UNIT - IX

CHAPTER – 11 : BIOTECHNOLOGY : PRINCIPLES AND PROCESSES

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

- The definition given by EFB (European Federation of Biotechnology) is as follows; 'The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'.

Principles of Biotechnology:

- Genetic engineering: Techniques to alter the chemistry of genetic material (DNA and RNA), to introduce these into host organisms and thus change the phenotype of the host organism.
- Maintenance of sterile ambience in chemical engineering processes to enable growth of only the desired microbe / eukaryotic cell in large quantities for the manufacture of biotechnological products like antibiotics, vaccines, enzymes, etc.

The techniques of genetic engineering which include creation of recombinant DNA, use of gene cloning and gene transfer, overcome this limitation and allow us to isolate and introduce only one or a set of desirable genes without introducing undesirable genes into the target organism.

In a chromosome there is a specific DNA sequence called the origin of replication, which is responsible for initiating replication. Therefore, for the multiplication of any alien piece of DNA in an organism it needs to be a part of a chromosome which has a specific sequence known as 'origin of replication'. Thus, an alien DNA is linked with the origin or replication, so that, this alien piece of DNA can replicate and multiply itself in the host organism. This is known as Cloning.

The construction of the first recombinant DNA emerged from the possibility of linking a gene encoding antibiotic resistance with a native Plasmid of *Salmonella typhimurium*.

The cutting of DNA at specific locations became possible with the discovery of the so-called 'Molecular scissors" – restriction enzymes. The cut piece of DNA was then linked with the plasmid DNA with the help of another enzyme called DNA ligase. These

plasmid DNA act as vectors to transfer the piece of DNA attached to it. A plasmid can be used as vector to deliver an alien piece of DNA into the host organism.

"Recombinant DNA technology" or also called "Genetic Engineering" deals about, the production of new combinations of genetic material (artificially) in the laboratory. These "recombinant DNA" (rDNA) molecules are then introduced into host cells, where they can be propagated and multiplied.

Basic steps in rDNA:

- 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:
Restriction Enzymes (Molecular Scissors):

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.

Example, the first restriction endonuclease – Hind II, always cut DNA molecules at a particular point by recognizing a specific sequence of six base pairs. This specific base sequence is known as the Recognition Sequence for Hind II.

Each restriction endonuclease recognizes a specific Palindromic Nucleotide Sequences in the DNA.

What are Palindromes?

These 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.

Restriction enzymes cut 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 called Sticky ends. The same enzyme cuts both DNA

(vector and foreign DNAs) strands at the same site with sticky ends and these can be joined together using DNA-ligase.

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.
- The DNA fragments are separated according to their size.
- 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*.
- These DNA fragments are purified and used in constructing recombinant DNA with cloning vector.

Cloning Vectors (Vehicles for Cloning):

Vector serves as a vehicle to carry a foreign DNA sequence into a given 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 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.
- The vector must also have atleast one unique restriction endonuclease recognition site to enable foreign DNA to be inserted into the vector during the generation of a recombinant DNA molecule.
- The vector should be relatively small in size.

The most commonly used vectors are – Plasmids and Bacteriophages.

Identification of recombinants:

Insertional inactivation:

The most efficient method of screening for the presence of recombinant plasmids is based on the principle that the cloned DNA fragment disrupts the coding sequence of a gene. This is termed as Insertional Inactiviation.

For example, the powerful method of screening for the presence of recombinant plasmids is referred to as Blue-White selection. This method is based upon the insertional inactivation of the lac Z gene present on the vector. The lac Z gene encodes the enzyme beta-galactosidase, which can cleave a chromogenic substrate into a blue coloured product. If this lac Z gene is inactivated by insertion of a target DNA fragment into it, the development of the blue colour will be prevented and it gives white coloured colonies. By this way, we can differentiate recombinant (white colour) and non-recombinant (blue colour) colonies.

Competent Host (Introduction of recombinant DNA into host cells):

In rDNA technology, the most common method to introduce rDNA into living cells is transformation, during which cells take up DNA from the surrounding environment.

1) Simple chemical treatment with divalent calcium ions increases the efficiency of host cells (through cell wall pores) to take up the rDNA plasmids.
2) rDNA can also be transformed into host cell by incubating both on ice, followed by placing them briefly at 42^{o}C (Heat Shock), and then putting them back on ice. This enables the bacteria to take up the recombinant DNA.
3) In Microinjection method, rDNA is directly injected into the nucleus of cells by using a glass micropipette.
4) Biolistics / Gene gun method, it has been developed to introduce rDNA into mainly plant cells by using a Gene / Particle gun. In this method, microscopic particles of gold / tungsten are coated with the DNA of interest and bombarded onto cells.
5) The last method uses *"Disarmed Pathogen"* Vectors (*Agrobacterium tumefaciens*), which when allowed to infect the cell, transfer the recombinant DNA into the host.

Processes of Reombinant DNA Technology:

rDNA technology involves several steps in specific sequence such as,

- Isolation of DNA
- Fragmentation of DNA by restriction endonucleases
- Isolation of desired DNA fragment
- Ligation of the DNA fragment into a vector
- Transferring the recombinant DNA into the host
- Culturing the host cells in a medium at large scale and extraction of the desired product

Isolation of DNA:

DNA should be isolated in pure form, without macromolecules. Hence cell wall can be broken down by treating the bacterial cells / plant or animal tissue with enzymes such as Lysozyme (bacteria), cellulose (plant cells), chitinase (fungus).

DNA should be removed from its histones proteins and RNAs. This can be achieved by using enzymes ribonuclease for RNA and Proteases for histone proteins. Finally purified DNA precipitates out after the addition of chilled Ethanol.

Fragmentation of DNA:

- Restriction enzyme digestions are performed by incubating purified DNA molecules with the restriction enzyme.
- DNA is a negatively charged molecule, hence it moves towards the positive electrode (anode).
- After having cut the source DNA as well as the vector DNA with a specific restriction enzyme, the cut out gene of interest from the source DNA and the cut vector with space are mixed and ligase is added. This results in the preparation of recombinant DNA.

Amplification of Gene of Interest using PCR:

PCR stands for Polymerase Chain Reaction. In this reaction, multiple copies of the gene of interest is synthesized in vitro using two sets of primers and the enzyme DNA polymerase.

The process of replication of DNA is repeated many times, the segment of DNA can be amplified to approximately billion times. Such repeated amplification is achieved by the use of a themostable DNA polymerase (*Taq* DNA Polymerase – isolated from a bacterium, *Thermus aquaticus*). The amplified fragment if desired can now be used to ligate with a vector for further cloning.

Insertion of Recombinant DNA into the Host Cell / Organism:

There are several methods of introducing the ligated DNA into recipient cells. If a recombinant DNA bearing gene for resistance to an antibiotic (ampicillin) is transferred into *E.coli* cells, the host cells become transformed into ampicillin-resistant cells. If we spread the transformed cells on agar plates containing ampicillin, only transformants will grow, untransformed recipient cells will die. The ampicillin resistance gene in this case is called a selectable marker.

Obtaining the Foreign Gene Product:

The cells harbouring cloned genes of interest may be grown on a small scale in the laboratory. The cultures may be used for extracting the desired protein and then purifying it by using different separation techniques.

Bioreactors :

To produce in large quantities, the development of bioreactors, where large volume of culture can be processed, was required. Thus, bioreactors can be thought of as vessels in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial plant, animal or human cells. A bioreactor provides the optimal conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).

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 so that small volumes of the culture can be withdrawn periodically.

Downstream Processing:

The processes include separation and purification, which are collectively referred to as downstream processing. Strict quality control testing for each product is also required.

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