BIOTECHNOLOGY: PRINCIPLES AND PROCESSES
TOOLS OF RECOMBINANT DNA TECHNOLOGY

Conceptual development of the Genetic Engineering Principles:

Sexual reproduction promotes variations while asexual reproduction preserves genetic information. The former is more advance then latter.

Traditional hybridisation procedures cause inclusion and multiplication of undesirable genes along with the desired genes.

The technique of genetic engineering involves formation of **recombinant DNA (rDNA)**, use of gene cloning and gene transfer. It examines this limitation and permits to isolate only one or a set of desirable genes without introducing undesirable genes into the target organism.

A piece of DNA is incorporated into the genetic material of the recipient where it may multiply and be inherited alongwith the host DNA. Thus alien DNA is linked with the **'Ori'** site or origin of replication (It is a site of initiation of replication) can replicate and multiply itself in the host organism that is called **cloning**. Thus **genetic engineering is alternately called recombinant DNA technology or gene cloning**. It is helpful in forming multiple identical copies of any template DNA.

Stanley Cohen and Herbert Boyer (1972) firstly constructed **recombinant DNA**. They isolated piece of DNA from a plasmid carrying **antibiotic-resistance gene** of the bacterium **Salmonella typhimurium** and fused it to the **plasmid of E. coli.** The plasmid is used as a vector to carry an alien piece of DNA into the host organism.The linking of these two is performed by **DNA ligase** resulting recombinant DNA is created in vitro. Now it is transferred into E.coli where it can replicate in the presence of the new host's **DNA polymerase enzyme** and form multiple copies that represent gene cloning.

Genetically modified organism (GMO) or Transgenic organism can be developed by the following three basic steps.

Identification of DNA with desirable genes.

Introduction of the identified DNA into the host.

Maintenance of introduced DNA in the host and transfer of the DNA to its progeny

Tools of Recombinant DNA Technology:

(1) Enzymes

(2) Cloning Vectors

(3) Competent host

(1) Enzymes:

Variety of specific enzymes are employed in genetic engineering.

(i) Restriction Enzymes:

They break DNA molecules. They are of three types.

(a) Exonucleases:

They separate nucleotides from the terminal ends of DNA in one strand of duplex.

(b) Endonucleases:

They produce cuts within the DNA.

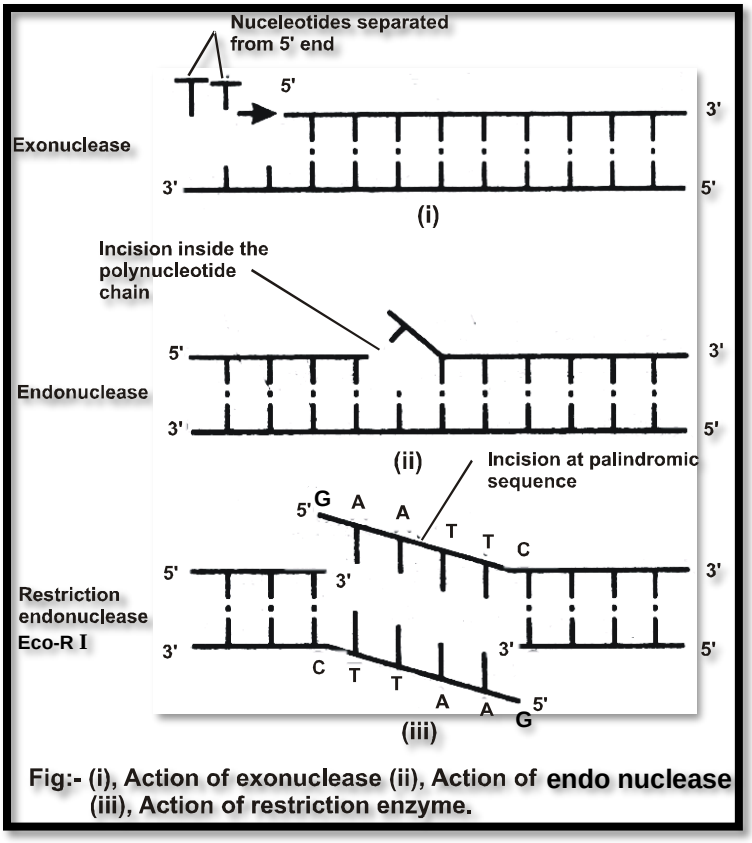


Fig:- (i), Action of exonuclease (ii), Action of endo nuclease (iii), Action of restriction enzyme.

(c) Restriction endonucleases :

They cut the DNA strands at specific base sequence in palindrome site.

They function as **'molecular scissors' or chemical scalpels**.

W. Arber, H. Smith and D. Nathans discovered these enzymes.

The first restriction endonuclease was **Hind II** isolated from **Haemophilus influenzae Rd**. It was found that Hind II always cut DNA molecules at a particular point by recognising a sequence of Hind II. It produces blunt ends.

The convention for naming these enzymes is the first letter of the name comes from the bacterium's genus name and the second two letters come from the species of the prokaryotic cell from which they were isolated, **e.g. EcoRI comes from Escherichia coli RY 13. In EcoRI, 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**.

The restriction endonuclease inspects the length of a DNA sequence. Once it recognises specific sequence, it binds to the DNA and cuts each of the two strands of the double helix

at specific points in their sugar phosphate back bones. Special sequence in the DNA recognised by restriction endonuclease is called palindromic nucleotide sequence.

The palindromes are groups of letters that form the same words when read in both directions forward and backward. **e.g.**

The palindromes in DNA are base pair sequences that are the same when read forward (left to right) or backward (right to left) from a central axis of symmetry. For **e.g.** the following sequences read the same on the two strands in 5'→3' direction. This is also true when we read in the 3' → 5' direction.

Fig : Palindromic sequence

Restriction enzymes cut the strand of DNA a little away from the centre of the palindrome sites but between the same two bases of the opposite strands. This leaves single stranded unpaired bases at cut ends. These ends with unpaired bases are called sticky ends or cohesive ends. The latter are named so because they form hydrogen bonds with their complementary cut counter parts. The sticky ends facilitate the action of the enzyme DNA ligase

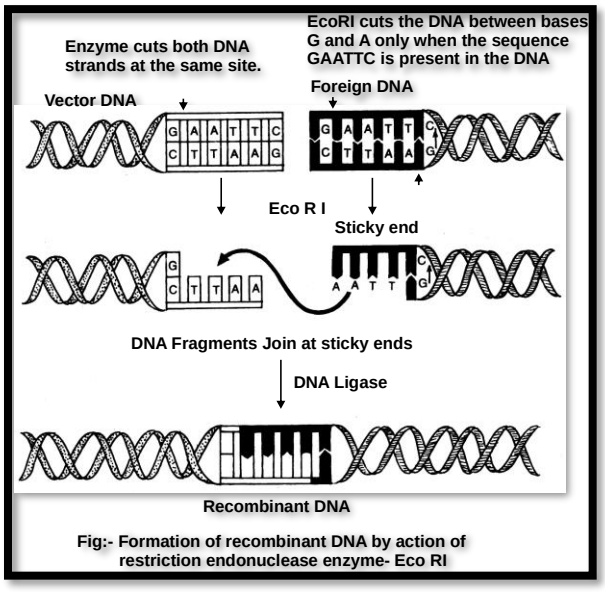


Fig:- Formation of recombinant DNA by action of restriction endonuclease enzyme- Eco RI

Some restriction enzymes, type II, their source, recognition sequence and site of cleavage.			
S.No.	Restriction Enzyme	Source	Recognition sequence and site of Cleavage
1.	Eco R I	*Escherichia coli RY* 13	5'-G↓A-A-T-T-C-3' 3'-C-T-T-A-A↑G-5
2.	Hin d II	*Haemophilus influenzae Rd*	5'G-T-C↓G-A-C-3' 3'-C-A-G↑C-T-G-5'
3.	Hin d III	*Haemophilus influenzae Rd*	5'-A↓A-G-C-T-T3' 3'-T-T-C-G-A↑A-5'
4.	Bam H I	*Bacillus amyloliquefaciens H*	5'-G↓G-A-T-C-C-5' 3'-C-C-T-A-G↑G-5'
5.	Sal I	*Streptomyces albus*	5'-G↓T-C-G-A-C-3' 3'-C-A-G-C-T↑G-5'
6.	Sma I	*Serratia marcescens*	5'-C-C-C↓G-G-G-3' 3'-G-G-G↑C-C-C-5'
7.	Alu I	*Arthrobacter luteus*	5'-A-G↓C-T-3' 3'-T-C↑G-A-5'
8.	Eco R II	*Escherichia Coli* R245	5'↓C-C-T-G-G-3' 3'-G-G-A-C-C↑5'
9.	Hae III	*Haemophilus aegyptius*	5'-G-G↓C-C-3' 3'-C-C↑G-G-5'
10.	Sca I	*Streptomyces caespitosus*	5'-A-G-T↓A-C-T-3' 3-T-C-A↑T-G-A-5'

Why type II restriction endonuclease are used in genetic engineering

Type II restriction endonucleases always cleave at or near their recognition sites. They produce small, well-defined fragments of DNA that help to characterize genes and genomes and that produce recombinant DNAs.

(ii) DNA Ligases (Molecular gum): They form **phosphodiester bonds** between adjacent nucleosides and covalently link two individual fragments of double-stranded DNA. They help in sealing gaps in DNA fragments. Thus they act as a **molecular glue**. **T_4 DNA ligase** is mostly utilized in the **rDNA technology**.

(iii) Alkaline Phosphatase: It is helpful to remove the phosphate group from the 5' end of DNA molecule, leaving a free 5' hydroxyl group. It can be isolated from calf intestine or bacteria. It is used to prevent unwanted self ligation of vector DNA molecules in process of rDNA technology.

(**iv**) **Reverse Transcriptase**: it is an enzyme used to generate complementary DNA (cDNA) from an RNA template, a process termed reverse transcription.it is mainly associated wth retrovirus.

(v) DNA Polymerase: It is employed to polymerize replication of DNA on DNA template or complementary DNA (cDNA). It catalyses in 5'→3' and 3'→5' exonucleolytic degradation of DNA. DNA polymerase firstly investigated by **A. Kornberg in *E.coli*** and is now known as **DNA polymerase I**. other two enzymes are **DNA polymerase II and DNA polymerase III**. The latter is more active than Polymerase II & I. It produces a parallel strand in the presence of ATP on DNA template.

(vi) Lysing enzymes: These enzymes are used to isolate the DNA from the cell in genetic engineering. There are three types of lysing enzymes-

i. Cellulase - are the enzymes that hydrolyze -1,4 glycosidic linkages in cellulose chains.
ii. Chitinase - chitin is an abundant biopolymer that is relatively resistant to degradation. Chitinase are hydrolytic enzymes that break down glycosidic bonds in chitin.
iii. Lysozyme - also called as glycosidase, is the enzyme responsible for cleaving the bond between N-acetyl muramic acid and N-acetyl glucosamine.

2. Vehicle DNA or Vector DNA. The DNA used as a carrier for transferring a fragment of foreign DNA into a suitable host is called vehicle or vector DNA.
You know that plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA.
The following are the features that are required to facilitate cloning into a vector.

(i) Origin Qf replication (ori):
This is a sequence from where replication starts and any piece of DNA when linked to this sequence can be made to replicate within the host cells. This sequence is also responsible

for controlling the copy number of the linked DNA. So. if one wants to recover many copies of the target DNA it should be cloned in a vector whose origin support high copy number.

(ii) Selectable marker: In addition to 'ori', the vector reguires a selectable marker, which helps in identifying and eliminating nontransformants and selectively permitting the growth of the transform ants.

Normally. the genes encoding resistance to antibiotics such as ampicilin. chloramphenicol. Tetracycline or kanamycin. etc., are considered useful selectable markers for E. coli.

Enzyme forming gene also act as selectable marker gene. eg. Lac Z gene

Note:

The normal E. coli cells do not carry resistance against any of these antibiotics.

(iii) Restriction sites/Cloning sites:

In order to link the alien DNA the vector needs. recognition sites for the commonly used restriction enzymes. The ligation of alien DNA is carried out at a restriction site present in one of the two antibiotic resistance genes. A vector should have restriction site for many enzyme but only one restriction site for each enzyme, otherwise vector will get fragmented.

Selection of vector depends on

(i) Size of desired gene

(ii) Type of host

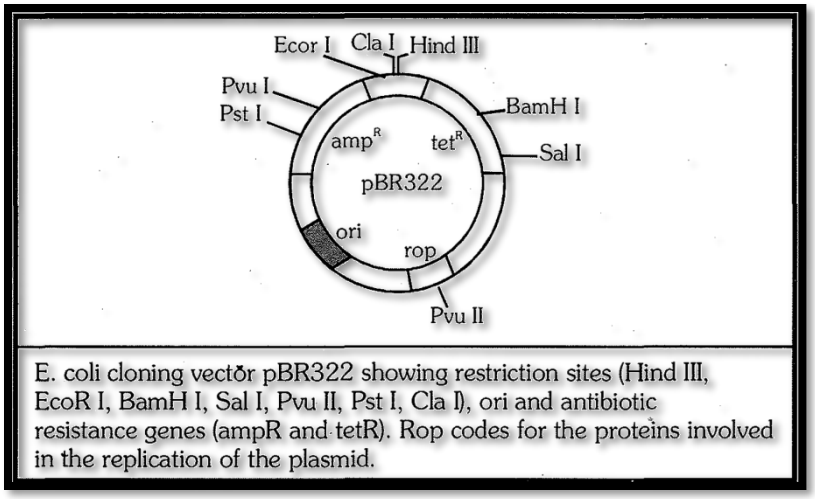
Some examples of vectors :-

(i) Plasmids:

They are double stranded, circular and extra chromosomal DNA segments found in bacteria which can replicate independently. Plasmids can be takeu out of bacteria and made to combine with desired DNA segments by means of restriction enzymes and DNA ligase. A plasmid carrying DNA of another organism integrated with it, is known as recombinant plasmid or hybrid plasmid or Chimeric plasmid.

eg. pBR 322, PUC 18 (used for gene transfer in bacteria)

Ti plasmid, Ri Plasmid (used for gene transfer in dicot plant)



E. coli cloning vector pBR322 showing restriction sites (Hind III, EcoR I, BamH I, Sal I, Pvu II, Pst I, Cla I), ori and antibiotic resistance genes (ampR and tetR). Rop codes for the proteins involved in the replication of the plasmid.

(ii) Viruses:

The DNA of certain viruses is also suitable for use as a vehicle DNA.

Bacteriophage DNA also used as a vector DNA for gene transfer.

Lambda phage (A, phage) has been used for transferring lac genes of E. coli into haploid callus of tomato.

Retro virus - useful for gene transfer in animal cell

	Vector type	Insert size kb
(1)	Plasmid	0.5 – 8
(2)	Bacterophage lambda	9 – 23
(3)	Cosmid	30 – 45
(4)	BAC	50 – 300
(5)	YAC	1000 – 2500