4.BIOTECHNOLOGY PRINCIPLES AND PROCESSES

TOOLS OF RECOMBINANT DNA TECHNOLOGY:

1.Restriction enzymes
2.Plymerase Enzyme
3.Ligase Enzyme
4.Cloning vector
5.Competent host (For transformation With Recombinant DNA)

Restriction Enzymes (Molecular Scissors):

- Restriction enzymes are the enzymes produced by certain bacteria and they have the property of cleaving DNA molecule at specific base sequences.
- A bacterium produces a restriction enzyme to defend against bacterial viruses called bacteriophages, or phages.
- The restriction enzyme prevents replication of the phage DNA by cutting it into many pieces.
- The restriction enzymes cut DNA at specific base pair sequence, and these specific base sequence is known as the **recognition sequence.**
  The first discovered restriction endonuclease - Hind II

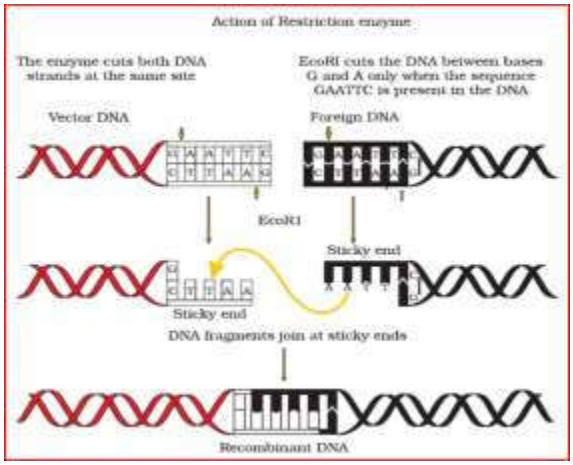
Nomenclature of Restriction Enzymes

- The naming of restriction enzymes are based on their origin and type of action
  For e.g. Eco RI
- E - The first letter of the enzyme name comes from the first letter of the genus of the prokaryotic cell from which enzyme were isolated (here 'E' stands for Escherichia)
- Co - The second two letters come from the species name.('co' stands for coli)
- R- Third letter indicates first letter of name of strain('R' stands for RY 13 strain of bacteria from which the enzyme obtained)
- I (roman numeral one)- Indicates the order of discovery of enzyme
- Restriction enzymes belong to a larger class of enzymes called Nucleases.
- These are of two kinds; Exonucleases and Endonucleases.
- Exonucleases remove nucleotides from the ends of the DNA whereas, endonucleases make cuts at specific position within the DNA.
- Each restriction endonuclease recognizes a specific palindromic nucleotide sequences in the DNA.
- Palindromes are groups of letters that form the same words when read both forward and backward.eg. "MALAYALAM".
- The palindrome in DNA is a sequence of base pairs that reads same on the two strands when orientation of reading is kept the same.
- The recognition site of Eco,RI is given below ; it is a palindromic sequence

G A A T T C
C T T A A G

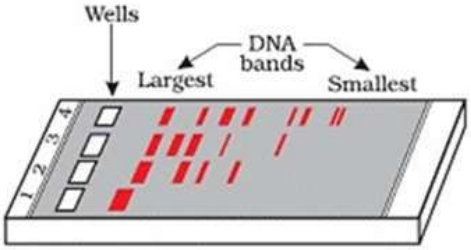
- Restriction enzymes cut the strand of DNA a little away from the center of the palindrome sites, but between the same two bases on the opposite strands.
- This leaves single stranded portions at the ends called Sticky ends.
- The stickiness of the strands facilitates the action of the enzyme DNA ligase.

- For e.g. EcoRI cut the DNA between G and A only when the sequence GATTC present in the DNA. It is the recognition site of EcoRI
- Restriction endonucleases are used in genetic engineering to form recombinant molecules of DNA which are composed of DNA from different sources or genome.



Separation and Isolation of DNA fragments (DNA of interest):

- The cutting of DNA by restriction endonucleases results in the fragments of DNA.
- These fragments can be separated by a technique known as **Gel Electrophoresis.**
- Negatively charged DNA fragments can be separated by forcing them to move towards the anode under an electric field through a medium.
- Nowadays most commonly used matrix/medium is Agarose Gel,which is a natural polymer extracted from sea-weeds.
- The DNA fragments are separated according to their size through sieving effect provided
  by the agarose .
- The particles with a smaller size have been reported to move faster and farther away from the
  well.
- As a result the molecules are separated by size.
- Electrophoresis enables you to distinguish DNA fragments of different lengths.
- The separated DNA fragments can be visualized only after staining the DNA with
- Ethidium bromide followed by exposure to UV radiation. Now DNA fragments appear bright orange coloured bands.
- The separated bands of DNA are cut out from the agarose gel and extracted from the gel
  piece. This step is known as **Elution**



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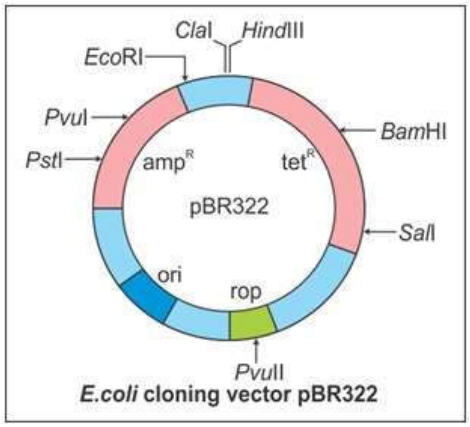
Cloning Vectors (Vehicles for Cloning):

- Vector serves as a vehicle to transfer a foreign gene (DNA sequence) into a given host cell.
- It has the ability to self replicate and integrate into the host cell

Salient features of a Vector:

- It should contain an origin of replication **(ori)** so that it is able to multiply within the host cell.
- It should have restriction sites for the insertion of the target DNA.
- It should incorporate a selectable marker (antibiotic resistance gene), which will allow to select those host cells that contain the vector from amongst those which do not.
- Some of the commonly used vectors are Plasmids, Bacteriophages, Plant or animal viruses Etc



E.coli cloning vector pBR322

- Plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA.
- The characteristic features of a plasmid are following

1.Origin of Replication

- It is the sequence from where replication starts.
- Any piece of DNA when linked to this sequence can be made replicate within the host cells.
- This sequence is responsible for controlling the copy number of the linked DNA.

2.Selectable Markers

- A vector requires a selectable marker which helps in identifying and eliminating transformants from non-transformants.
- **Transformation** is a procedure through which a piece of DNA is introduced in a host bacterium
- Normally, the genes encoding resistance to antibiotic such as ampicillin, chloramphenicol, tetracycline or kanamycin etc. are considered useful selectable marker for E.coli.

3.Cloning Sites

- A vector consists of some recognition sites where the alien DNA joins.
- The ligation of alien DNA is carried out at a restriction site present in one of the two antibiotic resistance genes, for e.g. we can ligate a foreign DNA at the Bam H1 site of tetracycline resistance gene in the vector **pBR 322** .
- The recombinant plasmids will lose tetracycline resistance due to insertion of foreign DNA but can still be selected out from non-recombinant ones by plating the transformants on tetracycline containing medium.
- The transformants growing on ampicillin containing medium are then transferred on a medium containing tetracycline.
- The recombinants will grow in ampicillin containing medium but not on that containing
- tetracycline.
- But, non- recombinants will grow on the medium containing both the antibiotics.
- In this case, one antibiotic resistance gene helps in selecting the transformants, whereas the other antibiotic resistance gene gets 'inactivated due to insertion' of alien DNA, and

helps in selection of recombinants.

Insertional inactivation:

- Recently, alternative selectable markers have been developed which differentiate recombinants from non-recombinants on the basis of their ability to produce colour in the presence of a chromogenic substrate.
- If a bacterial plasmid having a gene for *β-galactosidase enzyme*, the bacteria will produce that enzyme into the medium where it is grown.
- If this medium contain a chromogenic substrate the enzyme will react with this and a blue colour is produced.
- When a foreign gene is inserted into the β-galactosidase site of the bacterial plasmid, the bacteria will lose the ability to produce the β-galactosidase enzyme.
- Presence of insert results into insertional inactivation of the β-galactosidase gene and the colonies do not produce any colour,these are identified as recombinant colonies
- So the colonies of the bacteria having a foreign gene insert can be can be identified by noticing the colour.This process is called **insertional inactivation**.

Vectors for cloning genes in plants and animals :

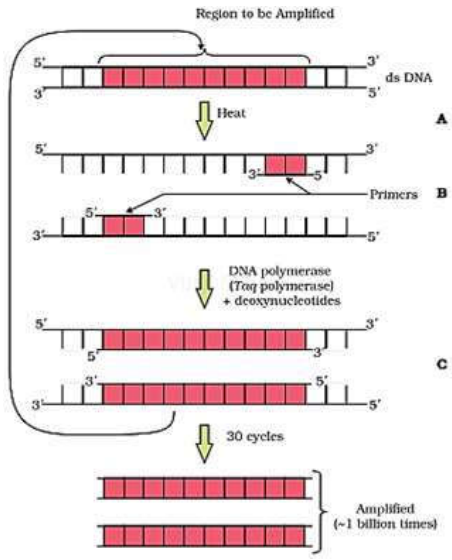
- *Agrobacterium tumefaciens* (pathogen of dicot plant) is able to deliver a piece of DNA known as '**T-DNA"** to transform normal plant cells into a tumorous one.
- Agrobacterium modify these tumor cells to produce the chemicals required by the pathogen.
- The tumor inducing (Ti) plasmid of Agrobacterium tumefaciens has been modified into cloning vector having no more pathogenic to plant.
- Retroviruses in animals have the ability to transform normal cells into cancerous cells.
- Nowadays retrovirus have been disarmed and are used to transfer desirable genes into animal cells

PROCESSES OF REOMBINANT DNA TECHNOLOGY:

- Recombinant DNA technology involves several steps in specific sequence such as
  1)Isolation of the Genetic material
  2)Cutting of DNA at specific locations
  3)Amplification of Gene of interest using PCR
  4)Insertion of Recombinant DNA Into the Host cell /organism
  5)Obtaining Foreign gene product
  6)Down stream Processing

3.Amplification of Gene of interest using PCR

- PCR stands for polymerase chain reaction. In this reaction, multiple copies of the gene of interest can be synthesized in vitro under three steps



Denaturation

- In this process the double stranded DNA is converted into the single stranded DNA.
- It is normally achieved by heating.

Annealing.

- The two sets of primers bind to their complementary sequences on single stranded DNA.
- Here primers are single-strand sequences of DNA around 20 to 30 bases in length.
- They serve as the starting point for the synthesis of DNA.

Extension

- The enzyme DNA polymerase extends the primers using the nucleotides provided in the reaction and the genomic DNA as template.
- If the process of replication of DNA is repeated many times segment of DNA can be amplified to approximately billion times.
- Such repeated amplification is achieved by the use of thermostable DNA polymerase (Taq polymerase enzyme obtained from bacteria called *Thermus aquaticus*)

Obtaining the Foreign Gene Product:

- The recombinant cells can be multiplied in large scale using a continuous culture system.
- Once the foreign DNA is inserted in to a host, it is multiplied and ultimately desirable protein is produced.
- For the production of the desired protein, the gene encodes for it needs to be expressed.

Bioreactors :

- Large scale production of desired proteins can be achieved by using bioreactors.
- In a bioreactor about 100 to1000 litres of cultures are processed.
- A bioreactor is a large culture vessel in which raw materials are biologically converted into specific products.
- The culture is done by using microbial , plant or human cells.
- In a bioreactor availability of optimum temperature , pH, substrate, salts, vitamins and oxygen are provided for culture.

Stirred-tank reactor:

- It is usually cylindrical or with a curved base to facilitate the mixing of the reactor contents.
- The stirrer facilitates even mixing and oxygen availability throughout the bioreactor.
- Alternatively air can be bubbled through the reactor.
- The bioreactor has an agitator system, an oxygen delivery system and a foam control system, a temperature control system, pH control system and sampling ports