Biotechnology: Principles and Processes

1 INTRODUCTION

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

Parameters	Traditional biotechnology	Modern biotechnology
Organisms involved	Microbes	Genetically modified organisms
Production	Small scale	Large scale
Examples/Technique include	Curd, bread or wine making	*In vitro* fertilisation leading to a 'test -tube' baby
		Synthesising a gene and using it
		Developing a DNA vaccine
		Correcting a defective gene

EFB (European Federation of Biotechnology)

- **'The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'.**
- It encompasses both traditional view and modern molecular biotechnology.

2 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 resistanse gene in *E.coli* was called **cloning** of antibiotic resistance gene in *E.coli*.

3 ADVANTAGES OF BIOTECHNOLOGY OVER OTHER TECHNIQUES

Methods	Advantage	Disadvantage
I. Asexual reproduction	Preserves genetic information	No variations
II. Sexual reproduction	Provides opportunities for variations and formulation of unique combinations of genetic setup	Some of which may be harmful to the organism as well as the population
III. Traditional hybridisation	Used in plant and animal breeding.	Very often lead to inclusion and multiplication of undesirable genes along with desirable genes.
IV. Genetic engineering	Allows us to isolate and introduce only one or a set of desirable genes without introducing undesirable genes into target organism.	—

4 THREE BASIC STEPS IN GENETICALLY MODIFYING ORGANISMS

(1) Identification of DNA with desirable genes (2) Introduction of the identified DNA into the host
(3) Maintenance of introduced DNA in the host and transfer of the DNA to its progeny

5 KEY TOOLS OF RECOMBINANT DNA TECHNOLOGY

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

Enzymes - Most commonly used enzymes in genetic engineering are
- **Nucleases**
- **DNA polymerase**
- **Ligases**

Nucleases - Catalyse the cleavage of nucleic acids.

Types

Exonucleases
Remove nucleotides from the ends of the DNA

Endonucleases
Make cuts at specific positions within the DNA *i.e.* at **recognition/palindromic sequence**

- In the year 1963, the two enzymes responsible for **restricting the growth of bacteriophage** in *Escherichia coli* were isolated

Methylase
Add methyl groups to bacterial DNA

Restriction endonuclease / Molecular scissors
Cut the DNA of bacteriophage

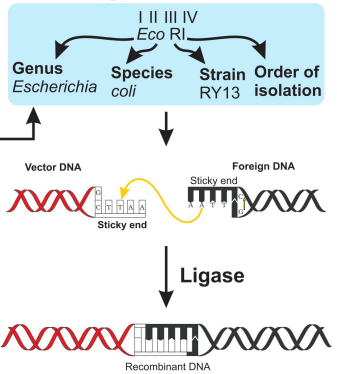
Palindromic sequence
reads same on the two strands (from 5′ → 3′ and 3′ → 5′ direction) when orientation of reading is kept same

6 ENZYMES

- **Restriction endonuclease**

More than **900** restriction enzymes have been isolated from over **230** strains of bacteria (**prokaryotic cell**) each of which recognise different recognition sequences.

- **Nomenclature/Naming of enzyme :**
- **Functions by:**
  - **'Inspecting'** the length of DNA sequence
  - **Binds** to the **"specific recognition sequence"**
  - **Cuts** the two strands of **ds DNA** at specific points in their **sugar-phosphate backbones** and leaves single stranded portions at the ends.
  - These **overhanging stretches** and called **sticky ends.**



Steps in formation of rDNA

- **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 and this stickiness facilitates the action of the enzyme **DNA ligase**.

- First restriction endonuclease - ***Hind* II :** Isolated and characterised five years later, recognises sequence of **6 bp**.
- First recombinant DNA was prepared by **Stanley Cohen and Herbert Boyer, 1972** :
  - Antibiotic resistant gene
  - Plasmid of *Salmonella typhimurium*

→ Recombinant plasmid —Introduced into→ *Escherichia coli*

7 CLONING VECTORS

- **Vectors are vehicles** for delivering foreign DNA into recipient cells.
- Vectors used at present are engineered in such a way that they help **easy linking of foreign** DNA and selection of recombinants from non recombinants

Features of cloning vectors:

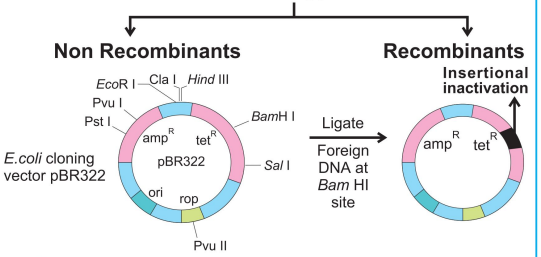
(1) **Origin of Replication (ori):**
- Sequence from where replication starts
- Responsible for **controlling copy number** of the linked DNA
- Those vectors are preferred which support **high copy number**

(2) **Selectable Marker:**
- Helps in selection of transformants
- Normally, the **genes encoding resistance to antibiotics** such as ampicillin, chloramphenicol, tetracycline or kanamycin, etc., are considered useful selectable markers for *E.coli*
- The **normal *E.coli* cells do not carry resistance against any of these antibiotics**

(3) **Cloning Sites/Restriction Sites**
- **Single recognition site** for a restriction enzyme within the vector is a **preferable** feature.
- Presence of more than one recognition sites within the vector will generate several fragments, which will complicate the gene cloning
- The ligation of alien DNA/**gene of interest (GOI)** is carried out at a restriction site present in one of the antibiotic resistant genes.

- **Transformation:** Procedure through which piece of foreign DNA is introduced in a host bacterium.
  - **Insertional inactivation:** Insertion of GOI within antibiotic resistance gene/selectable marker results in inactivation/formation of the coded product.
  - **Hypothesis:** Insertion of GOI at *Bam* HI site in tet^R.
  - If transformation **fail**s – Non transformants are obtained in antibiotic lacking agar medium but they don't grow on antibiotic rich medium.
  - If transformation **successful** – **Transformants** obtained are of **two types:**

- **All transformants are not recombinants but all recombinants are transformants.**
- One antibiotic resistant gene helps in selecting the transformants whereas the other antibiotic resistant gene helps in selection of recombinants
- *rop* → codes for the proteins involved in the replication of the plasmid



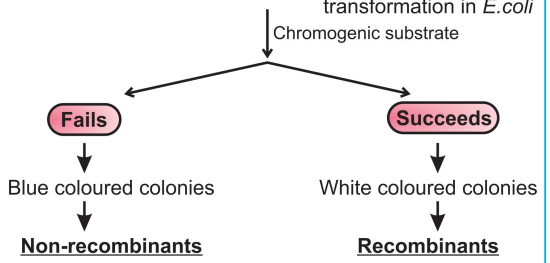
- **Plasmids as vectors:**
  - **Extra chromosomal**, circular, double stranded DNA.
  - Replicate independent of the control of chromosomal DNA (autonomously).
  - They may have 1 or 2 copies per cell or even15 - 100 copies per cell.

9 OTHER CLONING VECTORS

Selection of recombinants due to inactivation of antibiotic resistant gene as in pBR322 is a cumbersome procedure because it requires simultaneous plating of two plates having different antibiotics.

To **overcome the disadvantage of pBR322**, alternative selectable markers (***lac Z***) acting as **reporter enzyme** have been developed which differentiate recombinants from non-recombinants on the basis of their ability to produce colour in the presence of chromogenic substrate.

- *lac* Z gene coding for β-galactosidase acts as selectable marker in the plasmid
- **Experiment:** Insert foreign DNA at *lac* Z gene + transformation in *E.coli*



- **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**

10 METHODS OF TRANSFORMATION

I. Micro - injection

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

II. Biolistic/Gene gun

- **Plant cells** are bombarded with high velocity micro-particles of **gold or tungsten coated with DNA**.

III. Heat shock method

IV. "Disarmed pathogen" vector

11 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**.
- 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.

12 PROCESS OF RECOMBINANT DNA TECHNOLOGY

Isolation of DNA
↓
Fragmentation of DNA by restriction endonucleases
↓
Isolation of desired DNA fragment (**electrophoresis**)
↓
Amplification of gene of interest (**PCR**)
↓
Ligation of the DNA fragment into a vector
↓
Transferring the alien DNA/recombinant DNA into the host
↓
Culturing the host cells in a medium at large scale (**Bioreactors**)
↓
Extraction and **purification** of the desired product

I. Isolation of the Genetic Material (DNA)

- In majority of organisms, DNA is the genetic material
- Since DNA is enclosed within the membranes, we have to break the cell open to release DNA along with other macromolecules → **Bacteria → Lysozyme**; **Fungi → Chitinase**; **Plant** cell → **Cellulase**
- In order to get DNA in pure form (free from other macromolecules), it is treated with different enzymes like RNase, protease etc.

Pure DNA → Centrifuge → **Chilled ethanol to precipitate DNA**

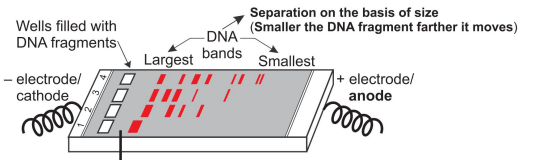
Process: **Spooling**

II. Fragmentation by restriction endonucleases

III. 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 → Natural polymer, obtained from **sea weeds**; Separate DNA fragments through **seiving effect**



Gel → Stained with **Ethidium Bromide** → Exposed to **U.V rays** → Appears: **Bright orange bands** → Removal of DNA fragment from gel → Process: **Elution**

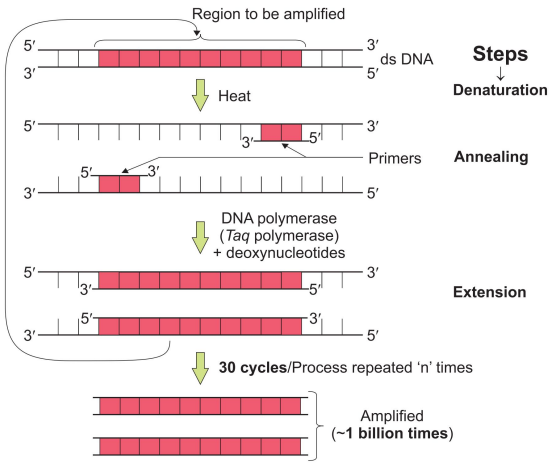
- Purified DNA fragments are generally amplified (PCR) before constructing rDNA by joining with cloning vector.

IV. PCR - Polymerase Chain Reaction

- *In vitro* amplification of DNA (gene of interest)

Reaction mixture	Work/Function
Nucleotides	Formation of DNA chain
Primers	2 sets of chemically synthesised oligonucleotides, complementary to the regions of DNA
***Taq* polymerase**	**Thermostable** DNA polymerase, isolated from bacterium, ***Thermus aquaticus***, remains active during high temperature induced denaturation of dsDNA. It extends the primers *i.e.* meant for chain elongation.
Genome DNA	Template DNA for gene of interest

- **Sequence of events**



- The amplified fragment if desired can now be used to ligate with a vector for further cloning.

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

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VI. Insertion of recombinant DNA into the host cell

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

VII. Culturing of recombinant host cells (Biosynthetic stage)

- The cells harbouring cloned genes of interest may be grown in **Laboratory/ Bioreactors**

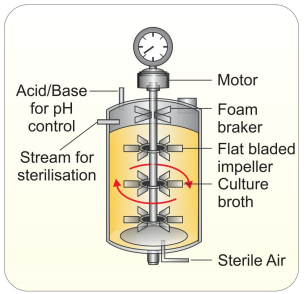
Parameters	Laboratory	Bioreactors
Culture	Small volume	Large volumes (100 - 1000 lts)
Maintaining optimal conditions	Not possible	✓
Growth rate of cell	Never optimal	Optimum
Production	Small scale	Large scale

- **Bioreactors:** Vessels in which raw materials are biologically converted into specific products using microbial plant, animal human cells and provide optimal growth conditions (temperature, pH, substrate, salts, vitamins, oxygen)

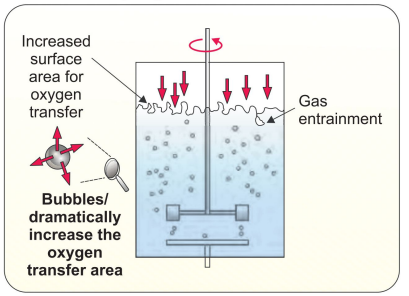
- Commonly used Bioreactors are **stirred type** having
  - Cylindrical or with curved base → Facilitate mixing of reactor contents
  - Stirrer → **Facilitate even mixing** and **oxygen availability** throughout the bioreactor
  - Agitator system
  - Oxygen delivery system
  - pH control system
  - Foam control system
  - Sampling ports → To withdraw small volumes of culture periodically

- **Types of stirred tanks**

Simple stirred tank



Sparged stirred tank



In Open Culture System/ Continuous Culture System

- 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**.
- Larger biomass → Higher yields of desired protein.

VIII. 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.

IX. Product is subjected for marketing as a finished product