the integrated use of biochemistry, microbiology and engineering sciences in order to achieve technological application of the capabilities of microorganisms, cultured tissues/cells and parts thereof'.

2. **Principles of biotechnology:** Biotechnology is based mainly on two core technologies-
   - **a. Genetic engineering:** It is the manipulation of genes by man i.e., artificial synthesis, isolation, modification, combination, addition and repair of the genetic material (DNA) to alter the phenotype of host organism to suit human needs.
   - **b. Biotechnological engineering processes** that help the growth of desired microbe/eukaryotic cell in large quantities in sterile medium (tissue culture technique) for the manufacture and multiplication of biotechnological products (antibiotics, vaccines, enzymes, hormones etc.

   The basic steps in genetic engineering include:

   i) Identification of the DNA with desirable gene
   ii) Introduction of the DNA into the host to form recombinant DNA (rDNA)
   iii) Maintenance of DNA in the host and gene cloning
   iv) Gene transfer

   In 1972, Stanley Cohen and Herbert Boyer constructed the first recombinant DNA.

   Steps carried out in constructing first rDNA:

   i) A gene encoding antibiotic resistance in the native plasmid of *Salmonella typhimurium V,* was identified. Plasmid is an autonomously replicating small circular extra-chromosomal DNA.

ii) The desired DNA was cut at specific locations by restriction endonuclease.

iii) The cut DNA was linked to plasmid DNA and transferred to *E.coli* for gene manipulation.

3. Tools of recombinant DNA technology

i) Restriction enzymes ii) polymerase enzymes iii) ligases iv) vectors v) host organism or cell vi) passenger DNA

4. **Restriction enzymes**: The restriction enzymes are called molecular scissors and are responsible for cutting DNA. They are present in bacteria to provide defence mechanism called **restriction modification system. The** first restriction endonuclease, Hind II, was isolated By Smith, Wilcox and Kelley in 1968 from *Haemophilus influenzae* bacterium. It was used to cut DNA molecules at a particular point by recognizing a specific sequence of 6 base pairs, known as **recognition site.**

Naming of restriction enzymes

The first letter is derived the genus name and the next two letters from the species name of the prokaryotic organism from where the enzyme was extracted. The fourth letter comes from the strain and the roman numeral indicates the order in which the enzyme was derived from that strain of bacteria For example *Eco*RI is derived from *Escherichia coli* RY13 strain, here '*E*' is from *Escherichia,' co'* is from *coli,'* R' is from RY13 and the roman numeral 'I' indicates that this enzyme is the first restriction enzyme derived from this very strain of bacteria. Some of the restriction enzyme are-*Bam*HI (*Bacillus amyloliquefaciens), Eco*RII (*Escherichia coli* R245) etc.

Restriction enzyme belongs to a class of enzymes called nucleases and are of two type:

A) Exonucleases – cut the DNA at terminal ends.

B) Endonucleases-cut the DNA at any other positions from within the DNA except at the terminal ends.

The recognition sequence of endonucleases is palindromic, i.e., the sequences of bases are same when read from both the DNA strands, when the orientation/direction of reading is kept the same e.g., 5’---GAATTC---3’

3’---CTTAAG---5’

Mechanism of action of Restriction Endonucleases

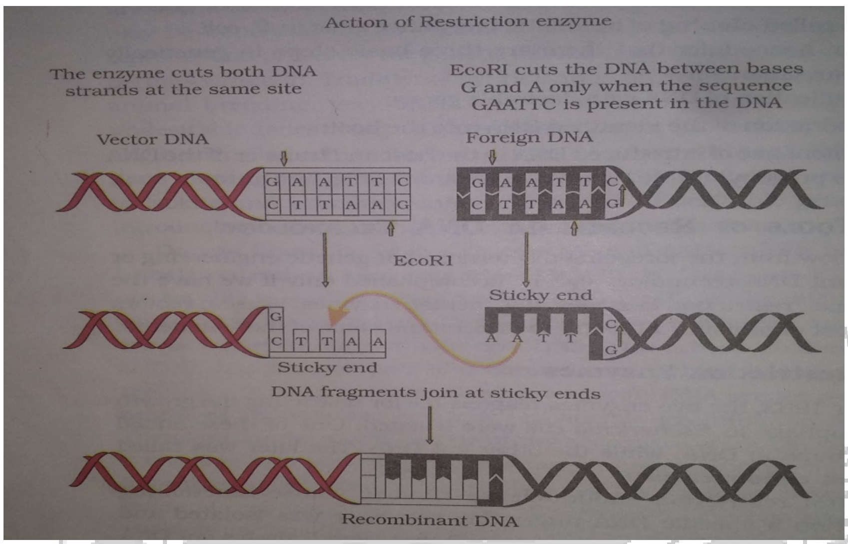

Figure: Diagrammatic representation showing action of restriction enzyme (*EcoRI)* on the substrate DNA producing sticky ends for *r*DNA formation.

Every restriction endonuclease inspects the entire DNA sequence for the palindromic recognition sequence.

On finding the palindrome, the restriction endonuclease binds to the DNA.

It cuts the opposite strands of DNA in sugar-phosphate backbone; a little away from the centre of the palindromic sites but between the same bases on both the strands.

The result is the formation of single stranded overhanging stretches at the end of each strand called sticky ends.

The sticky ends facilitate the action of the enzyme DNA ligase by readily forming hydrogen bonds with the complementary strands.

In genetic engineering, DNA from different sources are cut with the same restriction enzymes so that both DNA fragments have the same kind of sticky ends.

These sticky ends are complementary to each other and thus can be joined by DNA ligase (end-to-end) producing rDNA.

5. **Separation and Isolation of DNA fragments (Gel Electrophoresis)**
Gel electrophoresis is a technique for separating DNA fragments base on their sizes.
First, the DNA sample is cut into fragments by restriction endonucleases. The DNA fragments being negatively charged can be separated by forcing them to move towards the anode under electrical field through a medium/matrix.
Commonly used matrix is agarose, which is a natural linear polymer of D-galactose and 3,6-anhydrous L-galactose which is extracted from sea weeds.
The DNA fragments separate-out (resolve) according to their size because of the sieving property of agarose gel. Hence, smaller the fragment size, the farther it will move.
The separated DNA fragments are visualised after staining the DNA with ethidium bromide followed by exposure UV radiation. The DNA fragments are seen as orange coloured bands. The separated bands of DNA are cut out and extracted from the gel. This step is called elution. The purified DNA fragments are used to form rDNA which can be joined with cloning vectors.

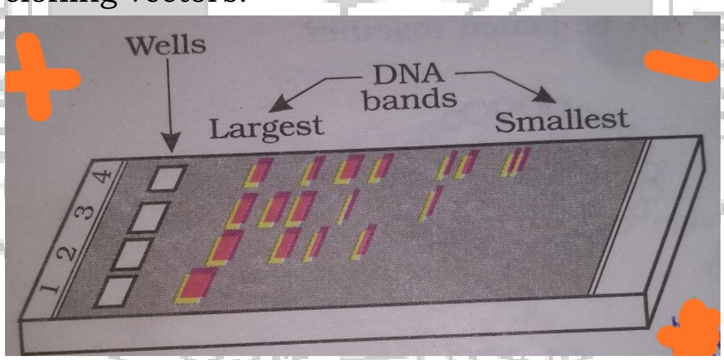

Figure: Agarose gel electrophoresis showing migration of Undigested (lane 1) and digested DNA fragments (lane 2-4)

6. **Cloning Vectors** : The vectors are the DNA molecules that can carry a foreign DNA segment into the host cell.
Vectors may be of: A) Plasmids: These are autonomously replicating small circular extra chromosomal DNA. B) Bacteriophages:-These are bacteria which can infect viruses.
Copy number: It is defined as the number of copies of vectors present in a cell. It varies from 1-100 copies per cell.

The best known vector is plasmid vector. **pBR322** is the first artificial vector developed in 1977 by Boliver and Rodriguez from *E. coli* plasmid.

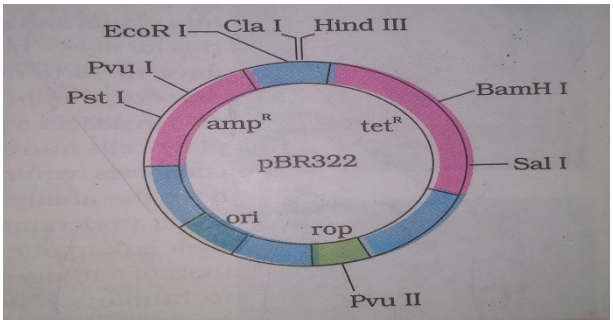

Figure 11.4 *E. coli* cloning vector pBR322 showing restriction sites (*Hind* III, *Eco*R I, *Bam*H I, *Sal* I, *Pvu* II, *Pst* I, *Cla* I), ori and antibiotic resistance genes (amp^R and tet^R). Rop codes for the proteins involved in the replication of the plasmid.

The following features are required to facilitate cloning into a vector:

i) Origin of replication (*ori)* : This is a DNA sequence that is responsible for initiating replication. Any piece of DNA when linked to this sequence can replicate within the host cells. *ori* also controls the copy number of the linked DNA.

ii) Selectable marker: It helps to select the host cells which contain the vector (transformants) and eliminate the non-transformants. **Transformation** is defined as the procedure by which a piece of DNA is introduced into a bacterial host. Genes encoding resistance to antibiotics like ampicillin, chloramphenicol, tetracycline or kanamycin, are useful selectable markers for *E. coli.* The normal *e. coli* cells do not carry resistance against these antibiotics.

iii) Cloning sites: To link the alien DNA, the vectors require very few (mostly single) recognition sites for restriction enzymes. More than one recognition sites within the vector, can complicate the cloning as it will generate several fragments. Ligation of alien DNA can be carried out at a restriction site present in one of the antibiotic resistance genes.

iv) Vectors for cloning genes in plants and animals: There are several vectors for cloning genes in plants and animals. In plants, the tumour inducing plasmid (Ti) of *Agrobacterium tumifaciens* is used as a cloning vector. *A. tumifaciens* is a pathogen of several dicot plants. It delivers a piece of DNA known as 'T' DNA in the Ti

plasmid which transforms normal plant cells into tumour cells to produce chemicals against pathogens. Retrovirus, adenovirus, papillovirus are also now used as cloning vectors for animals because of their ability to transform normal cells into cancerous cells.

Selection of recombinants formed can be done by one of the following methods:

a) Inactivation of antibiotics: If a foreign DNA ligates at *Bam*HI site of tetracycline resistance gene in the vector pBR322, the recombinant plasmid loses tetracycline resistance due to insertion of Foreign DNA. It can still be selected out from non-recombinant ones by plating the transformants on ampicillin containing medium. The transformants growing on ampicillin containing medium are then transferred on to a medium containing tetracycline. The recombinants can grow in ampicillin containing medium but not on that containing tetracycline, whereas non-recombinants can grow on medium containing both the antibiotics and thus recombinants are selected.
b) **Insertional inactivation:** On the basis of colour production in presence of a chromogenic substrate, the recombinants and non-recombinants can also be differentiated. Here, a recombinant DNA is inserted within the coding sequence of an enzyme β-galactosidase, which results into the inactivation of the enzyme. The bacterial colonies having inserted plasmids, show no colouration while those without inserted plasmid form blue colouration.

7. Competent host (for transformation with rDNA)-

DNA being a hydrophilic molecule cannot pass through cell membrane. Therefore, bacteria should be made competent to accept the DNA molecules. Competency is the ability of a cell to take up foreign DNA. The cell is made competent by the following methods: a) Chemical method, b) Physical method.

a) Chemical method; The cell is treated with specific concentration of divalent cation such as calcium to increase pore size of the cell wall. The cells are incubated with rDNA on ice, followed by placing them briefly at 42^0C and then putting them back on ice. This is called shock treatment. The bacteria can now take up recombinant DNA.

b) Physical method: It includes- Micro-injection method where rDNA is directly injected into the nucleus of the animal cell. Biolistic/gene gun method: the cells are bombarded with high velocity micro-particles of gold or tungsten coated with rDNA (mostly in plant cells). Disarmed pathogen vectors are also used to transfer rDNA.

8. Process of recombinant DNA technology

Recombinant DNA technology involves the following steps:

i) Isolation of DNA
ii) Fragmentation of DNA by restriction endonuclease.
iii) Isolation of a desired DNA fragment/gene of interest
iv) Amplification of gene of interest
v) Ligation of gene of interest to a vector
vi) Insertion of recombinant DNA into the host
vii) Culturing of the host cells on a suitable medium on a large scale
viii) Extraction of the desired gene product.
ix) Downstream processing of the product as finished product, ready for marketing.

i) **Isolation of DNA**: The bacterial/fungal/plant/animal cell is broken down by enzymes to release DNA, along with RNA, proteins, polysaccharides and lipids. Bacterial cell is treated with lysozyme, fungal cell with Chitinase, plant cell with cellulose. RNA is removed by treating with ribonuclease and proteins are removed by treating with protease. After several treatments, the purified DNA is precipitated by adding chilled ethanol.

ii) **Cutting of DNA at specific locations:** the DNA is cut using restriction endonuclease. The purified DNA is incubated, with specific restriction enzymes at conditions optimum for enzymes to act.

iii) **Isolation of desired DNA fragment/gene of interest:** Using agarose gel electrophoresis, the activity of the restriction enzymes can be checked. Since DNA is negatively charged, it moves towards the Anode or positive electrode and in the process, DNA fragments separate out based on their sizes. The desired DNA fragment is eluted out.

iv) **Amplification of the gene of interest:** The polymerase chain reaction (PCR) is a reaction in which amplification of specific DNA sequences is carried out *in vitro.* The technique was developed by Kary Mullis in 1985, and for this he received Nobel Prize for Chemistry in

1993. PCR requires the following- a) DNA template: The double stranded DNA that need to be amplified. b) Primers: Small chemically synthesized oligonucleotides of about 10-18 nucleotides that are complementary to a region of template DNA. c) Enzyme: Two commonly used enzymes are *Taq* polymerase (isolated from thermophilic bacterium, *Thermus aquaticus*) and DNA polymerase (vent) (isolated from *Thermococcus litoralis).*

PCR is carried out in the following three steps:

A. Denaturation-The double stranded DNA is denatured subjecting to high temperature of abut 95^{0}C for about 15 second. Each separated single stranded DNA now acts as a template for DNA replication.

B. **Annealing-** Two sets of primers are added which anneal to the 3' end of each separated DNA strand. Primers act as initiators of replication.

C. Extension- DNA polymerase extends the primers by adding nucleotides complementary to the template provided in the reaction. A thermostable DNA polymerase (*Taq* polymerase) is used in the reaction which can tolerate the high temperature of the reaction. All the steps are repeated many times to obtain several copies of the desired DNA.

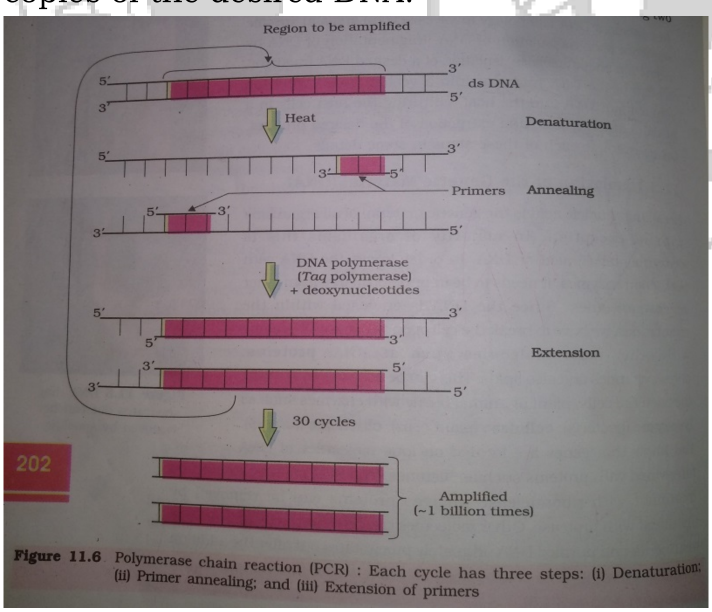

Figure 11.6 Polymerase chain reaction (PCR) : Each cycle has three steps: (i) Denaturation; (ii) Primer annealing; and (iii) Extension of primers

v) Ligation of DNA fragment into a vector: The vector DNA and the source DNA are cut out with the same restriction endonuclease to

obtain sticky ends. These are then ligated by mixing vector DNA, gene of interest and enzyme DNA ligase to form a recombinant DNA.

vi) Insertion of rDNA into the host cell/organism: Introduction of ligated DNA into recipient cells occurs by several methods, before which the recipient cells are made competent to receive the DNA. if rDNA carrying antibiotic resistance (e.g., ampicillin) is transferred into *E. coli* cells, the host cell is transformed into ampicillin resistant cells. The ampicillin resistant gene in this case is called a selectable marker. On growing the transformed cells on agar plates containing ampicillin, only transformants will grow and others will grow.

vii) Culturing the host cells- The transformed cells are grown in appropriate nutrient medium at optimal conditions. The DNA gets multiplied and expresses itself to form desired product.

viii) Extraction of the desired gene product: When a protein encoding gene is expressed in a heterologous host, it is called a recombinant protein. The cells having gene of interest can be grown on a small scale or a large scale. On small scale, the cells are grown on cultures in laboratory and then the expressed protein is extracted and purified by different separation techniques. On the large scale, the cells are grown in a continuous culture system in which fresh medium is added from one side to maintain cells in exponential growth phase and the desired protein is collected from the other side. In large scale method, larger biomass is produced which leads to high yield.

ix) Downstream processing: All the processes to which a product is subjected to before being marketed as a finished product are called downstream processing.
It includes: a) separation of the product from the reactor. b) Purification of the product. c) Formulation of the product with suitable preservatives. D) Quality control testing and clinical trials in case of drugs.

9. Bioreactors

Bioreactors are vessels of large volumes (100-1000 litres) in which raw materials are biologically converted into specific products. It provides all the optimal conditions for achieving the desired product by providing optimal growth conditions like temperature, pH, substrate, salt, vitamins and oxygen. Stirred-tank bioreactors are commonly used bioreactors. A

bioreactor has the following components: i) An agitator system, ii) An oxygen delivery system, iii) foam control system, iv) Temperature control system, v) pH control system, vi) Sampling ports to withdraw cultures periodically.

Bioreactors are cylindrical with curved base to facilitate proper mixing of contents. The stirrer mixes the contents and makes oxygen available throughout the bioreactor. Sparged stirred-tank reactor is a stirred type reactor in which air is bubbled.

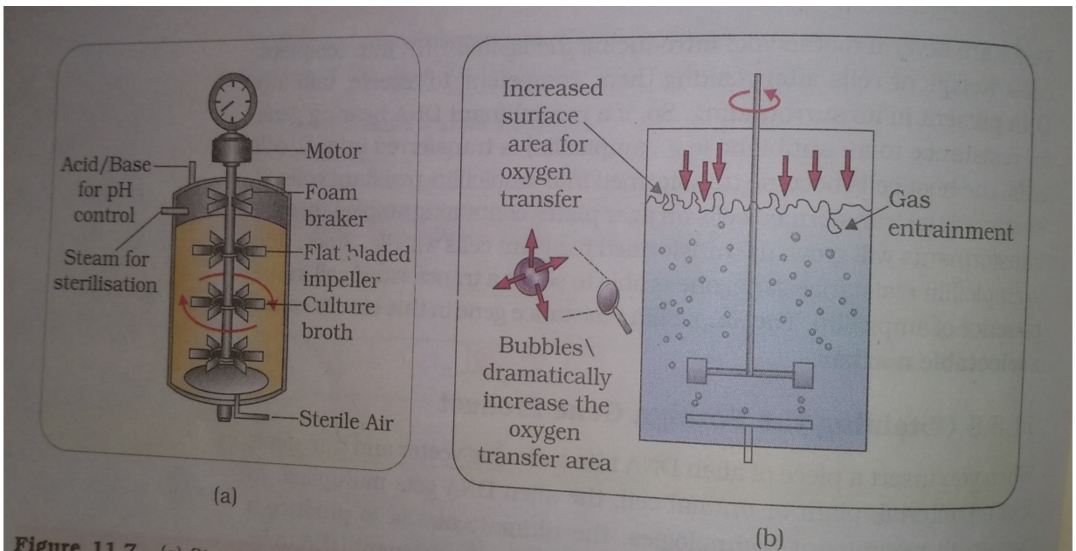

Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged