Biotechnology : Principles and Processes

Introduction to Biotechnology

What is biotechnology?

- Biotechnology refers to the technology using biology, which has applications in agriculture, food processing industry, medicine diagnostics, bioremediation, waste treatment, and energy production.
- The European Federation of Biotechnology (EFB) defines biotechnology as "the integration of natural science and organisms, cells, parts thereof and molecular analogues for products and services".

Basis of Modern Biotechnology

- **Genetic engineering –** Introduction of foreign genetic material (DNA/RNA) into the host's genome and altering its phenotype
- **Aseptic techniques –** Involves maintenance of contamination-free ambience in chemical engineering processes for manufacture of products such as antibiotics, vaccines, etc.This is done so as to enable the growth of only desired microbes responsible for a bioprocess.

Genetic Engineering

- Asexual reproduction preserves the genetic information while sexual reproduction preserves variations.
- Plant and animal hybridization procedures often result in introduction of undesirable genes along with desirable ones.
- Genetic engineering overcomes this limitation.
- Genetic engineering includes:
- Creation of recombinant DNA
- Gene cloning
- Gene transfer into host organism
- The introduced piece of DNA does not replicate in the host unless it is integrated with the chromosome of host.

- For getting replicated, the foreign DNA must integrate into the host DNA sequence having 'origin of replication'. When this integration occurs, foreign DNA is replicated and many copies are formed. This process is called **cloning** (the process of formation of multiple identical copies of DNA).

Construction of a Recombinant DNA

- Plasmid (autonomously replicating, circular, extra-chromosomal DNA) is isolated.
- Plasmid DNA acts asa **vector** since it is used to transfer the piece of DNA attached to it to the host.
- Plasmid DNA also contains genes responsible for providing antibiotic resistance to the bacteria.
- Plasmid DNA was cut with a specific restriction enzyme ('molecular scissors' – that cut a DNA at specific locations).
- The DNA of interest (to be inserted) was also cut with the same restriction enzyme.
- The DNA of interest is hybridised with the plasmid with the help of DNA ligase to form a **Recombinant DNA**.
- Recombinant DNA is then transferred to a host such as *E.coli*, where it replicates by using the host's replicating machinery.
- When *E.coli* is cultured in a medium containing antibiotic, only cells containing recombinant DNA will be able to survive due to antibiotic resistance genes and one will be able to isolate the recombinants.

Restriction Enzymes as Tools of RDT

- Restriction enzymes are specialised enzymes that recognise and cut a particular sequence of DNA.
- Nucleases are of two types:
- Endonucleases – Cut the DNA at specific positions within the DNA
- Exonucleases – Cut the DNA at the ends (Remove the nucleotides at the ends of the DNA)
- Every restriction enzyme identifies different sequences (Recognition sequences). Over 900 restriction enzymes have been isolated, all of which recognise different sequences.
- Recognition sequences are **pallindromic-** Pallindromes are the sequence of base pairs that read same both backwards and forwards (i.e., same $\text{in } 5' \rightarrow 3'$ and $3' \rightarrow 5'$ direction).
  Example:

$5' - GAATTC - 3'$

$3' - CTTAAG - 5'$

- Restriction enzymes cut a little away from the centre of pallindrome site, but between the same two bases on the opposite strands.

$$\downarrow$$

$5' - GAATTC - 3'$

$3' - CTTAAG - 5'$

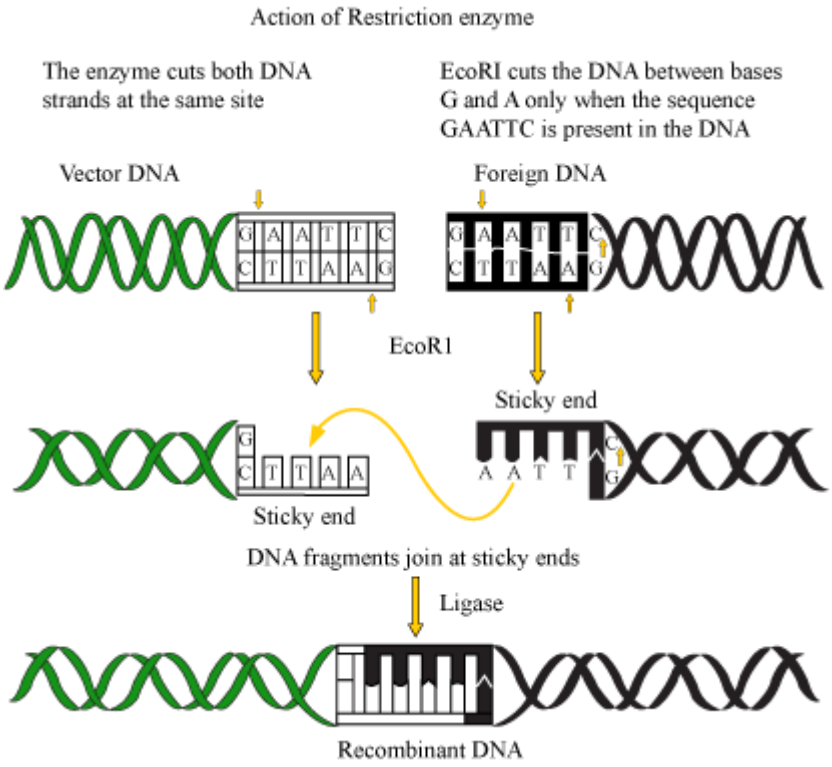
$$\uparrow$$

- As a result, overhangs (called sticky ends) are generated on each strand.

$5' - G$ and $AATTC\,3'$

$3' - CTTAA$ $G5'$

- Sticky ends form hydrogen bonds with their complementary counterparts with help of DNA ligases.
- All these processes form the basis of RDT.



- Naming restriction enzyme
- Ist letter – Genus of the organism from which the enzyme is derived

- IInd and IIIrd letters – Species of the organism
- IVth letter – Name of the strain
- Roman number – Order of isolation
  E.g., In EcoRI – Derived from *E.coli*, strain R.
  It is the Ist to be discovered.

Gel Electrophoresis

- The fragments obtained after cutting with restriction enzymes are separated by using gel electrophoresis.
- Electric field is applied to the electrophoresis matrix (commonly agarose gel) and negatively charged DNA fragments move towards the anode.
- Fragments separate according to their size by the sieving properties of agarose gel. Smaller the fragment, farther it moves.
- Staining dyes such as ethidium bromide followed by exposure to UV radiations are used to visualise the DNA fragments.
- DNA fragments are visible as bright orange coloured bands in the agarose matrix.
- These bands are cut from the agarose gel and extracted from the gel piece (elution).
- DNA fragments are purified and these purified DNA fragments are used in constructing recombinant DNAs.

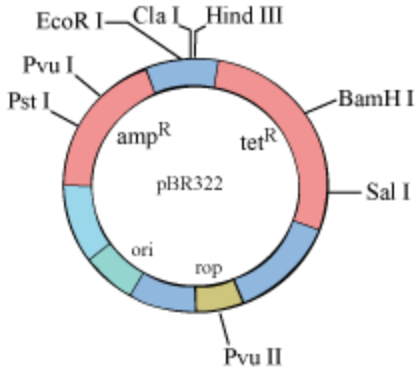
Cloning vectors & host as tools of RDT

Cloning Vectors

- Plasmids and bacteriophages are commonly used as cloning vectors.
- Both of these have the ability to replicate within the bacterial cells independent of the chromosomal DNA.
- Bacteriophages – Have high copy number (of genome) within the bacterial cell
- Plasmids – May have 1 – 2 copy number to 15 – 100 copy number per cell
- If foreign DNA is linked to these vectors, then it is multiplied to the number equal to the copy number of vector.

- Features present in the vector itself help in the easy isolation of recombinants from the non-recombinants.

Components of a plasmid cloning vector



- **Origin of replication (*ori*)**
- Replication starts from *ori*. Any fragment of DNA when linked to *ori* can be made to replicate.
- With the help of this, the genetic engineer may control copy number of the recombinant DNA. To recover a high number, suitable origin of replication must be chosen.
- **Selectable marker**
- These genes help to select recombinants over non-recombinants.
- Antibiotic resistance genes such as amp^R (ampicillin resistant), tet^R (tetracycline resistant) serve as selectable markers usually.
- **Cloning sites**
- These sites refer to the recognition sites for restriction enzymes (such as EcoRI, Hind III, PvuI , BamHI, etc.)
- These are the sites where restriction enzymes cut the DNA.
- Cloning process becomes complicated when more than one recognition sites are present.
- Therefore, ligation is carried out only at the restriction sites present on the antibiotic resistance genes.

How antibiotic resistance genes help in selecting recombinants?

- Suppose tet^R gene has Bam HI recognition site.

- When BamHI is used for restriction, foreign DNA fragment is inserted within the tet^R gene.
- Hence, tetracycline resistance is not present in the recombinants.
- Recombinants will grow on the media containing ampicillin, but will die on media containing tetracycline.
- On the other hand, non-recombinants will grow on medium containing ampicillin as well as on medium containing tetracycline.
- In this way, antibiotic resistance gene helps in selecting transformants.

Alternate selectable marker

- Other than antibiotic resistance genes, alternative markers can be used.
- One of them is gene coding for $\hat{a}$ galactosidase.
- When foreign gene is inserted within $\hat{a}$-galactosidase gene, the enzyme $\hat{a}$-galactosidase gets inactivated (insertional inactivation).
- Then the bacteria are grown on a chromogenic substrate.
- Non-recombinants will produce blue-coloured colonies.
- Recombinants will produce colourless colonies.

Cloning vectors for plants and animals

- Ti plasmid (tumour-inducing plasmid) refers to the plasmid of *Agrobacterium tumefaciens*.
- *A. tumefaciens* is a plant pathogen. It produces tumours in the plants it infects.
- Ti plasmid can be modified into a cloning vector by removing the genes responsible for pathogenicity.
- Retrovirus – These are the viruses that infect animals. They produce cancers in animals.
- Retroviruses can be disarmed to be used as a cloning vector.

Competent host

- Competent host refers to the bacterial cells that have the ability to take up the vector (containing Recombinant DNA).
- Methods to introduce recombinant DNA into competent host:

- Cells are treated with divalent cations (e.g. Ca^{2+}). Then, these cells are incubated with recombinant DNA on ice, followed by heat shock (at 42º), and then putting them back on ice. By this, bacteria are able to take up recombinant DNA.

- **Microinjection** – Recombinant DNA is directly injected into the nucleus of animal cell.

- **Biolistics (Gene Gun)** – Cells are bombarded with high velocity micro particles of gold or tungsten.

- Disarmed vector as in case of *A. tumefaciens* and retrovirus

Processes of RDT

Isolation of Genetic Material (DNA)

- For the processes of RDT, DNA must be available in its pure form.

- First of all, cells are treated with specific chemicals to break open the cell to release cellular components such as DNA, RNA, proteins, etc.
  This is done by enzymes such as lysozymes (bacterial cell), cellulase (plant cell), and chitinase (fungal cell).

- Contaminants such as RNA and proteins are digested with the help of ribonucleases and proteases respectively.

- Addition of chilled ethanol ultimately precipitates out the purified DNA, which can be seen as collection of fine threads in the suspension.

Cutting of DNA at Specific Locations

- DNA is cut into fragments with the help of restriction enzymes.

- Fragments generated after restriction are isolated with the help of gel electrophoresis.

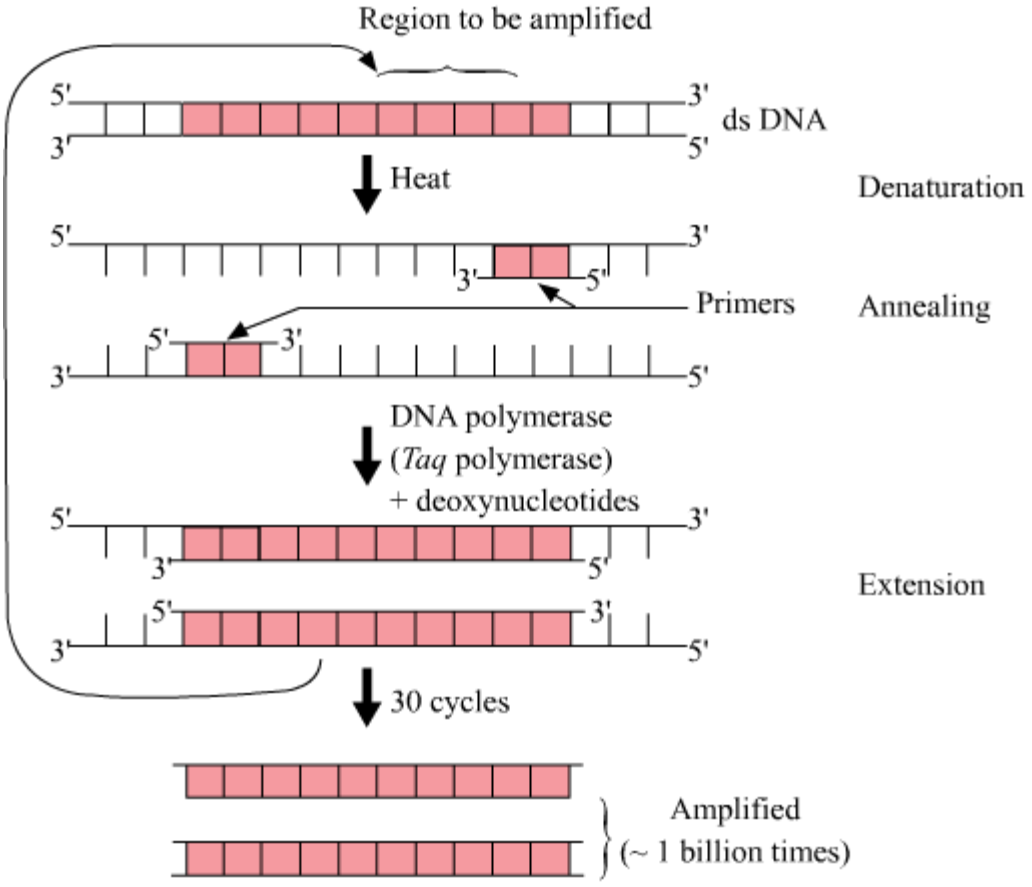
- Recombinant DNA is obtained by hybridising 'gene of interest' with vector, with the help of enzyme DNA ligase.

Polymerase Chain Reaction (PCR)

- Recombinant DNA can be amplified by PCR. Several identical copies of it can be synthesised in vitro.

- Two sets of **primers** (chemically synthesised oligonucleotide stretches that are complementary to a region of DNA), enzyme **DNA polymerase**,and deoxynucleotides are added.

- PCR consists of 3 steps:
- **Denaturation** – Double helical DNA is denatured by providing high temperature. DNA polymerase does not get degraded in such high temperatures since the DNA polymerase used in this reaction is thermostable as it is isolated from thermophilic bacteria, *Thermus aquaticus* (*Taq*).
- **Annealing-** It is the step in which primers are annealed to single stranded DNA templates.Two sets of primers (small chemically synthesised oligonucleotides that are complementary to the regions of DNA) are used. The temperature of reaction mixture is lowered to 50- 65°C for some seconds to allow annealing of primers. DNA polymerase extends the primer in 5' to 3' direction.
- **Extension** – Replication of DNA occurs in vitro.
- This cycle is repeated several times to generate up to 1 billion identical copies of the DNA.

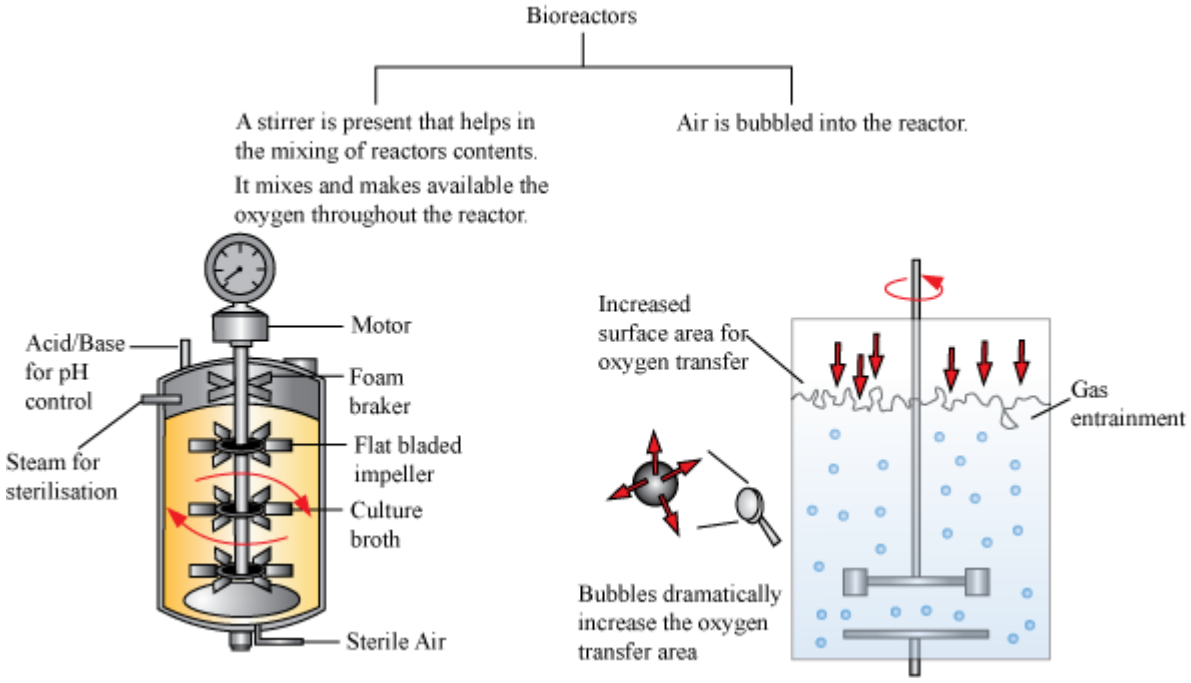


Insertion of Recombinant DNA into Competent Cells

- Insertion of recombinant DNA into host is done by several methods:
- Transformation in case of bacteria
- Disarmed vectors, biolistics, and micro-injections in case of plant and animal cells
- The cells bearing recombinant DNA are selected because the recombinants exclusively have selectable marker present in them (similar to antibiotic resistance).

Obtaining the Foreign Gene Product

- This is the stage for which the recombinant DNA was produced.
- The cell containing recombinant DNA will produce a novel protein product (desirable product/Recombinant protein).
- For large scale production of the desirable product (antibiotics, vaccines, enzymes), optimum conditions are to be provided.
- Continuous culture − Used culture media is drained from one side and fresh culture media is added from the other side.
- Cells are kept throughout in their log/exponential phase.
- Larger biomass is produced by this method leading to higher yield.
- Bioreactors − Large vessels in which large volumes (100 − 1000 litres) of culture can be produced
- Optimal growth conditions for microbes are present (temperature, pH, substrate, salts, vitamins, etc.).



- A bioreactor has the following components - agitator system, oxygen delivery system, foam control system, temperature and pH control system, sampling ports.

Downstream Processing

- Biosynthesis of many compounds such as enzymes, alcohols, and antibiotics take place within the bioreactor.
- The products so obtained are crude and require separation, purification, and finishing, which is done under downstream processing (DSP).
- DSP makes a crude bio product marketable.
- After proper separation and purification, preservatives are added and the finished product is made to undergo clinical trials and quality checks before being sent to market.