BIOTECHNOLOGY - PRINCIPLES AND PROCESSES

The definition given by EFB is as follows: **'The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'.**

Stanley Cohen and Herbert Boyer : First made recombinant DNA by linking an antibiotic resistance gene with a plasmid of ***Salmonella typhimurium.***

Paul bergh (Father of genetic engineering). He transferred gene of SV-40 virus (simian virus) in to E.coli with the help of λ – phage. (Nobel prize - 1980)

Principles of Biotechnology

Genetic Engineering
Formation of transgenic organism or genetically modified organism **(GMO)**

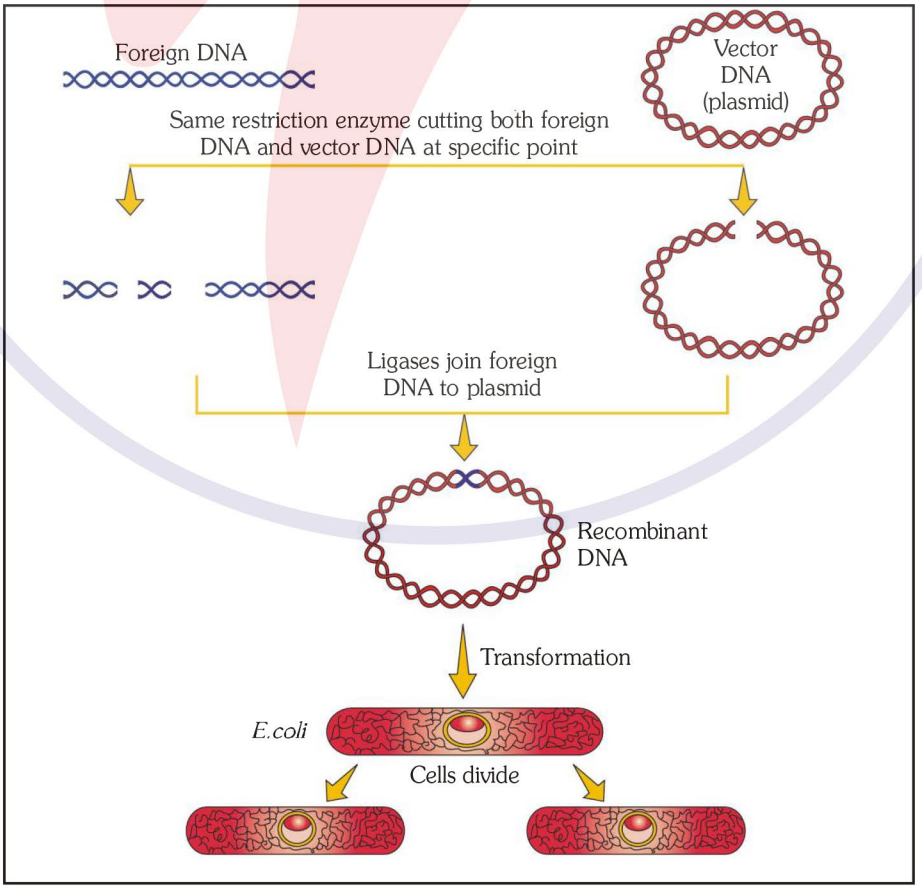
Maintenance of GMO to obtain desired products.

Genetic Engineering: Techniques to alter the chemistry of genetic material to introduce it into host organisms and thus change the phenotype of host organisms.

This involves Recombinant DNA Technology.

Recombinant DNA Technology (RDT): Involve techniques to form new combinations of heritable genetic material followed by the incorporation of that material from different organisms.

RDT having at their core the process of Gene Cloning.



TOOLS AND TECHNIQUES OF GENETIC ENGINEERING

Tools

1. Enzymes
2. Vehicle DNA or Vector DNA
3. Passenger DNA
4. Host Cell

1. **Enzymes :**

These include lysing enzymes, cleaving enzymes and joining enzymes.

(i) **Lysing enzymes:** These enzymes are used for opening the cells to get DNA for genetic experiment.

- Bacterial cell : is commonly digested with the help of lysozyme.
- Plant cell : is commonly digested with the help of **cellulase** and **pectinase**.
- Fungul cell : is commonly digested with the help of **chitinase**.

(ii) **Joining enzymes (molecular glues):** These enzymes help in joining the DNA fragments.

(iii) **Cleaving enzymes:** These enzymes are used for DNA molecules.

Types of cleaving enzymes :

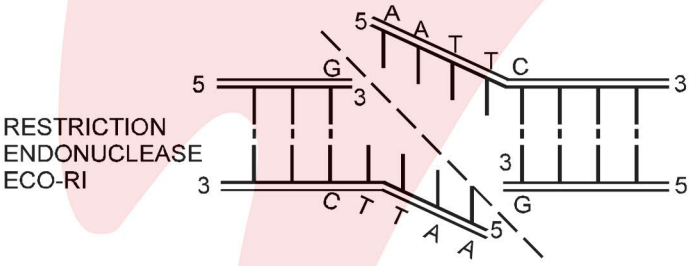
(a) **Exonucleases** cut off nucleotides from 5' or 3' ends of DNA molecule.

(b) **Endonucleases** break DNA duplex at any point except the end.

(c) **Restriction endonucleases :** A restriction enzyme or restriction endonuclease (also known as 'molecular scissors) is an enzyme that cleaves DNA into fragments at or near specific recognition sites within the molecule known as restriction sites or recognition sites.

Restriction site is 4-8 bp long pallindromic sequence

Every restriction enzyme has their own restriction site.



The first isolated and characterized restricticton endonuclease was HindII, isolated by Smith from bacterium Haemophilus influenzae.

At present more than 900 restriction enzymes have been isolated from 230 strains of bacteria.

Restriction enzyme is so named as it restricts the growth of bacteriophage in bacteria cell.

Naming of Restriction Enzymes :

1st Letter : Genus

2nd and 3rd Letter : Species

4th Letter : Strain

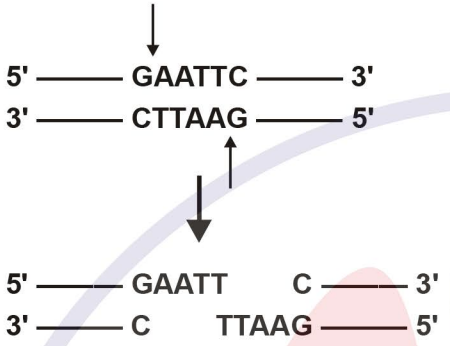
Roman number : Order of Isolation

Example: Restriction enzyme EcoRI

Abbreviation	Meaning	Description
E	Escherichia	Genus
co	coli	species
R	RY-13	strain
I	First isolated	Order of isolation from that bacterial strain

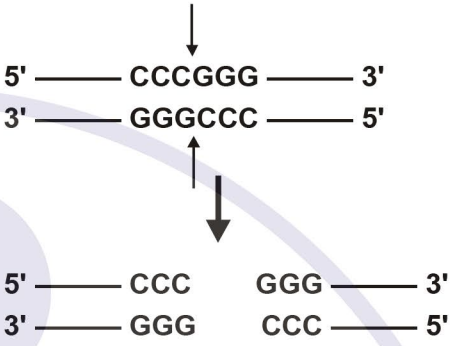
Mode of cutting of Restriction enzymes:

1. **Oblique cut:** Restriction enzyme cuts the DNA slightly away from the center of pallindromic sequence. It produces **Sticky ends**. Eg. ***EcoRI***



Example :- Hind-III , Bam HI, Sal I

2. **Straight cut:** Restriction enzym cuts the DNA in the center c pallindromic sequence. It produce **Blunt ends.** Eg. ***SmaI***



Example :- ECoR-V, Hae-III

2. **Vehicle DNA or Vector DNA.** The DNA used as a carrier for transferring a fragment of foreign DNA into a suitable host is called **vehicle or vector DNA.**

The following are the features that are required to facilitate cloning into a vector.

(i) **Origin of replication (ori) :** This is a sequence from where replication starts.

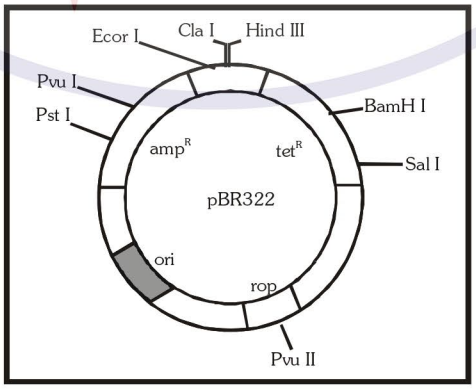
(ii) **Selectable marker :** In addition to 'ori', the vector requires a selectable marker. Normally, the genes encoding resistance to antibiotics such as ampicilin, chloramphenicol, tetracycline or kanamycin, etc., are considered useful selectable markers for E. coli.

(iii) **Cloning sites :** In order to link the alien DNA, the vector needs, **recognition sites** for the commonly used restriction enzymes.

Some examples of vector :-

(I) Plasmid :- Double stranded, circular, extra chromosomal DNA Segment.

Ex. :- pBR-322, pUC 18



Agrobacterium tumifaciens, a pathogen of several dicot plants deliver a piece of DNA known as 'T-DNA' to transform normal plant cells into a **tumour**.

(II) Virus :- Retrovirus for animal cell.

(III) BAC and YAC (For large DNA fragment)

3. **Passenger DNA.** It is the DNA which is transferred from one organism into another by combining it with the vehicle DNA. The passenger DNA can be complementary, synthetic or random.

4. **Host Cell :-** This organism is used for DNA cloning.

Host may be plant, animal, bacteria (E.coli), fungi (Yeast).

Processes of Recombinant DNA Technology :

1. **Isolation of the Genetic Material (DNA)**

DNA Extraction :- Cells are lysed using a enzymes that disrupts the plasma membrane.

↓

Cell contents are treated with protease to destroy protein, and RNAase to destroy RNA.

↓

Cell debris is pelleted in a centrifuge. The supernatant (liquid) containing the DNA is transferred to a clean tube.

↓

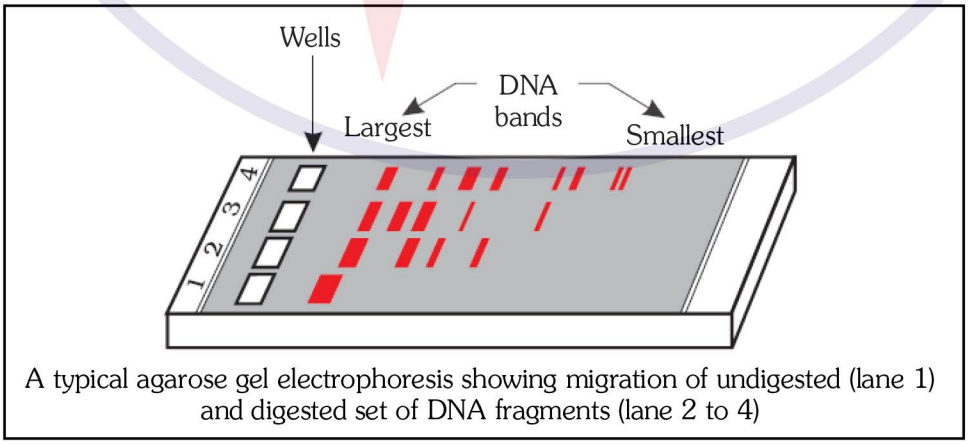
The DNA is precipitated with ethanol. It forms viscous strands that can be spooled on a glass rod.

2. **Fragmentation of DNA by restriction endonucleases :-**

Restriction enzyme digestions are performed by incubating purified DNA molecules with the restriction enzyme, at the optimal conditions for that specific enzyme.

Agarose gel electrophoresis is employed to check the progression of a restriction enzyme digestion.

Both the passenger and vehicle DNAs are then, cleaved by using the same restriction endonuclease so that they have complementary sticky ends.

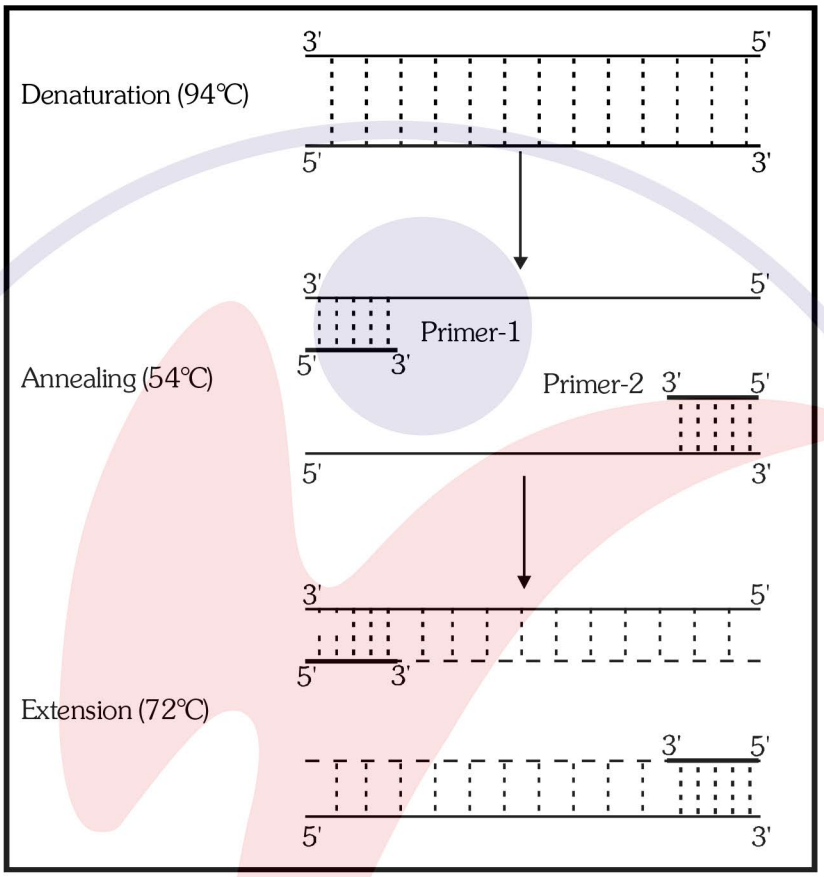


A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested set of DNA fragments (lane 2 to 4)

3. Amplification of gene of interest using PCR (Polymerase chain reaction technology)

PCR reaction takes place in **Eppendorf tube**.

Taq polymerare enzyme is used in PCR which is a special type of DNA polymerare enzyme which is resistant to high temperature.



4. Ligation of the DNA fragment into a vector :-

The complementary sticky ends of the passenger and vehicle DNAs are joined with ligase enzyme. This gives rise to a recombinant DNA.

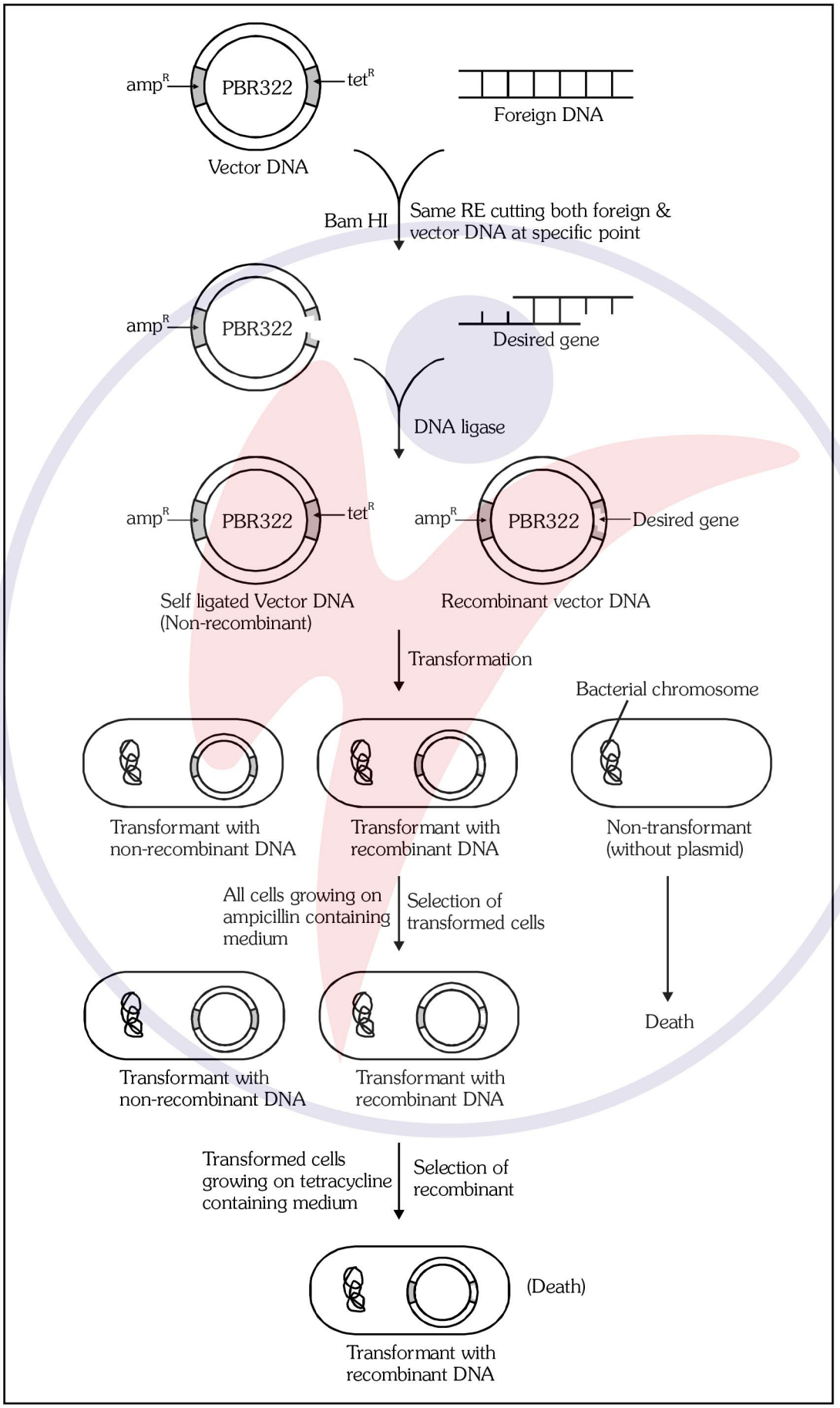
5. Transformation - The Uptake of DNA by Host

A. Gene Transfer in bacterial cell : Since DNA is a hydrophilic molecule, it cannot pass through cell membranes. In order to force bacteria to take up the plasmid, the bacterial cells must first be made 'competent' to take up DNA. This is done by treating them with a specific concentration of a divalent cation, such as calcium, which increases the efficiency with which DNA enters the bacterium through pores in its cell wall. Recombinant DNA can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 42°C (heat shock), and then putting them back on ice. This enables the bacteria to take up the recombinant DNA.

B. Microinjection: makes use of a very fine pipette to inject recombinant DNA molecules directly into the nucleus of the cells to be transformed

C. Biolistics or Gene Gun: cells are bombarded with high velocity micro-particles of gold or tungsten coated with DNA.

6. Screening and Selection of Recombinant



A. **Selection by two antibiotic resistant gene :-** You can ligate a foreign DNA at the Bam H I site of tetracycline resistance gene in the vecter pBR322. The recombinant plasmids will loss tetracycline resistance due to insertion of foreign DNA (insertional inactivation) now, it can be selected out from non-recombinant ones by plating the transformants on ampicillin containing medium. The transformants (plasmid transfer) 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.

Note :

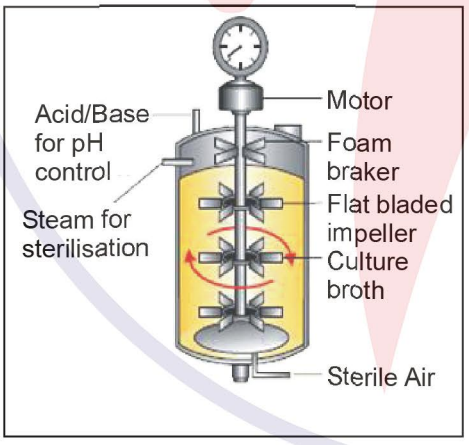
Insertional inactivation : Due to insertion of desired gene within selectable marker gene of vector, selectable marker gene become inactive or loose their function. This is called Insertional inactivation.

(B) **Selection by one Lac Z gene and one antibiotic resistant gene :-**

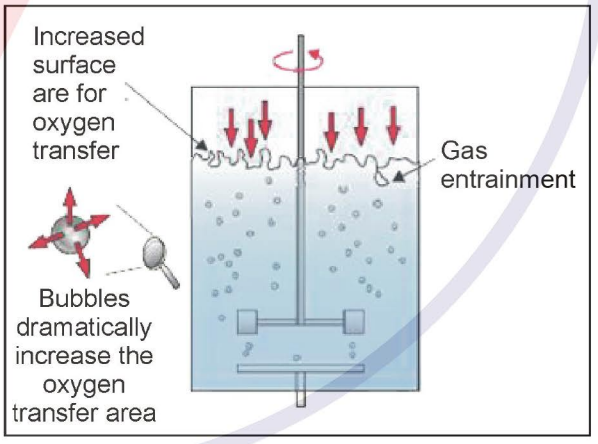
Selection of recombinants due to inactivation of antibiotics is a cumbersome (troublesome) procedure because it requires simultaneous plating on two plated having different antibiotics. Therefore, alternative selectable markers have been developed which differentiate recombinants from non-recombinants on the basis of their ability to produce colour in the presence of a chromogenic substrate. In this, a recombinant DNA is inserted within the coding sequence of an enzyme, which is referred to as **insertional inactivation.** The presence of a chromogenic substrate X–gal gives blue coloured colonies if the plasmid in the bacteria does not have an insert. Presence of insert results into insertional inactivation of the β-galactosidase (reporter enzyme) and the colonies do not produce any colour, these are identified as recombinant colonies.

7. **Obtaining the Foreign Gene Product :-**

Small volume cultures cannot yield appreciable quantities of products.To produce in large quantities, the development of bioreactors, where large volumes (100-1000 litres) 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.



Simple-Stirred Tank Bioreactor



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. Alternatively air can be bubbled through the reactor.

8. **Downstream Processing :-** After completion of the biosynthetic stage, the product has to be subjected through a series of processes before it is ready for marketing as a finished product.

The processes include **separation and purification**, which are collectively referred to as downstream processing.

BIOTECHNOLOGY AND ITS APPLICATIONS

BIOTECHNOLOGICAL APPLICATION IN AGRICULTURE :

Plants, bacteria, fungi and animals whose genes have been altered by manipulation are called **Genetically Modified Organisms** (**GMO**). GM plants have been useful in many ways. Genetic modification has:

(i) made crops more tolerant to abiotic stresses (cold, drought, salt, heat).

(ii) reduced reliance on chemical pesticides (pest-resistant crops).

(iii) helped to reduce post harvest losses. Ex :- Flavr savr tomato.

(iv) increased efficiency of mineral usage by plants (this prevents early exhaustion of fertility of soil).

(v) enhanced nutritional value of food, e.g., Vitamin 'A' enriched rice (Golden rice).

In addition to these uses, GM has been used to create tailor-made plants to supply alternative resources to industries, in the form of starches, fuels and pharmaceuticals.

Genetically modified crops –

Bt Cotton :

Some strains of Bacillus thuringiensis produce proteins that kill certain insects such as lepidopterans (tobacco budworm, armyworm), coleopterans (beetles) and dipterans (flies, mosquitoes). B. thuringiensis forms protein crystals during a particular phase of their growth. These crystals contain a toxic **insecticidal protein** . The Bt toxin protein exist as inactive protoxins but once an insect ingest the inactive toxin, it is converted into an active form of toxin due to the alkaline pH of the gut which solubilise the crystals. The activated toxin binds to the surface of midgut epithelial cells and create pores that cause cell swelling and lysis and eventually cause death of the insect.

The **proteins encoded by the genes cryIAc and cryIIAb control the cotton bollworms, that of cryIAb controls corn borer.**

Pest Resistant Plants :

Several nematodes parasitise a wide variety of plants and animals including human beings. A nematode Meloidegyne incognitia infects the roots of tobacco plants and causes a great reduction in yield. A novel strategy was adopted to prevent this infestation which was based on the process of RNA interference (RNAi). RNAi takes place in all eukaryotic organisms as a method of cellular defense. This method involves silencing of a specific mRNA due to a complementary dsRNA molecule that binds to and prevents translation of the mRNA (silencing).

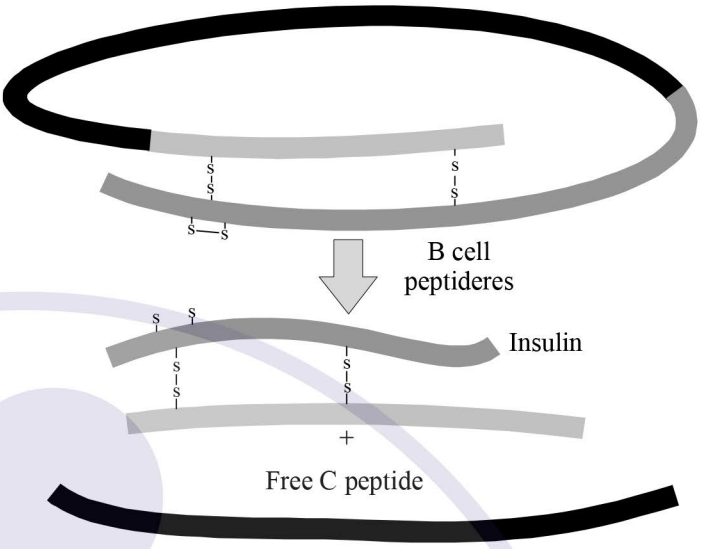
Using Agrobacterium vectors, nematode-specific genes were introduced into the host plant. The introduction of DNA was such that it produced both sense and anti-sense RNA in the host cells. These two RNA's being complementary to each other formed a double stranded (dsRNA) that initiated RNAi and thus, silenced the specific mRNA of the nematode. The consequence was that the parasite could not survive in a transgenic host expressing specific interfering RNA. The transgenic plant therefore got itself protected from the parasite figure.

BIOTECHNOLOGYCAL APPLICATIONS IN MEDICINE

(1) Genetically Engineered Insulin

The first genetically engineered insulin obtained by **recombinant DNA technique** with the help of **E-Coli** was produced by the American firms, **Eli-Lilly** on July 5, 1983. It has been given the trade name **humulin** and has been approved for clinical use.

This C peptide is not present in the mature insulin and is removed during maturation into insulin. The main challenge for production of insulin using rDNA techniques was getting insulin assembled into a mature form.



Maturation of pro-insulin into insulin after removal of C-peptide (to be simplified)

In 1983, Eli Lilly an American company prepared two DNA sequences corresponding to A and B, chains of human insulin and introduced them in plasmids of *E. coli* to produce insulin chains. Chains A and B were produced separately, extracted and combined by creating disulfide bonds to form human insulin.

(2) Gene Therapy :

– Gene therapy is a collection of methods that allows correction of a gene defect that has been diagnosed in a child/embryo. Here genes are inserted into a person's cells and tissues to treat a disease. Correction of a genetic defect involves delivery of a normal gene into the individual or embryo to take over the function of and compensate for the non-functional gene.

The first clinical gene therapy was given in 1990 to a 4-year old girl with adenosine deaminase (ADA) deficiency.

(3) Medical Diagnosis of Disease

Using conventional methods of diagnosis (serum and urine analysis. etc.) early detection is not possible. Recombinant DNA technology, Polymerase Chain Reaction (PCR) and Enzyme Linked Immuno-sorbent Assay (ALISA) are some of the techniques that serve the purpose of early diagnosis.

TRANSGENIC ANIMALS

Animals that have had their DNA manipulated to possess and express an extra (foreign) gene are known as **transgenic animals.** Transgenic rats, rabbits, pigs, sheep, cows and fish have been produced, although over **95 percent of all existing transgenic animals are mice.**

(i) **Normal physiology and development**

(ii) **Study of disease**

(iii) **Biological products : In 1997, the first transgenic cow, Rosie, produced human protein-enriched milk (2.4 grams per litre). The milk contained the human alpha-lactalbumin and was nutritionally a more balanced product for human babies that natural cow-milk.**

(iv) **Vaccine safety**

(v) **Chemical safety testing**

ETHICAL ISSUES

The manipulation of living organisms by the human race cannot go on any further, without regulation. Some ethical standards are required to evaluate the morality of all human activities that might help or harm living organisms.

Therefore, **the Indian Government has set up organisations such as GEAC (Genetic Engineering Approval Committee), which will make decisions regarding the validity of GM research and the safety of introducing GM-organisms for public services.**

Biopiracy: is the term used to refer to the use of bio-resources by multinational companies and other organisations without proper authorisation from the countries and people concerned without compensatory payment.