BIOTECHNOLOGY: PRINCIPLES AND PROCESSES

- ✓ **Biotechnology** – Field of biology that deals with the use of live organisms or biological systems to make products or processes that are useful to human beings.
- ✓ Biotechnology as per *European Federation of Biotechnology* (EFB) – The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services.

➢ PRINCIPLES OF BIOTECHNOLOGY

Genetic Engineering:

- Techniques to ***modify genetic material***.
- It allows the introduction of these modified genetic material into the host organisms.
- Can change the phenotype of the organism.

Bioprocess Engineering:

- Maintenance of sterile condition to enable the growth of only desired microbes.
  - Sterile: Free from microbial contamination.
- Prevents the growth of undesirable microbes.

➢ SOME BASIC CONCEPTS

- *What happens when a DNA molecule is somehow introduced into a host cell?*
  - The DNA cannot replicate within the host cell.
  - Most probably it will be degraded.
- *How can you make the foreign DNA multiply or make copies of itself within the host cell?*
  - The foreign DNA must become part of the host organism's genome by integrating with it.
    - Genome: All the genetic material of an organism.
  - The foreign DNA now gets multiplied along with the host chromosome as the host chromosome contains the ***origin of replication***.
    - The origin of replication is essential to initiate the process of DNA replication.
  - This process is called the ***cloning*** or making multiple identical copies of any template DNA.

Construction and Cloning of the first artificial rDNA molecule:

- Scientists: Stanley Cohen and Herbert Boyer (1972)
- Native Plasmid of *Salmonella typhimurium* was linked to antibiotic resistant gene.
  - *Plasmid*: Double stranded, autonomously replicating, extra chromosomal circular DNA that provides additional features to the bacterial cell if present.
  - *Antibiotic resistant gene*: Gene that enables the bacteria to survive in a medium that contains antibiotics.
- **Steps involved:**
  - Identification and cutting of a piece of DNA from a plasmid that provides resistance to antibiotics.
    - The DNA was cut at a specific position.
    - Enzyme involved: ***Restriction Enzymes*** – Molecular scissors.
  - The cut piece of DNA was linked to the native plasmid of *S. typhimurium*.
    - Enzyme involved: ***DNA Ligase***
  - This modified circular DNA is a *recombinant DNA* (rDNA) and acts as a *vector*.
    - Vector – carrier of foreign DNA/gene of interest.
    - rDNA – A DNA molecule that has been modified and may have a foreign DNA segment.

- Upon introduction of this rDNA into *Escherichia coli* bacteria, the rDNA was able to replicate within the new host (*E. coli*) and multiply in number using the new host's DNA polymerase enzyme.
- As the original antibiotic resistant gene has been multiplied within the *E. coli* host, this process is referred to as the cloning of the antibiotic resistance gene in *E. coli*.

Three basic steps in genetically modifying an organism

I. Identification of DNA with desirable genes;
II. Introduction of the identified DNA into the host;
III. Maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

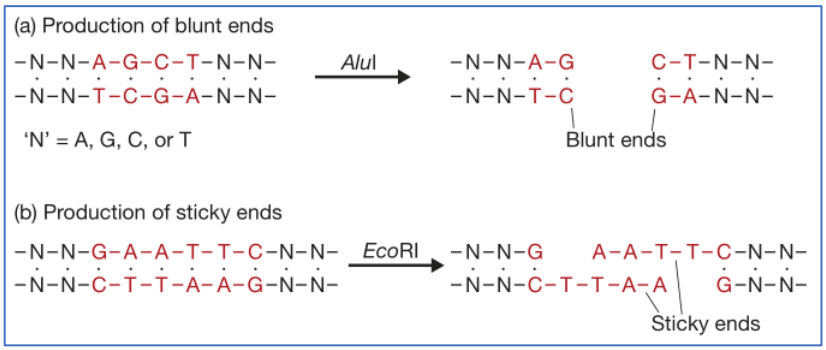
TOOLS OF RECOMBINANT DNA TECHNOLOGY

➢ RESTRICTION ENZYMES

- 1963 : Two enzymes were discovered that could restrict the growth of bacteriophage in E. coli.
  - One enzyme added methyl group to DNA.
  - The other enzyme could cut the DNA- ***restriction endonuclease***.
- The first identified restriction endonuclease is ***Hind II***.
- Restriction enzymes comes under ***Nucleases*** (larger class of enzyme).
  - Two types of nuclease
    - ***Exonuclease***
      - Removes nucleotides from the periphery/ends of the DNA.
    - ***Endonuclease***
      - It cuts the DNA at specific positions away from the periphery within the DNA.
- The restriction endonuclease cuts the DNA at a specific position by recognizing a specific sequence of DNA referred to as ***recognition sequence***.
  - Each restriction endonuclease recognizes a specific ***palindromic nucleotide sequences*** in the DNA.
    - ***Palindrome*** in DNA is a sequence of base pairs that *reads same* on the two strands when *orientation of reading is kept the same.*
    - For example, the following sequences reads the same on the two strands in 5' → 3' direction. This is also true if read in the 3' → 5' direction.
      5' – GAATTC – 3'
      3' – CTTAAG – 5'
- Upon identification of the recognition sequence the RE binds with the DNA and cuts each of the two strands of the DNA double helix at specific points in their sugar-phosphate backbones.

Blunt end and Sticky end

- Many RE make a simple double-stranded cut in the middle of the recognition sequence resulting in a ***blunt end***.
  - Examples of RE producing blunt end: *PvuII* and *AluI.*



- In other RE the two DNA strands are not cut at exactly the same position. Instead the cut is staggered, usually

by two or four nucleotides, so that the resulting DNA fragments have short *single-stranded overhangs* at each end.

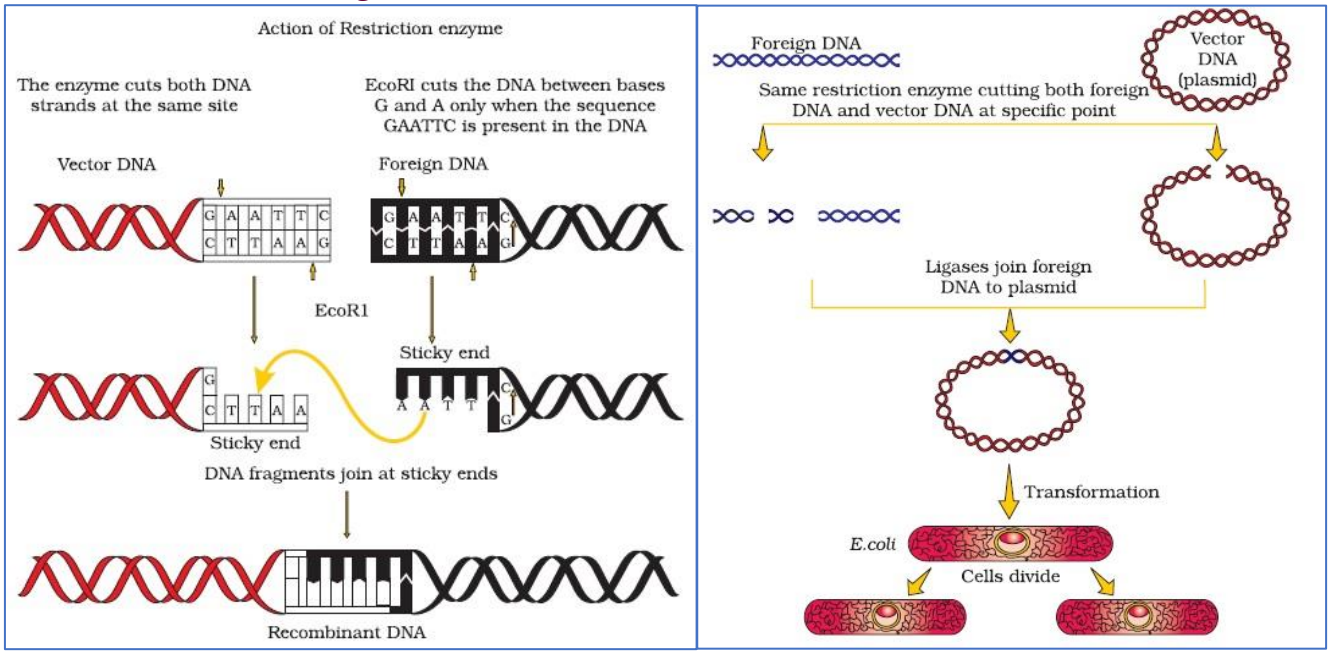
- These are called ***sticky ends***, as base pairing between them can stick the DNA molecule back together again.
- This stickiness of the ends facilitates the action of the enzyme DNA ligase.

Nomenclature of Restriction Enzymes

- Rules of nomenclature of RE :
  - The first letter of the name comes from the genus name.
  - The second two letters come from the species of the prokaryotic cell from which they were isolated
  - Following the three letters, the strain is sometimes represented.
  - Following the strain, the Roman numbers following the names indicate the order in which the enzymes were isolated from that strain of bacteria.
- Examples: (need to remember the EcoRI nomenclature only)

Enzymes Name	Genus name of Source organism	Species name of Source organism	Strain	Order of isolation from the bacteria strain
EcoRI	Escherichia	coli	RY 13	I
HindII	Haemophilus	influenzae	d	II
HindIII	Haemophilus	influenzae	d	III
BamHI	Bacillus	amyloliquefaciens	H	I

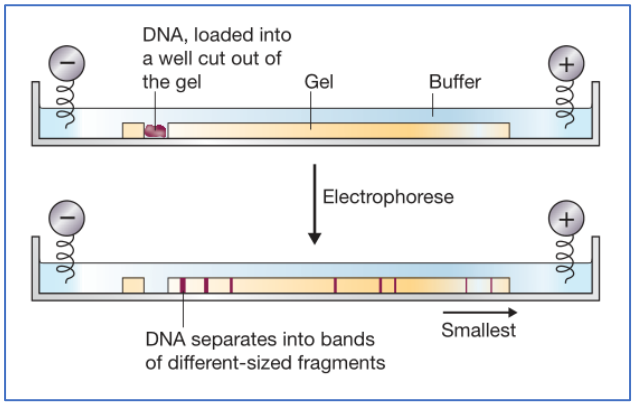
Steps in formation of recombinant DNA by action of restriction endonuclease enzyme – EcoRI



Recombinant DNA (rDNA)

- RE are used in genetic engineering to form 'recombinant' molecules of DNA, which are composed of DNA from different sources/genomes.
- When cut by the same restriction enzyme, the resultant DNA fragments have the same kind of 'sticky-ends' and, these can be joined together (end-to-end) using DNA ligases.
- Unless one cuts the vector and the source DNA with the same restriction enzyme, the recombinant vector molecule cannot be created.

Agarose Gel Electrophoresis



- Used for the separation and isolation of DNA fragments that is formed after a restriction digestion.
- Gel electrophoresis separates DNA molecules according to their size.
- DNA molecules are negatively charged.
  - due to the presence of phosphate moiety.
- DNA molecules can be separated by forcing them to move towards anode under the influence of electric field through a medium/matrix.
  - anode- positive pole.
- Matrix used: ***Agarose***
  - Agarose is extracted from ***sea weeds***.
- The DNA fragments separate (resolve) according to their size through sieving effect provided by the agarose gel.
  - Smaller DNA fragments: Moves the farthest.
  - Larger DNA fragments: Moves the least and typically will be nearer to the loading wells of the agarose gel.

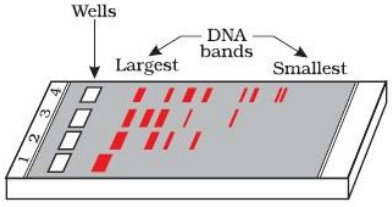
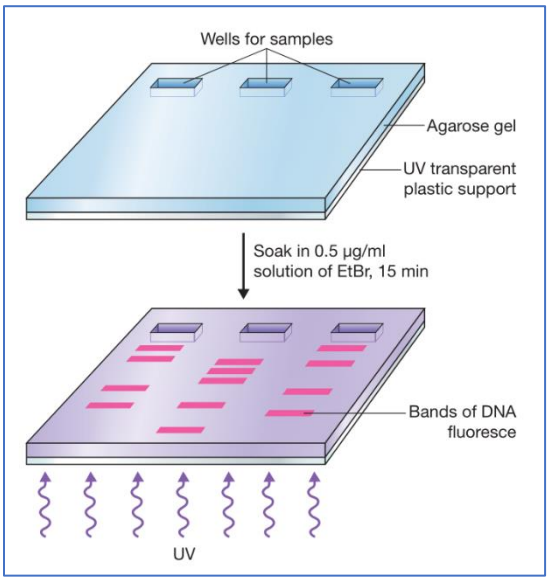


Figure 11.3 A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested set of DNA fragments (lane 2 to 4)

Visualization of separated DNA fragments

- DNA is not visible under normal light without staining with a compound that makes the DNA visible.
- ***Ethidium bromide (EtBr)*** is used for staining the DNA in the agarose gel.
- The different DNA bands in the gel is clearly visible under ***ultraviolet light*** after staining with EtBr.
  - This procedure is very hazardous because ethidium bromide is a powerful mutagen.
- The DNA bands appear as ***bright orange*** colored bands.



Elution

- The process of cutting out the required separated bands of DNA from the agarose gel and extraction of the DNA from the gel piece after the gel electrophoresis is called elution.
- This purified DNA fragments then is used in constructing recombinant DNA by joining them with cloning vectors.

CLONING VECTORS

- Plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA.

Copy Number in Vector

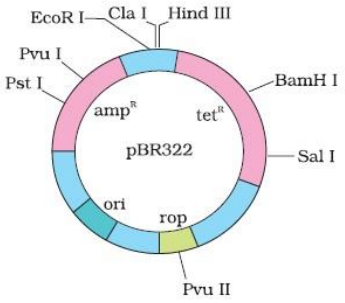
- The copy number refers to the number of molecules of an individual plasmid/phage that are normally found in a single bacterial cell.

- Some plasmids may have only one or two copies per cell whereas others may have 15-100 copies per cell.
- Bacteriophages because of their high number per cell, have very high copy numbers of their genome within the bacterial cells.
- If the foreign DNA (alien DNA) can be linked to this plasmid DNA or bacteriophage, the number of foreign DNA becomes equal to the copy number of the plasmid or bacteriophage.
- In other words higher the copy number, more will be the gene expression, and hence more will be product obtained.

Features of a Cloning Vector

- Certain features are essential for the plasmid for the cloning to take place. These are as follows:

1. Origin of Replication (ori)



- Sequence where ***replication starts***.
- If a DNA sequence is linked with the *'ori'*, it gets replicated.
- *'Ori'* also regulates copy number of this linked DNA.

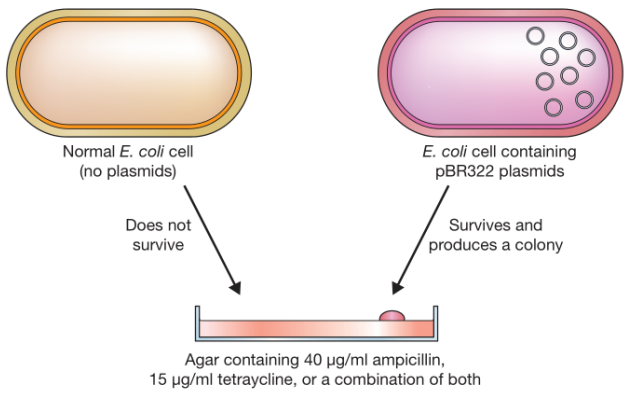
2. Selectable Marker

- It helps in identifying the transformants from the non-transformants that can be eliminated.
- It helps in selectively growing the ***transformants***.
  - Transformation – The process through which a foreign DNA (plasmid/vector/rDNA) is introduced into a host bacterial cell.
  - Transformants – Bacterial cells that have successfully undergone the process of transformation and contains the foreign DNA.
- Examples:
  - Antibiotic resistant gene
    - Ampicillin resistant gene (***amp^R***)
    - Tetracycline resistant gene (***tet^R***)
    - Kanamycin resistant gene
    - Chloramphenicol resistant gene

rop – it is the gene that expresses proteins that are essential for the replication of the plasmid.

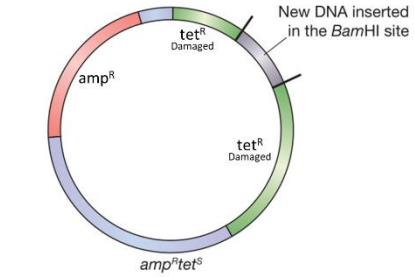
Use of selectable Marker (Just for your Understanding!!)

- It helps to distinguish a cell that has taken up a plasmid (transformant) from the many thousands that have not taken up the plasmid (non-transformants).
- E. coli cells are normally sensitive to the antibiotics ampicillin and tetracycline.
- However, cells that contain the plasmid pBR322 (one of the first cloning vectors to be developed) are resistant to these antibiotics.
- This is because pBR322 carries genes, that makes the host cell (*E. coli*) resistant to ampicillin and tetracycline when expressed.
- After transformation with pBR322, only those E. coli cells that have taken up a plasmid are $amp^R tet^R$ and able to form colonies on an agar medium that contains ampicillin or tetracycline.
- Non-transformants, which does not contain the pBR322, cannot express the antibiotic resistant genes, hence do not produce colonies on the agar medium that contains ampicillin or tetracycline.
- Transformants and non-transformants are therefore easily distinguished.



3. Cloning Sites

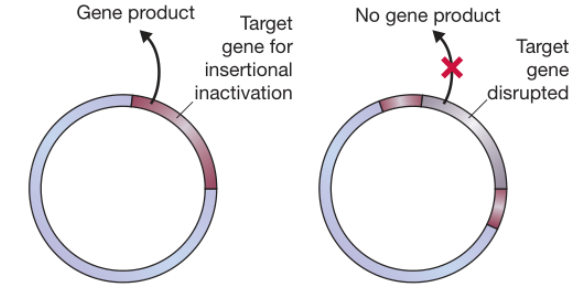
- It refers to the segment of DNA in the plasmid where the alien (foreign) DNA can be inserted.
- The vector/plasmid should ideally have one or very few recognition sites for the commonly used RE.
- For the process of cloning, an RE is chosen that is generally part of the selection marker.
  - For example a foreign DNA can be ligated at the **BamH I** site of tetracycline resistance gene in the vector **pBR322**.



A recombinant pBR322 molecule

Insertional Inactivation

- When we grow bacterial cells on a selective medium (agar medium that contains ampicillin or tetracycline), we can differentiate between the transformants and non-transformants.
- But we still have no idea if the transformants contains the recombinant plasmid DNA or the original plasmid DNA.
- This technique is used to identify the recombinants from the non-recombinants.
  - Recombinants – Plasmid DNA with the inserted foreign/alien/target DNA.

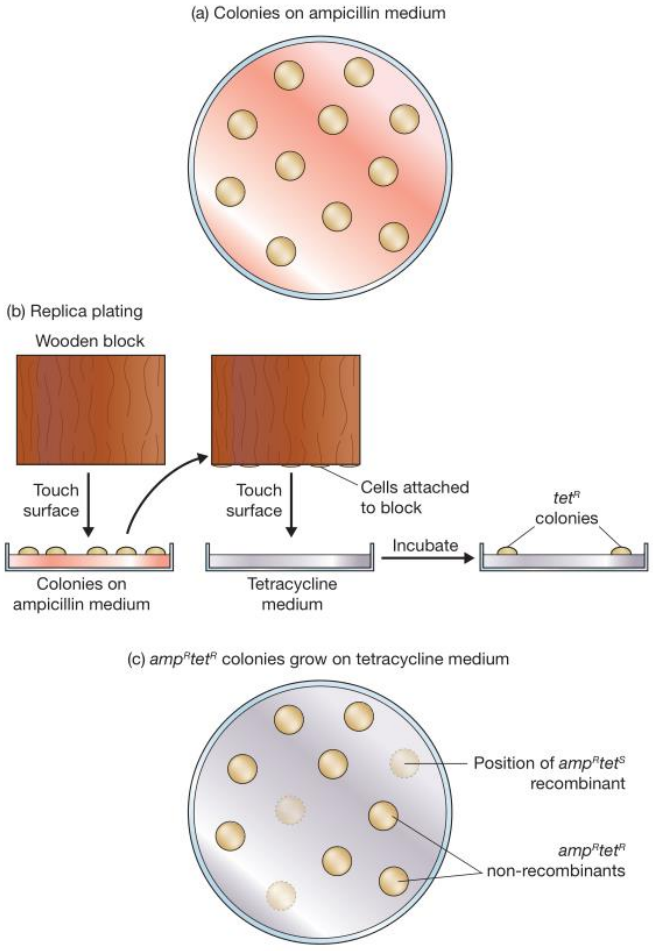


(a) Normal vector molecule (b) Recombinant vector molecule

- The insertion of a foreign DNA fragment into the plasmid destroys the integrity of one of the genes (*selectable marker gene*) present on the molecule.
- Recombinants can therefore be identified because the characteristic coded by the inactivated gene is no longer displayed by the host cells.

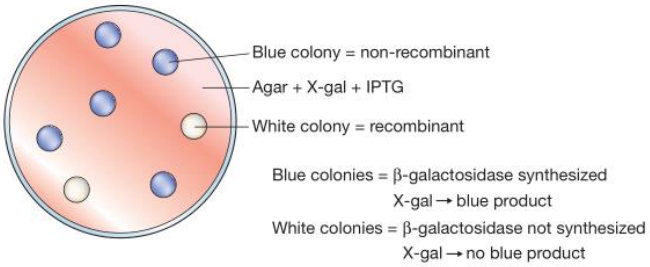
Insertional inactivation of an antibiotic resistance gene

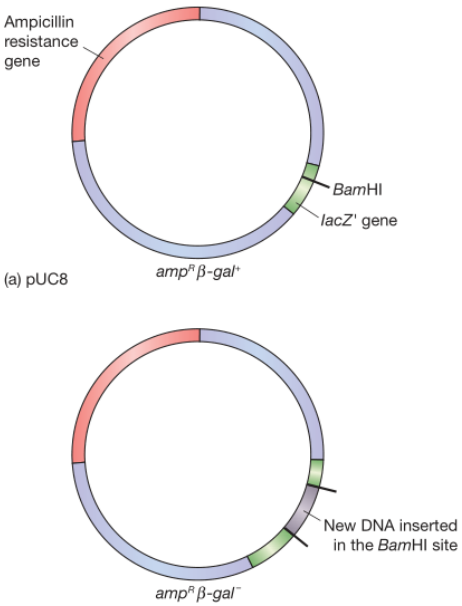
- When a foreign DNA at the *BamH I* site of tetracycline resistance gene in the vector pBR322 is ligated, the recombinant plasmids will lose tetracycline resistance due to insertion of foreign DNA.
- It can still be selected out from non-recombinant ones by plating the transformants on tetracycline 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.



Insertional inactivation without antibiotic resistance gene (β-galactosidase gene)

- Here the recombinants and the non-recombinants are differentiated based on the basis of their ability to produce color in the presence of a *chromogenic substrate*.
- In this method the foreign/target DNA is inserted within the coding sequence of an enzyme, ***β-galactosidase***.
- This inactivated the β-galactosidase gene expression.
- When chromogenic substrate (X-gal) is added, bacterial colonies with functional β-galactosidase gives blue color, while bacterial colonies without the functional β-galactosidase gives no color.





Diagrams only for Reference

Nomenclature of pBR322 ***(Extra information)***

- "p" indicates that this is indeed a plasmid.
- "BR" identifies the laboratory in which the vector was originally constructed (BR stands for Bolivar and Rodriguez, the two researchers who developed pBR322).
- "322" distinguishes this plasmid from others developed in the same laboratory (there are also plasmids called pBR325, pBR327, pBR328, etc.).

Vector for Cloning genes in Plants and Animals

- For Plants
  - ***Agrobacterium tumifaciens*** : It delivers 'T – DNA' in the several dicot plants and transforms the normal cells into tumor and direct these tumor cells to produce the chemicals required by the pathogen.
  - The 'Ti' Plasmid of A tumifaciens has been modified into a cloning vector.
    - It is no more pathogenic to plants.
    - It can deliver the foreign gene into a large number of plants.
- For Animal cells
  - ***Retroviruses*** : It can transform the normal animal cells into cancerous cells.
  - It now has been modified as follows:
    - It is no more pathogenic to animal cells.
    - It can deliver the foreign gene into animal cells.

INTRODUCTION OF ALIEN DNA INTO HOST CELL

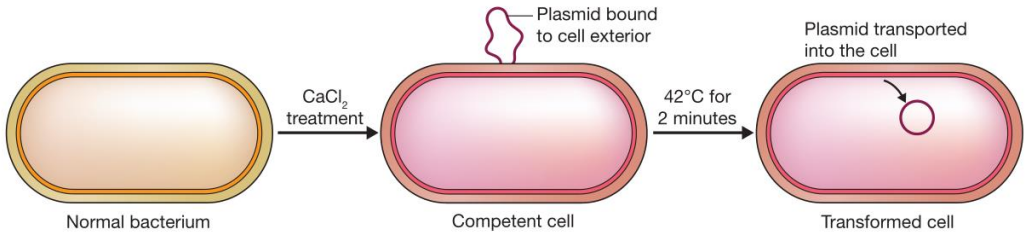
Bacterial Transformation

- Process of uptake of DNA by bacteria.

Competent Cells

- As DNA is hydrophilic in nature, it cannot pass through the cell membranes.
- All bacterial cells can not take up the desired DNA.
- Only ***competent bacterial cells*** can take up the DNA.

- These cells are prepared by treating them with a specific concentration of a ***divalent cation***, such as ***calcium***.[50 mM calcium chloride (CaCl2)]
- It increases the efficiency with which DNA enters the bacterium through pores in its cell wall.



Process of Transformation (Heat Shock Treatment)

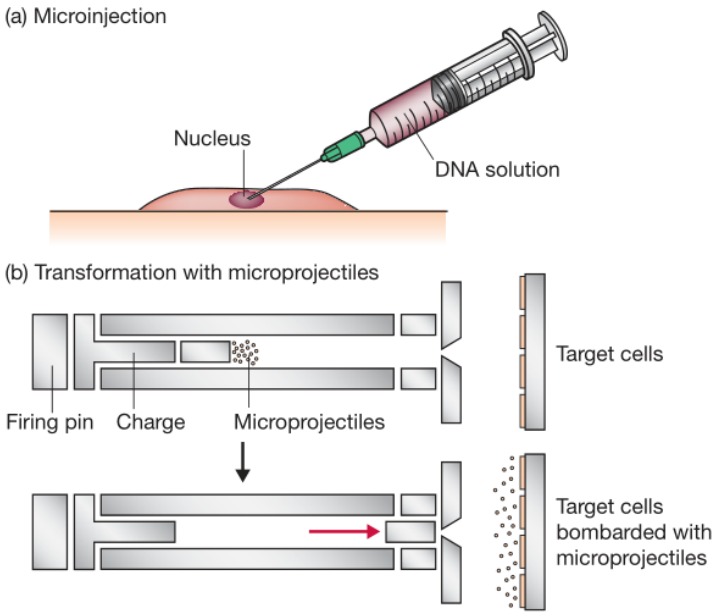
- Competent cells are incubated along with the rDNA (foreign/target DNA) in an ***ice-cold*** condition.
- A ***heat shock*** is given to the cells by briefly placing them at ***42°C***.
- The cells are then again placed back on the ice.
- This allows the competent cells to take up the foreign DNA.

Microinjection

- Here the rDNA is directly injected into the nucleus of the host cell.
- It makes use of a very fine pipette to inject DNA molecules.
- This method is generally used for the animal cells.

Biolistic / Gene gun

- The host cells are bombarded with high velocity microprojectiles, usually particles of gold or tungsten ***coated with DNA.***
- This method is more suitable for the plant cells.



PROCESSES OF RECOMBINANT DNA TECHNOLOGY

- Recombinant DNA technology involves several steps in specific sequence such as isolation of DNA, fragmentation of DNA by restriction endonucleases, isolation of a 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 is isolated by first degrading all the membranes enclosing it with specific enzymes
  - For bacterial cell – ***Lysozyme***
  - For plant cells – ***Cellulase***
  - For fungal cells – ***Chitinase***
- RNA is removed by treatment with RNA digesting enzymes – ***Ribonuclease***.
- Proteins are removed by treatment with protein digesting enzymes – ***Protease***.
- Other biomolecules are removed by appropriate treatments.

- Finally, the DNA is precipitated out by the addition of chilled ethanol.
  - This is seen as collection of fine threads in the suspension.
  - This DNA can be removed by spooling.

➢ CUTTING OF DNA AT SPECIFIC LOCATIONS

- DNA can be cut at specific location by digesting the purified DNA with specific Restriction enzyme.
- Agarose gel electrophoresis can be used to check the progress of restriction digestion.
- After the source DNA and the vector DNA have been cut with a specific RE, the gene of interest is cut out and is ligated with the cut vector DNA (plasmid).
  - The ligation of the gene of interest and the vector DNA is mediated by an enzyme named – ***DNA ligase***.

➢ AMPLIFICATION OF GENE OF INTEREST USING PCR

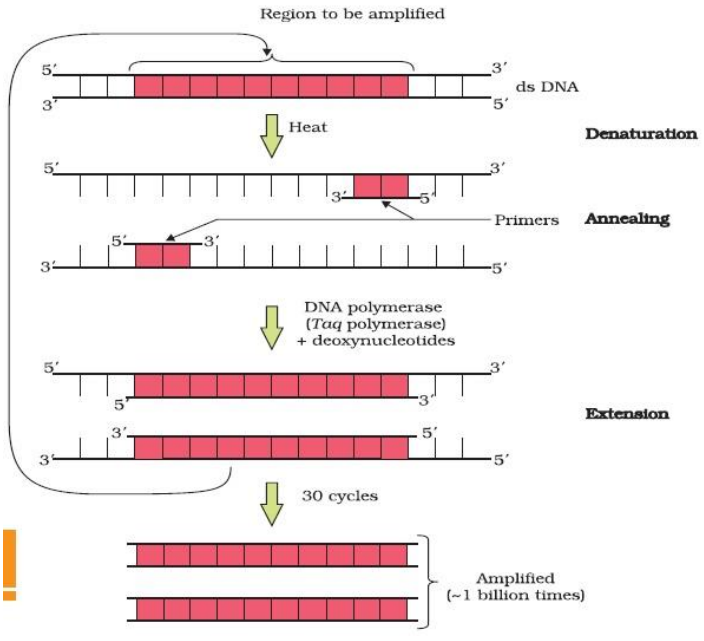
- The Polymerase Chain Reaction (PCR) results in the selective amplification of a chosen region of a DNA molecule.

Requirements for PCR

- Template DNA
- Deoxynucleotides/deoxyribonucleotides
- Thermostable enzyme: ***Taq* Polymerase**
  - Source: Bacterium - ***Thermus aquaticus***
  - Property**:** The enzyme remains active during the high temperature.
- Sets of ***Primers:***
  - Primers are small chemically synthesized oligonucleotides that are complementary to the regions of DNA.
  - They delimit the region of DNA to be amplified.

Step involved in PCR

- PCR is carried out in a single test tube simply by mixing DNA with a set of reagents and placing the tube in a thermal cycler.



Denaturation

- The mixture is heated to 94°C, at which temperature the hydrogen bonds that hold together the two strands of the double-stranded DNA molecule are broken, causing the molecule to denature.
- It forms single stranded DNA (ssDNA).

Annealing

- The mixture is cooled down to 50–60°C.
- The primers anneal (joins) with the ssDNA molecules at specific positions.

Extension

- The temperature is raised to 74°C.
- The *Taq* DNA polymerase works best at this temperature.
- It attaches to one end of each primer and synthesizes new strands of DNA, complementary to the template DNA molecules.
- This results in four stands of DNA instead of the two that there were to start with.

➢ INSERTION OF RECOMBINANT DNA INTO THE HOST CELL/ORGANISM

- Recipient cells after making them 'competent' to receive, take up DNA present in its surrounding.
- So, if a recombinant DNA bearing gene for resistance to an antibiotic (e.g., 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.
- Since, due to ampicillin resistance gene, one is able to select a transformed cell in the presence of ampicillin. The ampicillin resistance gene in this case is called a selectable marker.

➢ OBTAINING THE FOREIGN GENE PRODUCT

- In most of the recombinant technologies, the ultimate aim is to produce a desirable protein.
- The foreign gene gets expressed and produces the maximum protein in appropriate conditions.
- ***Recombinant protein***: If any protein encoding gene is expressed in a heterologous host.

Culturing of Host cell

- The host cells with the rDNA is grown on an appropriate condition (proper nutrients, temperature, pH, etc.)
- Small scale culture: Cells are grown in the laboratory in cultures. The protein are then extracted and purifies by using different separation techniques.
- Large scale culture: Cells are grown in ***continuous culture system*** wherein the used medium is drained out from one side while fresh medium is added from the other to maintain the cells in their physiologically most active log/exponential phase.

Bioreactors

- These are large vessels of large volumes (100-1000 litres), in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial, plant, animal or human cells.
- It provides the optimal conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).

Components of a bioreactor:

- an agitator system
- an oxygen delivery system
- a foam control system
- a temperature control system
- pH control system
- sampling ports so that small volumes of the culture can be withdrawn periodically

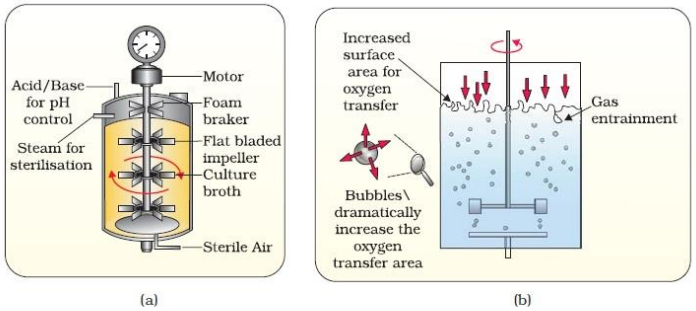


Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

Stirred Tank Bioreactor

- A stirred-tank reactor 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.

Sparged Stirred Tank Bioreactor

- It is a stirred-tank reactor type bioreactor where the air is bubbled.

➢ DOWNSTREAM PROCESSING

- It involves all the stages after the expression of the gene product in the culture system by the host cells (biosynthetic stage).
- The processes include ***separation*** and ***purification***, which are collectively referred to as downstream processing.
- Suitable ***preservatives*** if required are added.
- If the product is a drug, then it undergoes ***clinical trials***.
- The downstream processing and quality control testing vary from product to product.