BIOTECHNOLOGY PRINCIPLES AND PROCESSES

Objectives

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

➢ Microbe mediated processes like making of curd, bread ,vine etc are considered as primitive form of biotechnology.

➢ Nowadays many processes/techniques are included under biotechnology. For example, in vitro fertilisation leading to a „test-tube[cr] baby, synthesising a gene and using it, developing a DNA vaccine or correcting a defective gene, are all part of biotechnology.

➢ The European Federation of Biotechnology (EFB) has defined biotechnology as „The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services.

PRINCIPLES OF BIOTECHNOLOGY:

1 . GENETIC ENGINEERING:

introduce these into host organisms and thus change the phenotype of the host organism.

2. BIOPROCESS ENGINEERING:

➢ 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.

➢ Traditional hybridization procedures used in plant and animal breeding, very often lead to inclusion and multiplication of undesirable genes along with the desired genes.

➢ In genetic engineering the genes of desirable qualities are taken from an organism and transfer it to the host organism. So we will get an organism having desirable qualities. ➢ The techniques of genetic engineering include creation of recombinant DNA, use of gene cloning and gene transfer.

➢ 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 of replication, can replicate and multiply itself in the host organism.

➢ This is known as Cloning or making multiple identical copies of any template DNA.

RECOMBINANT DNA TECHNOLOGY

➢ It is introduced by Stanley Cohen and Herbert Boyer in 1972.

➢ They isolated an antibiotic resistant gene from Salmonella typh1MURr(JM and introduced to another bacterium called Escherichia coli.

➢ They cut the antibiotic resistant gene from Salmonella by usingrestriction endonuclease enzyme.

➢ They used plasmids as vectors to transfer this alien DNA to E,coli.

➢ The linking of antibiotic resistant gene to plasmid is done by using DNA ligase enzyme. ➢ By using DNA polymerase enzyme it is able to multiply the copies of antibiotic resistance gene. This ability to multiply copies of a gene is called Gene Cloning.

➢ Three Basic steps in rDNA technology:

o 1.Identification of DNA with desirable genes 0

2.Introduction of the identified DNA into the host

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

TOOLS OF RECOMBINANT DNA TECHNOLOGY:

1 . Restriction enzymes 2.Polymerase enzymes 3.Ligase enzymes 4.Clonig vectors

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.

Bacterium produces a restriction enzyme to defend against bacterial viruses call 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 this specific base sequence is known as the recognition sequence.

The first discovered restriction endonuclease - Hind Il Nomenclature of Restriction Enzymes

The naming of restriction enzymes are based on their origin and type of action

For e.g. Eco RI o 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) o Co - The second two letters come from the species name. („co et stands for coli)

O R- Third letter indicates first letter of name of ;train(„R" stands for RY 13 strain of bacteria from which the enzyme obtained) o I (roman numeral one)- Indicates the order of discovery ofenzyme ➢ 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.

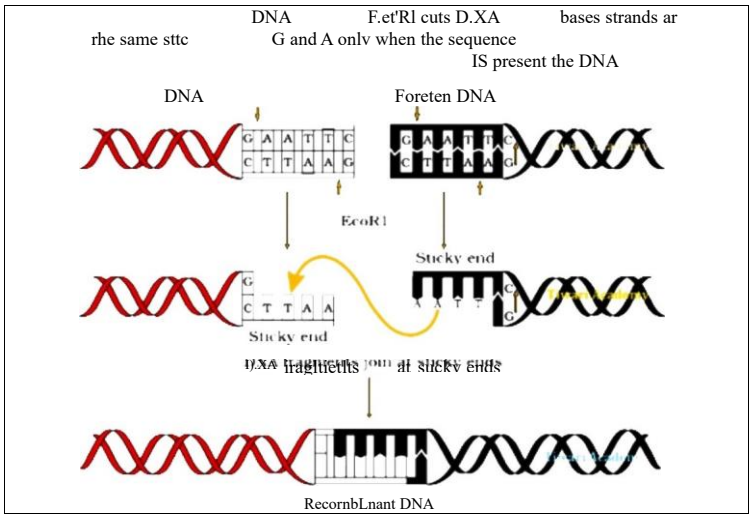
➢ Each restriction endonuclease recognizes a specific palindromic nucleotide sequences in the DNA. ➢ Palindromes are groups of letters that form the same words when read both forward and backward.eg. "MALAYALAM".

➢ 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

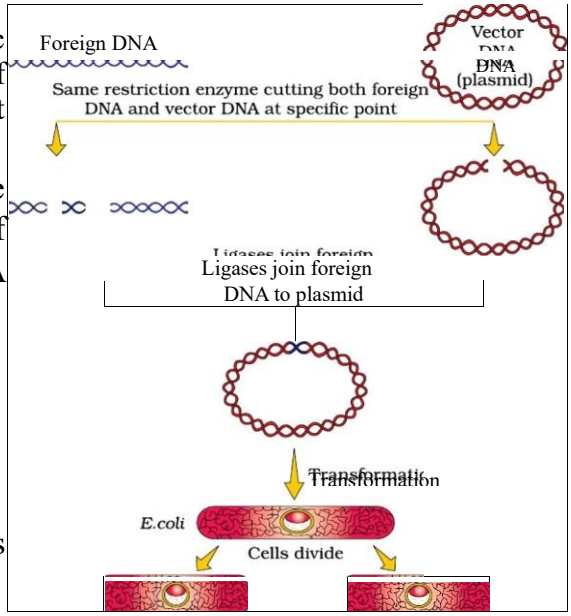
. G A A T T C
. C T T A A G
-

- 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 GA TTC 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.

➢ C] When cut the same restriction enzyme the resultant DNA fragments have the same kind ➢ of Sticky-ends and can be joined together using DNA ligases.



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 polmer 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.

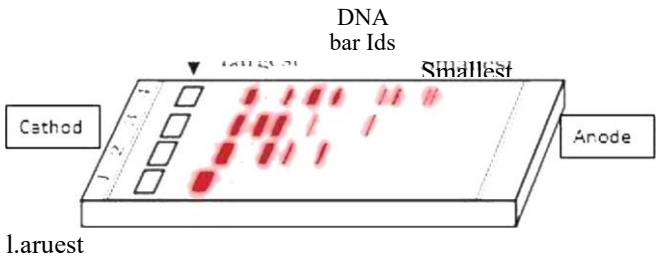


Figure 1 1.3 A typical agarose electrophoresis showtng migration of undik^{S}ested (lane I) and digested set of I).NA fragrnent.s (lane 2 to -I)

Cloning Vectors (Vehicles for Cloning):

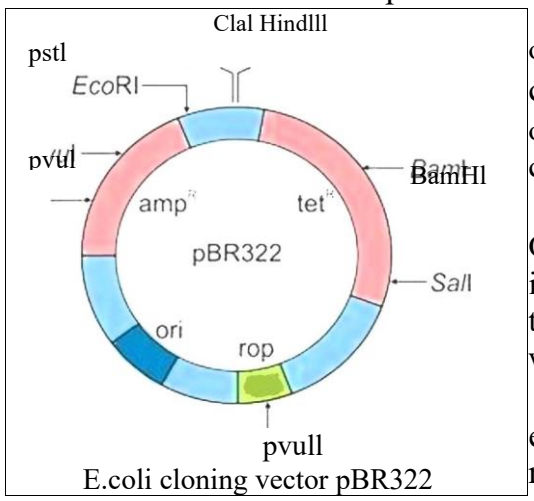
Vector serves as a vehicle to transfer a foreign gene (DNA sequence) into a given host cell. It has the ability to self replicate and integrate into the host cell Salient features of a Vector:

o 0 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.

O Some of the commonly used vectors are Plasmids, Bacteriophages, Plant or animal viruses etc o Plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA.

The characteristic features of a plasmid Vectors are



E.coli cloning vector pBR322

o Any piece of DNA when linked to this sequence can be made replicate within the host cells.

o This sequence is responsible for controlling the copy number of the linked DNA.

2.SeIectable Markers

O A vector requires a selectable marker which helps in identifying and eliminating transformants from non-transformants. o Transformation is a procedure through which a piece of DNA is introduced in a host Bacterium.

1 . Origin of Replication o It is the sequence from where replication starts.

o Normally, the genes encoding resistance to antibiotic such as ampicillin, 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 HI site of tetracycline resistance gene in the vector pBR 322 o 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[et] 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 P-galactdosidase 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 B-galactosidase site of the bacterial plasmid, the bacteria will lose the ability to produce the ß-galactosidase enzyme.
- Presence of insert results into insertional inactivation of the B-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**:**

- AgrobacteRrUM 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.

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

- In r-DNA technology, the most common method to introduce r-DNA into living cells is transformation, during which cells take up DNA from the surrounding environment.
- At first the bacterial cells must made competent to take up the foreign DNA .
- This is done by treating them with a specific concentration of divalent cations like calcium. ➢ It increases the efficiency of bacteria to take DNA through the pores on the cell wall. ➢ The recombinant DNA is then forced into bacterial cell by incubating the cells with rDNA on ice, followed by forcing them briefly at 42 ^{0}C (heat shock) and then putting back on ice.
- This enables the bacteria to take up the recombinant DNA. Micro injection o The host cell is made immobilized and then recombinant DNA is injected into the nucleus of the host cell. (It is done with the help of a micro needle). o This technique is now used to produce transgenic animals. Gene Gun o It is the new technology where vector less direct gene transfer is made in plants.
  - o High velocity micro particles of gold or tungsten coated with DNA are bombarded on the host cells. This method is known as biolistics or gene gun.

PROCESSES OF REOMBINANT DNA TECHNOLOGY:

- Recombinant DNA technology involves several steps in specific sequence such as

  l)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

l.Isolation of the Genetic material

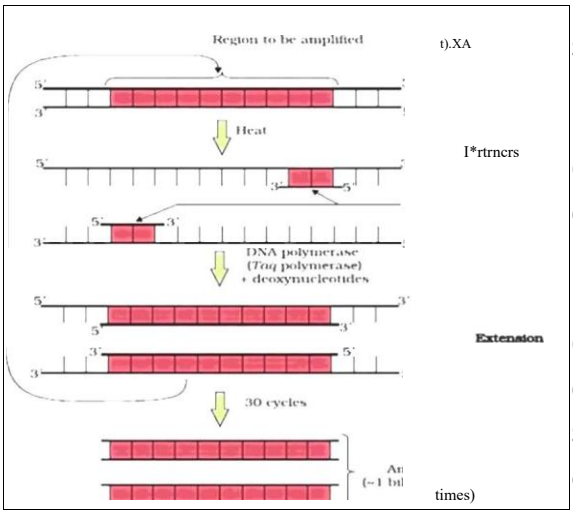
- DNA or RNA may be the genetic material; in majority of the organisms DNA is the genetic material
- The cells are broken and opened to release DNA along with other macromolecules such as RNA, proteins, polysaccharides and lipids.
- It is done by treating the cells with enzymes such as lysozyme (bacteria), cellulase (plant cells) or chitinase (fungus).
- The RNA can be removed by treatment with ribonuclease whereas proteins can be removed by treatment with protease.
- After the treatment of appropriate enzymes the DNA can be precipitated by adding chilled ethanol
- DNA can be seen as collection of fine threads in the suspension.
- Use spooling to extract the precipitated DNA. Spooling involves winding the fine threads of DNA on to a reel.

Cutting of DNA at specific locations ➢ For cutting the desired DNA or gene, the purified DNA molecules are incubated with the restriction endonuclease enzymes.

- The agarose gel electrophoresis is done to analyze the progression of a restriction enzyme digestion.
- DNA is a negatively charged molecule so it moves towards the positive electrode (anode).
- The vector DNA is also cut by using the same restriction enzyme.
- After cutting the DNA as well as vector DNA with restriction enzyme, the desired gene and cut vector are mixed together and DNA ligase enzyme is added to join the two DNA molecules.
- This results in the formation of recombinant DNA.

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 s nthesized in vitro under three steps



a. Denaturation

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

b. Anneal ing.

O The two sets of primers bind to their complementary sequences on single stranded DNA.

o Here primers are single-strand sequences of DNA around 20 to 30 bases in length.

O They serve as the starting point for the synthesis of DNA

c. Extension

- The enzyme DNA polymerase extends the prdmers 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 a thermostable DNA polymerase O (Taq polymerase, enzyme obtained from bacteria called ThermU5 AQUatrCUS)

4. Insertion of Recombinant DNA into the Host Cell / Organism:

- In this step, the recombinant DNA is introduced into a recipient host cell. This process is termed as Transformation.
- Once the recombinant DNA is inserted into the host cell, it gets multiplied and is expressed in the form of the desired product under optimal conditions.
- There are several methods of introducing the ligated DNA into recipient cells.

5. Obtaining the Foreign Gene Product:

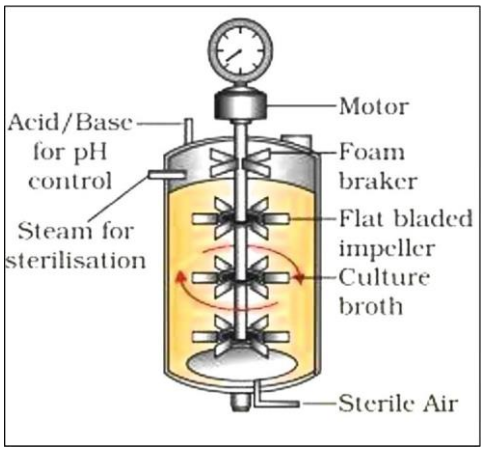
- The recombinant cells can be multiplied in large scale using a continuous culture system.
- Once the foreign DNA is inserted in to host, it is multiplied and ultimately desirable protein is produced.
- For the production of the desired protein, the gene encodes for it needs to be expressed.

BIOREACTORS

- Large scale production of desired proteins can be achieved by using bioreactors.
- In a bioreactor about 100 tolOOO 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.
- 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



6. Downstream Processing:

➢ The product obtained in a culture or a bioreactor is not in pure form.

➢ The downstream processing involves those processes and methods that are responsible for the separation and purification of the desired product.

➢ The desired product has to be separated from the culture and formulated with suitable preservatives.
