Biotechnology Principles

Introduction

- **Biotechnology** deals with techniques of using live organisms or enzymes from organisms to produce products and processes useful to humans.

Principles of Biotechnology/Core Techniques Involved in Modern Biotechnology

Parameters	Genetic engineering	Bioprocess engineering
Definition	Techniques to alter the chemistry of genetic material to introduce these into host organisms and thus change the phenotype of host organism	Maintenance of sterile ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell in large quantities
Include	Creation of rDNA Gene cloning Gene transfer	Manufacture of biotechnological products like antibiotics, vaccines, enzymes, etc.
The ability to multiply copies of antibiotic resistance gene in E. coli was called cloning of antibiotic resistance gene in E. coli.		

Three Basic Steps in Genetically Modifying Organisms (GMO):

- 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

Key Tools of Recombinant DNA Technology:

- **(1) Enzymes**
- **(2) Vectors**
- **(3) Competent host cells**

ENZYMES

Restriction Endonuclease :

- More than 900 restriction enzymes have been isolated from over 230 strains of bacteria each of which recognise different recognition sequences.
- First restriction endonuclease-Hind II: Isolated and characterised in 1968 later, recognises sequence of 6 bp. The first recombinant DNA was constructed by Stanley Cohen and Herbert Boyer, 1972.

Functions

- Cuts the two strands of dsDNA at specific points in their sugarphosphate backbones and leaves single stranded portions at the ends.

Ligase :

- When source DNA and vector DNA are cut by the same restriction enzyme, the resultant DNA fragments have the same kind of sticky-ends.
- Sticky ends are named so because they form hydrogen bonds with their complementary cut counterparts.
- Stickiness facilitates the action of the enzyme DNA ligase.

Cloning Vectors :

Vectors are vehicles for delivering foreign DNA into recipient cells.

Features of cloning vectors:

- Origin of Replication (ori)
- Selectable Marker
- Cloning Sites/Restriction Sites

Transformation: Procedure through which piece of foreign DNA is introduced in a host bacterium.

- Insertional inactivation: Insertion of gene of interest within antibiotic resistance gene/selectable marker results in inactivation.
  All transformants are not recombinants but all recombinants are transformants.
- Non-Transformants: Hosts that do not take up the vector DNA (Non-recombinant).
- Transformants: Hosts that take up the vector DNA (Recombinant or Non-recombinant).
- Recombinants: Transformant hosts that take up the recombinant DNA (Vector DNA with desired DNA).
- Non-Recombinants: Transformant hosts that take up the nonrecombinant DNA (Vector DNA without desired DNA)
- rop → Codes for the proteins involved in the replication of the plasmid.

Plasmids as vectors:

- Extra-chromosomal, circular, double-stranded DNA.
- Replicate independent of the control of chromosomal DNA (autonomously).
- They may have 1 or 2 copies per cell or even 15-100 copies per cell.

OTHER CLONING VECTORS

Ti-plasmid of Agrobacterium tumefaciens

- Agrobacterium tumefaciens, a pathogen of several dicot plants is able to deliver a piece of DNA known as 'T-DNA' to transform normal plant cells into a tumor and direct the tumor cells to produce the chemicals required by the pathogen.
- Disarmed tumour inducing (Ti) plasmid is used which is no more pathogenic to the plants but is still able to use the mechanism to deliver the genes of our interest into varieties of plants.

Bacteriophages

- High copy number than plasmid.

Retroviruses

- Retroviruses in animals have the ability to transform normal cells into cancerous cells.
- Disarmed retroviruses are used to deliver desirable genes into animal cells.

Methods of Transformation

1. **Micro-injection**
   - Recombinant DNA is directly injected into the nucleus of an animal cell.
2. **Biolistic/Gene gun**
   - Plant cells are bombarded with high velocity microparticles of gold or tungsten coated with DNA.
3. **Heat-shock method**
4. **Disarmed pathogen vectors**

Competent Host for Transformation with recombinant DNA

- DNA is hydrophilic, so it can not pass through cell membranes.
- In order to force cell to take up alien DNA/rDNA, it must first be made 'competent' by treating with ice cold calcium chloride (CaCl2).
- Entry of rDNA in host cell is due to transient pores created by heat shock (42°C) and not due to Ca^{2+} ions.
- Divalent cations increases the efficiency with which DNA enters the bacterium through pores in its cell wall.

Process of Recombinant DNA Technology

1. Isolation of the Genetic Material (DNA)
2. Fragmentation by restriction endonucleases
3. Separation and isolation of DNA fragments

Gel electrophoresis:

- Separation of negatively charged DNA molecules under an electric field through a medium/matrix.
- Most commonly used matrix for DNA separation is agarose.

4. PCR-Polymerase Chain Reaction

- In vitro amplification of DNA (gene of interest)
- The amplified fragment if desired can now be used to ligate with a vector for further cloning.

5. Ligation of the DNA fragment into a vector by DNA ligase

6. **Insertion of recombinant DNA into the host cell**

- Transformed host cells are selected with the help of selectable marker genes.

7. Culturing of recombinant host cells (Biosynthetic stage)

- The cells harbouring cloned genes of interest may be grown in laboratory/ bioreactors.
- **Bioreactors:** Vessels in which raw materials are biologically converted into specific products using microbial plant, animal or human cells and provide optimal growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).

8. Downstream processing

- Separation and purification of the desired product/recombinant protein from heterologous host (non native host).
- Product has to be formulated with suitable preservatives.
- Strict quality control testing is done for each product.
- The downstream processing and quality control testing vary from product to product.

9. Product is subjected for marketing as a finished product

In Open Culture System/Continuous Culture System

- Used medium is drained out from one side.
- Fresh medium is added from the other to maintain the cells in their physiologically most active log/exponential phase.
- Larger biomass → Higher yields of desired protein
- Restriction enzymes belong to a class of enzymes called nucleases.
- The nucleases include exonucleases and endonucleases.

(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.

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.

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 nonrecombinants 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, b-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.