02 Biotechnology: Principles and Processes

1. Introduction :

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

(A) Old or Traditional Biotechnology:

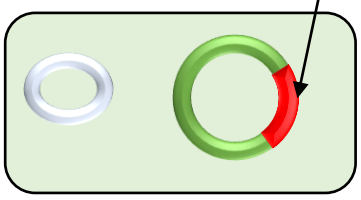
- Based on the natural capabilities of micro organisms. In this sense, making **curd, bread** or **wine**, which are all microbe-mediated processes, could also be thought as a form of biotechnology **(Early age biotechnology)**.

(B) New or Modern Biotechnology:

- Based on Recombinant DNA technology. e.g. Human gene producing Insulin has been transferred and expressed in bacteria like *E. coli*. However, it is used in a restricted sense today, to refer to such of those processes which use genetically modified organisms to achieve the same on a larger scale.
- Further, many other processes/techniques are also included under biotechnology. For example, synthesising a gene and using it, developing a DNA vaccine or correcting a defective gene, all are part of biotechnology.
- The European Federation of Biotechnology **(EFB)** has given a definition of biotechnology that encompasses both traditional view and modern molecular biotechnology.
- 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'*).**
- **Paul berg** (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)

2. Principles of Biotechnology :

Among many, the two core techniques that enabled birth of modern biotechnology are:



Genetic modified Organism (GMO)

(A) Genetic Engineering/Recombinant DNA Technology : Techniques to alter the chemistry of genetic material (DNA and RNA), to introduce these into host organisms and thus change the phenotype of the host organism. (i.e. formation of genetically modified organism).

(B) Bioprocess Engineering : Maintenance of sterile (microbial contamination-free) ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell in **large quantities** for the manufacture of biotechnological products like antibiotics, vaccines, enzymes, etc.

The concept of genetic engineering was the outcome of two very significant discoveries made in bacterial research. These were :

- Presence of extrachromosomal DNA fragments called **plasmids** in the bacterial cell, which replicate independent of chromosomal DNA of the bacterium.
- Presence of enzymes **restriction endonucleases** which cut DNA at specific sites.
- These enzymes are, therefore, called **'molecular scissors'**.

The conceptual development of the principles of genetic engineering :

- The techniques of genetic engineering which include creation of recombinant DNA, use of gene cloning and gene transfer, overcome this limitation and allows us to isolate and introduce only one or a set of desirable genes without introducing undesirable genes into the target organism. Most likely, this piece of DNA would not be able to multiply itself in the progeny cells of the organism. But, when it gets integrated into the **genome** of the **recipient**, it may multiply and be inherited along with the host DNA. This is because the alien piece of DNA has become part of a chromosome, which has the ability to replicate.

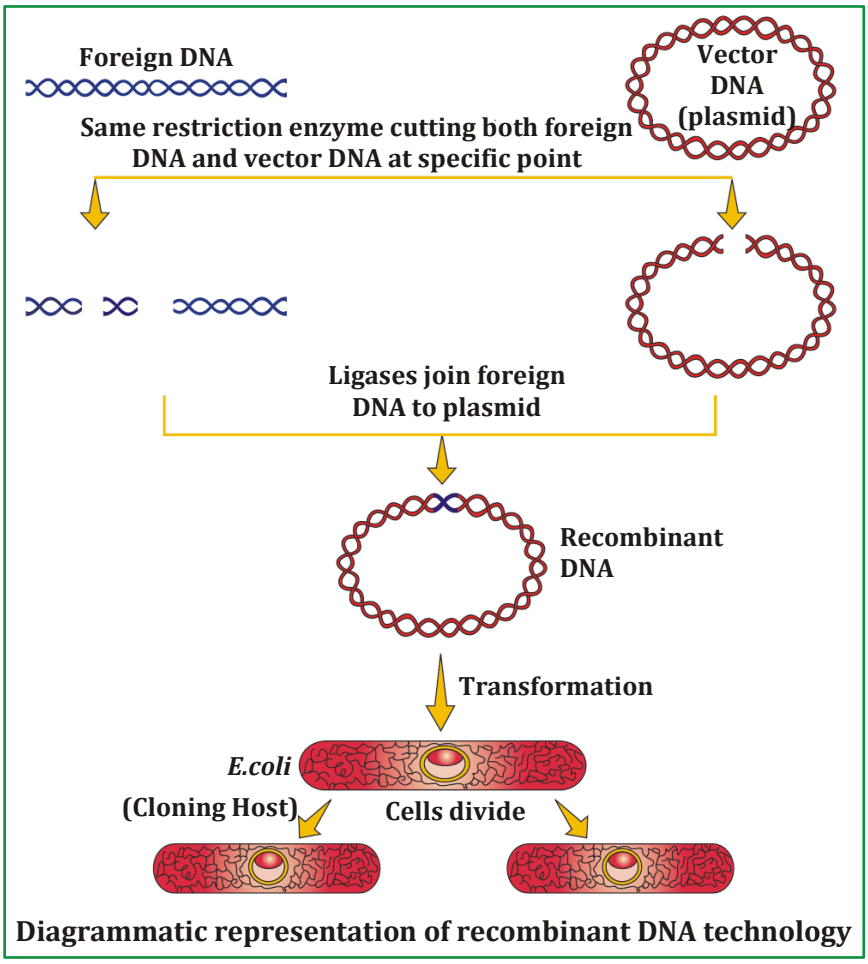
Construction of an artificial recombinant DNA molecule :

- The construction of the **first recombinant DNA** emerged from the possibility of linking a gene encoding **antibiotic resistance** with a native **plasmid** (autonomously replicating circular extra-chromosomal DNA) of ***Salmonella typhimurium*****.**
- **Stanley Cohen** and **Herbert Boyer** accomplished this in 1972 by isolating the antibiotic resistance gene by cutting out a piece of DNA from a plasmid which was responsible for conferring antibiotic resistance. The cutting of DNA at specific locations became possible with the discovery of the so-called **'molecular scissors'– restriction enzymes**.
- The cut piece of DNA was then linked with the plasmid DNA. These plasmid DNA act as **vectors** to transfer the piece of DNA attached to it, (A plasmid can be used as vector to deliver an alien piece of DNA) into the host organism.
- **Recombinant DNA** - The linking of antibiotic resistance gene with the plasmid vector became possible with the enzyme **DNA ligase**, which acts on cut DNA molecules and joins their ends. This makes a new combination of circular autonomously replicating DNA created in vitro and is known as **recombinant DNA**.
- When this DNA is transferred into *Escherichia coli*, a bacterium closely related to *Salmonella*, it could replicate using the new host's DNA polymerase enzyme (Enzyme used in DNA synthesis) and make multiple copies.
- **Cloning** - The ability to multiply copies of antibiotic resistance gene in *E. coli* was called cloning of antibiotic resistance gene in *E. coli*.

  The main basis of Recombinant DNA Technology is DNA cloning :- It is making multiple identical copies of any template DNA.

  Basic steps in genetically modifying an organism (of DNA cloning):

  (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.



Diagrammatic representation of recombinant DNA technology

3. Tools of Recombinant DNA Technology :

- Genetic engineering involves cutting of desired segments of DNA and pasting of this DNA in a vector to produce a recombinant DNA **(rDNA).**
- The **'biological tools'** used in the synthesis of recombinant DNA include **enzymes, vehicle or vector DNA, desired DNA and host cells**.

(A) Enzymes :

- A number of specific kinds of enzymes are employed in **genetic engineering**.
- These include lysing enzymes, cleaving enzymes, synthesising enzymes and joining enzymes.

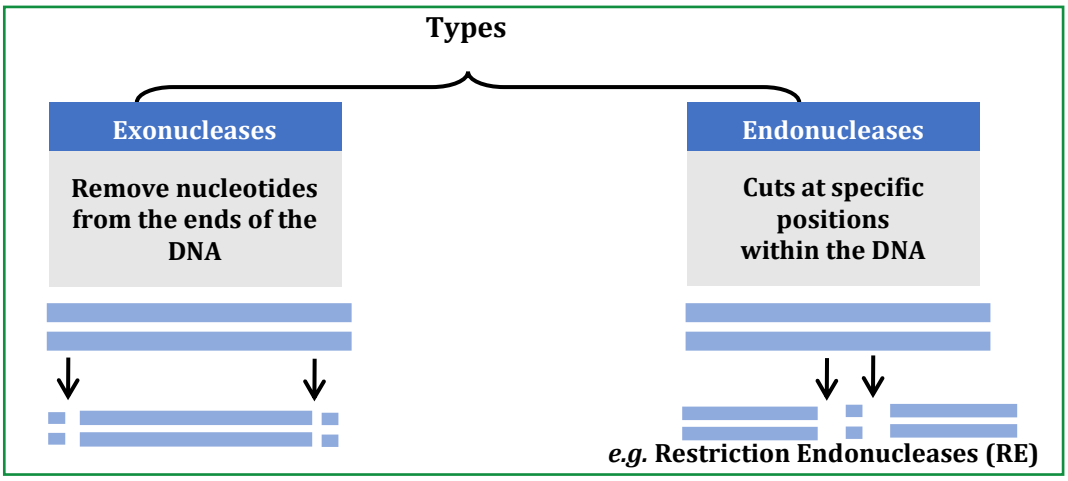
Lysing Enzymes

Lysozyme (Digests Bacterial cell wall)	**Cellulase/ Pectinase** (Digests Plant cell wall)	**Chitinase** (Digests fungal cell wall)	**Lipase** (Digests cell membrane)

(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**.
- **Fungal cell** : is commonly digested with the help of **chitinase**.

(ii) **Cleaving enzymes** - These enzymes are used to cleave DNA molecules. Cleaving enzymes are of two types;



Restriction Endonuclease Enzymes :

- It is also called as **molecular scissor** or **molecular knife** due to **cutting** of DNA molecule.
- Restriction enzymes belong to a larger class of enzymes called endonucleases.
- Restriction enzymes are used in recombinant DNA technology because they can be used *in vitro* to recognise and cleave within specific DNA sequence typically consisting of **4 to 8 nucleotides**.
- This specific 4 to 8 nucleotide sequence is called **restriction site** and is usually **palindromic**, this means that the DNA sequence is the same when read in a 5'-3' direction on both DNA strands.
- Restriction enzymes are obtained from bacteria.

5' —— GAATTC —— 3'
3' —— CTTAAG —— 5'

Function of restriction enzymes in bacteria :

- They are useful to bacteria because the enzyme bring about **fragmentation of viral DNA** without affecting the bacterial genome. This is an adaptation against **bacteriophages**.
- These enzymes exist in many bacteria beside cleavage, some restriction endonuclease also have capability of modification.
- Modification in the form of **methylation**, by methylation the bacterial DNA modifies and therefore protects it's own chromosomal DNA from cleavage by these restriction enzymes.
- The first restriction endonuclease **Hind II**, whose functioning depended on a specific DNA nucleotide sequence was isolated and characterised five years later.
- It was found that Hind II always cut DNA molecules at a particular point by recognising a specific sequence of six base pairs. This specific base sequence is known as the recognition sequence for Hind II. Restriction enzyme (EcoR I) was discovered by **Arber, Smith & Nathans (1978 Nobel prize)**.
- Besides Hind II, today we know more than 900 restriction enzymes that have been isolated from over 230 strains of bacteria each of which recognise different recognition sequences.

Nomenclature of Enzyme :

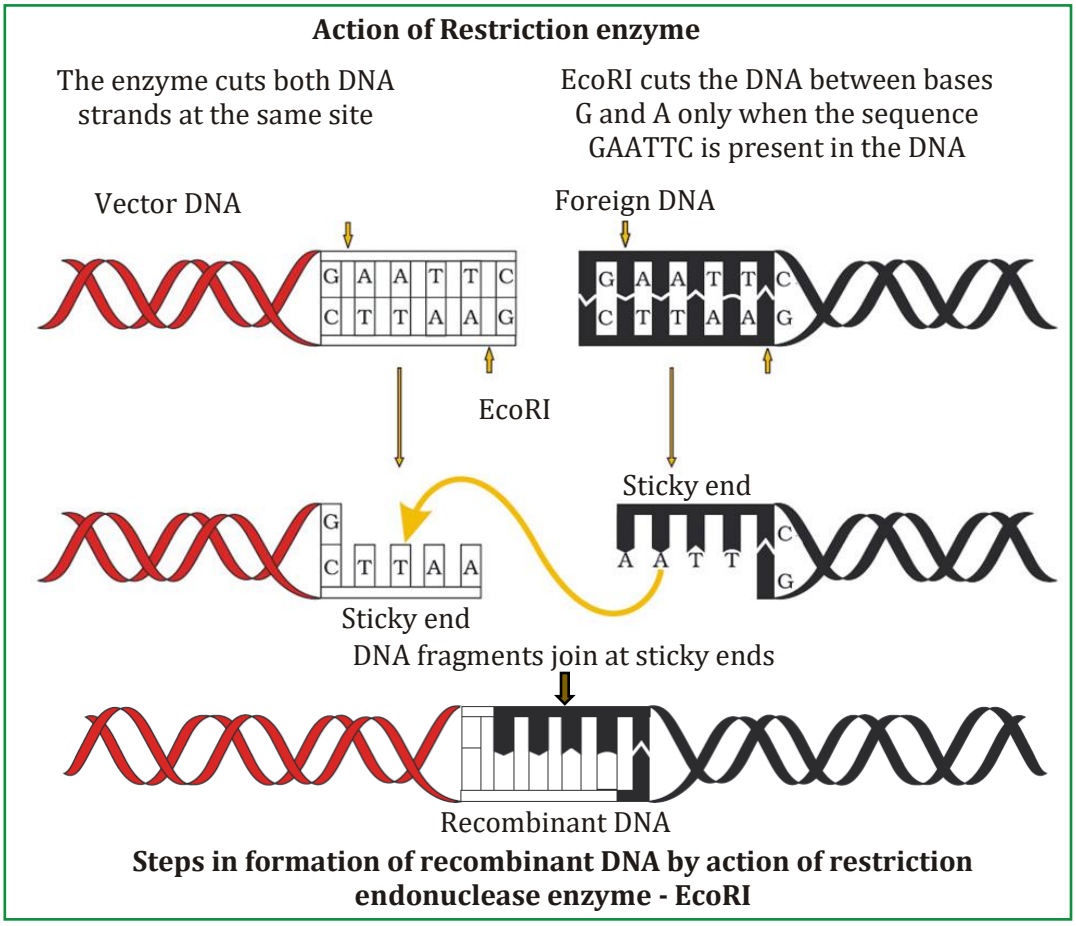
- **The first letter** : indicates bacterial genus (In italic)
- **Second and third letter** : indicate species of bacteria (In italic)

Fourth letter : indicates strain of bacteria (optional)

- **Roman numeral** : signifying the order in which the enzymes were isolated from that strain of bacteria.
  e.g. **EcoR I** comes from ***Escherichia coli* RY 13.** In EcoR I, the letter 'R' is derived from the name of strain. Roman numbers following the names indicate the order in which the enzymes were isolated from that strain of bacteria.

Restriction enzymes forms two types of ends on the basis of mode of cutting :

(a) **Sticky end (Free end)**: Restriction enzymes cut the strand of DNA a little away from the centre of the palindrome sites, but between the same two bases on the opposite strands. This leaves single stranded portions at the ends. There **overhanging stretches** called sticky ends on each strand. These are named so because they form hydrogen bonds with their complementary cut counterparts. This **stickiness** of the ends facilitates the action of the enzyme DNA ligase.
e.g. *Eco*R I, *Hin*d III, BamH I, Sal I



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

(b) **Blunt end (non-sticky end)**: some enzymes cleave both strand of DNA at exactly the same nucleotide position, typically in the centre of the recognition sequence resulting in **blunt end** or **flush end**. ***e.g.*** Sma I, EcoR V, Hae III, Sma I

Sma I (*Serratia marcescens*)

$$\begin{array}{l} 5'\ C\ C\ C\ G\ G\ G\ 3' \\ 3'\ G\ G\ G\ C\ C\ C\ 5' \end{array} \Rightarrow \begin{array}{l} 5'\ C\ C\ C\ 3' \\ 3'\ G\ G\ G\ 5' \end{array} + \begin{array}{l} 5'\ G\ G\ G\ 3' \\ 3'\ C\ C\ C\ 3' \end{array}$$

Examples of restriction enzyme
Recognition sequences of some restriction endonucleases

Name	Recognition sequence	End after cleavage		Source
EcoR I	– G A A T T C – – C T T A A G –	– G – C T T A A	A A T T C – G –	*Escherichia coli*
Hind III	– A A G C T T – – T T C G A A –	– A – T T C G A	A G C T T – A –	*Haemophilus influenzae*
BamH I	– G G A T C C – – C C T A G G –	– G – C C T A G	G A T C C – G –	*Bacillus amyloliquefaciens*
Hae III	– G G C C – – C C G G –	– G G – C C	C C – G G –	*Haemophilus aegyptius*
EcoR V	– G A T A T C – – C T A T A G –	– G A T – C T A	A T C – T A G –	*Escherichia coli*
Hind II	– G T C G A C – – C A G C T G –	– G T C – C A G	G A C – C T G –	*Haemophilus influenzae*

(iii) **Synthesizing enzymes :** These enzymes are used to synthesize new strands of DNA, complementary to existing DNA or RNA template. They are of two types; reverse transcriptases and DNA polymerases.

- **Reverse transcriptases** help in the synthesis of complementary DNA strands on RNA templates.
- **DNA polymerases** help in the synthesis of complementary DNA strands on DNA templates.

(iv) **Joining enzymes :-** These enzymes help in joining the DNA fragments. For example **DNA ligase** is used to join DNA fragments. Joining enzymes are, therefore, called **molecular glues**.

(B) 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**.

- **Plasmids** (extra chromosomal DNA in bacterium) and **bacteriophages** have the ability to replicate within bacterial cells independent of the control of chromosomal DNA. Bacteriophages because of their high number per cell, have very high copy numbers of their genome within the bacterial cells.
- Some plasmids may have only one or two copies per cell whereas others may have 15-100 copies per cell. Their numbers can go even higher.
- If we are able to link an alien piece of DNA with bacteriophage or plasmid DNA, we can multiply its numbers equal to the copy number of the plasmid or bacteriophage.

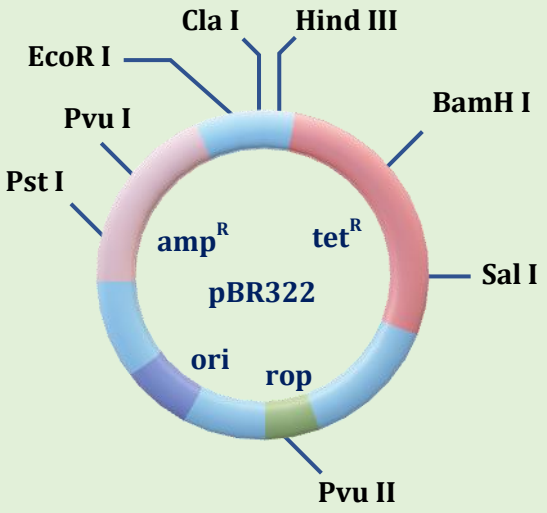
The following are the features that are required to facilitate cloning into a vector:

(i) Origin of replication (ori) :

- This is a sequence from where replication starts and any piece of DNA when linked to this sequence can be made to replicate within the host cells.
- This sequence is also responsible for **controlling the copy number** of the linked DNA. So, if one wants to recover many copies of the target DNA it should be cloned in a vector whose origin support high copy number.

(ii) Selectable Marker :

- In addition to 'ori', the vector requires a selectable marker, which helps in identifying and eliminating non-transformants and **selectively permitting the growth of the transformants**.
- Transformation is a procedure through which a piece of DNA is introduced in a host bacterium.



E. coli cloning vector pBR322 showing restriction sites (Hind III, EcoR I, BamH I, Sal I, Pvu II, Pst I, Cla I), ori and antibiotic resistance genes (amp^R and tet^R). rop codes for the proteins involved in the replication of the plasmid.

- Normally, the genes encoding resistance to antibiotics such as **ampicillin**, **chloramphenicol**, **tetracycline or kanamycin**, etc., are considered useful selectable markers for *E. coli*.
- Enzyme forming gene also act as selectable marker gene. ***e.g.*** **Lac Z gene**. The normal *E. coli* cells do not carry resistance against any of these antibiotics.

(iii) Restriction sites/Cloning sites :

- In order to link the alien DNA, the vector needs, recognition sites (***e.g.*** Hind III, EcoR I, BamH I, Sal I, Pvu II, Pst I, Cla I in pBR322) for the commonly used restriction enzymes.
- The ligation of alien DNA is carried out at a restriction site present in one of the two antibiotic resistance genes.
- A vector should have restriction site for many enzymes but only one restriction site for each enzyme, otherwise vector will get fragmented.

Selection of vector depends on :

(a) Size of desired gene

(b) Type of host

Some examples of vectors :

(a) Plasmids:

- They are double stranded, circular and extra chromosomal DNA segments found in bacteria which can replicate independently.
- Plasmids can be taken out of bacteria and made to combine with desired DNA segments by means of restriction enzymes and DNA ligase.
- A plasmid carrying DNA of another organism integrated with it, is known as **recombinant plasmid** or **hybrid plasmid** or **Chimeric plasmid**.

  e.g. **pBR322**, **PUC18** (used for gene transfer in bacteria)
  Ti plasmid, **Ri** Plasmid (used for gene transfer in dicot plant)

(b) Viruses :

- The DNA of certain viruses is also suitable for use as a vehicle DNA.
- Bacteriophage DNA is also used as a vector DNA for gene transfer.

e.g. **λ**-Bacteriophage, **M-13**-Bacteriophage

Retro virus – useful for gene transfer in animal cell

	Vector type	Insert size kb
(1)	Plasmid	0.5 – 8
(2)	Bacteriophage lambda	9 – 23
(3)	Cosmid	30 – 45
(4)	BAC	50 – 300
(5)	YAC	1000 – 2500

(C) Desired DNA / Alien DNA / Foreign DNA / 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.

(i) Complementary DNA (cDNA):

- It is synthesized on mRNA template with the help of reverse transcriptase and necessary nucleotides.
- cDNA formed through reverse transcription is shorter than the actual or *in vivo* gene because of the absence of introns or non-coding regions.

(ii) Synthetic DNA (sDNA) :

- It is artificially synthesized with the help of DNA polymerase.
- **Kornberg** (1961) synthesized first synthetic DNA.
- **Khorana** (1968) synthesized first artificial gene (DNA) without a template. They synthesized the gene coding for yeast alanine *t-RNA,* which contained only 77 base pairs. However, it did not function in the living system.
- In 1979, **Khorana** was able to synthesize a functional tyrosine *t-RNA* gene of *E. coli* with 207 nucleotide pairs. Since then a number of genes have been synthesized artificially.

(D) Host Cell:

- This cell is used for DNA cloning.
- Host cell may be plant cell, animal cell, bacterial cell *(E. coli)*, fungal cell (Yeast).

4. Processes of Recombinant DNA Technology:

(A) Isolation of DNA :

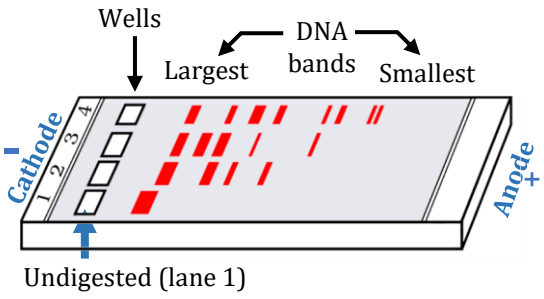
- The DNAs which are to be used as passenger DNA and the vehicle DNA are extracted out of their cells by lysing the cells with the suitable enzyme.
- Since the DNA is enclosed within the membranes, we have to **break** the cell open to release DNA along with other macromolecules such as RNA, proteins, polysaccharides and also lipids.

DNA spooling Method

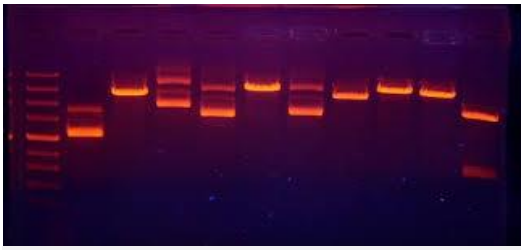
- This can be achieved by treating the bacterial cells/plant or animal tissue with **lysing enzymes** such as **lysozyme** (bacteria), cellulase (plant cells), chitinase (fungus). You know that genes are located on long molecules of DNA interwined with proteins such as histones.
- The RNA can be removed by treatment with ribonuclease whereas proteins can be removed by treatment with protease. Other molecules can be removed by appropriate treatments and purified DNA ultimately precipitates out after the addition of **chilled ethanol**. This can be seen as collection of fine threads in the suspension.
- In the last step DNA is spooled over a glass rod, this step is called **spooling.**

(B) 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.
- These fragments can be separated by a technique known as **gel electrophoresis**.
- Since DNA fragments are **negatively charged** molecules they can be separated by forcing them to move towards the anode under an electric field through a medium/matrix.
- Nowadays the most commonly used matrix is **agarose** which is a natural polymer extracted from **sea weeds**. The DNA fragments separate (resolve) according to their size through **sieving effect** provided by the agarose gel. Hence, the smaller the fragment size, the farther it moves **(towards anode).**



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



You can see bright orange coloured bands of DNA in a ethidium bromide stained gel exposed to UV light

- **Staining-** The separated DNA fragments can be visualised (Bright orange coloured bands) only after staining the DNA with a compound known as **ethidium bromide** followed by exposure to **UV radiation** (you cannot see pure DNA fragments in the visible light and without staining).
- The separated bands of DNA are cut out from the agarose gel and extracted from the gel piece, this step is known **as elution**. The DNA fragments purified in this way are used in constructing recombinant DNA by joining them with cloning vectors.

(C) Amplification of Gene of Interest using PCR :

- PCR stands for **Polymerase Chain Reaction**. This technique was invented by **Kary mullis (1983)**.
- In 1993, Karry Mullis got nobel prize for PCR (for chemistry). PCR is a method for amplifying a specific region of DNA molecule without the requirement for time consuming cloning procedures.

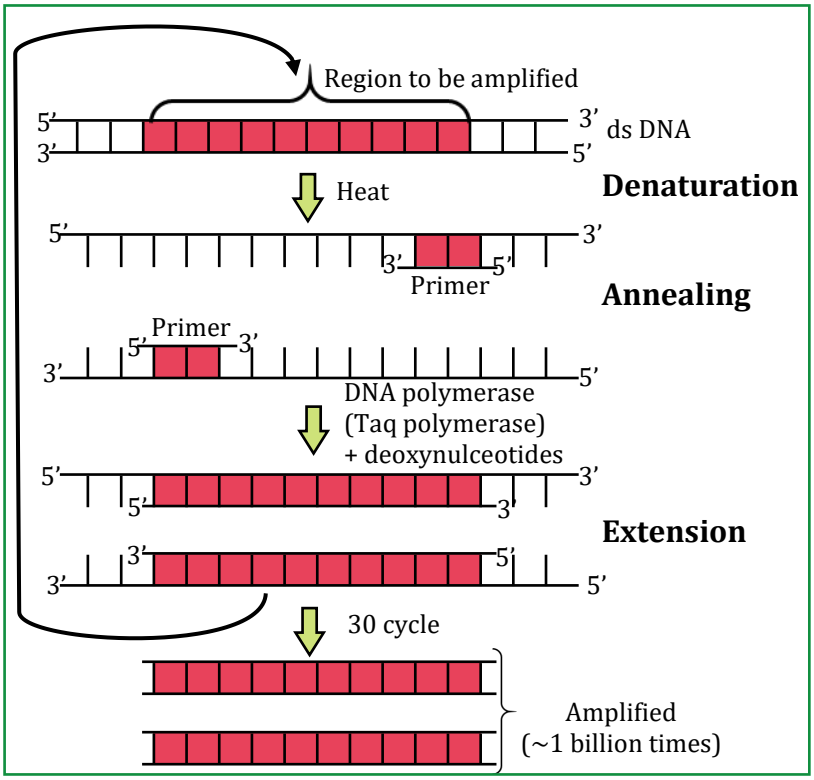
- In this reaction, multiple copies of the gene (or DNA) of interest is synthesised *in vitro* using two sets of **primers** (small chemically synthesised oligonucleotides that are complementary to the regions of DNA) and the enzyme DNA polymerase. PCR reaction takes place in **Eppendorf tube**. PCR occurs in **Thermocycler**.
- Using PCR-technique very low content of DNA available from samples of blood or semen or any other tissue or hair cell can be amplified many times and analysed. In this technique ***Taq*-*Polymerase*** is used. *Taq* polymerase enzyme is used in PCR which is a special type of DNA polymerare enzyme which is resistant to high temperature.
- *Taq* Polymerase is isolated from ***Thermus aquaticus*** bacterium.
- Some other examples of polymerase which are used in PCR are –
- **Pfu DNA Polymerase** - Isolated from *Pyrococcus furiosus* bacterium.
- **Vent DNA Polymerase** - Isolated from *Thermococcus litoralis* bacterium.

Steps:-

(a) **Denaturation (94°C) :** In this step a double stranded DNA molecule is placed at 94°C. So double stranded DNA becomes single stranded & each single stranded DNA functions as a template.

(b) **Annealing/Primer annealing (54°C):** In this step two primer DNA are attached at 3' end of single stranded DNA.

(c) **Extension (72°C):** In this process ***Taq* polymerase** enzyme synthesizes DNA strand over template. PCR is automatic process because *Taq* polymerase enzyme is heat resistant.



(D) 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.

(E) Insertion or Transferring the Recombinant DNA into the host cell/organism (Gene Transfer) :

Transfer of desired genes from one organism into another is an important aspect of genetic engineering.

Gene transfer is achieved by two kinds of transfer methods:

(i) Indirect Method :

(a) Gene Transfer in bacterial cell :

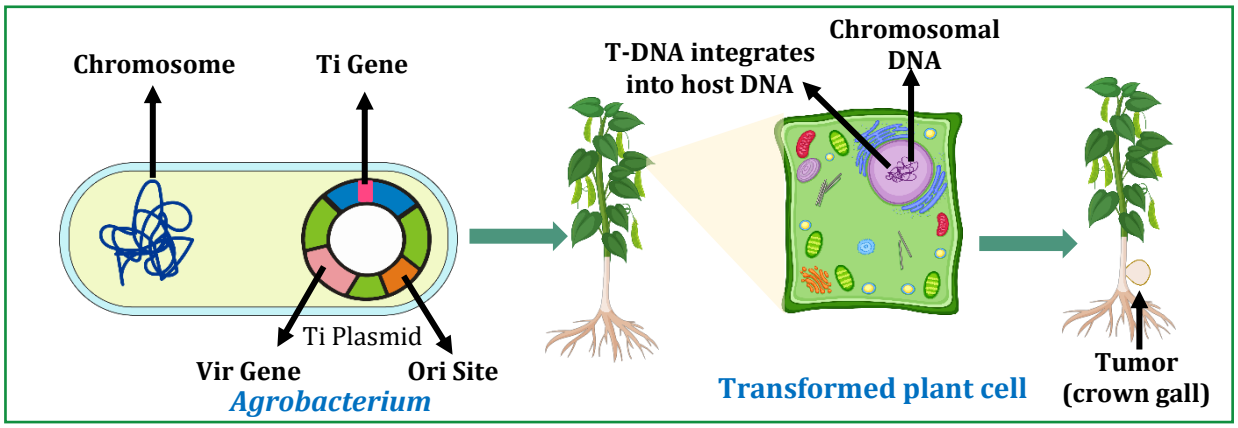
- Since DNA is a hydrophilic molecule, it cannot pass through cell membranes.
- In order to force bacteria to take up the plasmid, the bacterial cells must first be made **'competent'** to take up DNA. This is done by treating them with a specific concentration of a divalent cation, such as calcium, 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.

Note:-

- The bacteria to be used as host cell should be without plasmids.
- The host cells are treated with calcium chloride.

(b) Gene Transfer in plant cell :

- A plant pathogenic bacterium ***Agrobacterium tumefaciens*** produces crown galls or plant tumours in almost all dicotyledonous plants.
- This bacterium infects all broad leaved agricultural crops such as tomato, soyabean, sunflower and cotton but not cereals.
- Tumour formation is induced by it's plasmid which is therefore called **Ti plasmid** (Ti = Tumour inducing)
- Agrobacterium tumefaciens naturally transfers some part of Ti-plasmid in to host plant DNA without any human effort so it is called natural genetic engineer of plant.
- **In the transformation process two essential component in Ti-plasmid**
  T-DNA – (Transfer DNA)
  Vir-region – (Virulence region)
- Inside the host plant cell, T-DNA is separated from Ti-plasmid, and integrated into host plant DNA that causes crown gall tumour.
- Vir-region contains genes which are essential for T-DNA transfer and integration to host plant DNA.
- When we use Ti plasmid as a vector, first we remove the tumour causing gene from T-DNA region. Then desired gene is inserted in place of it. Now, this plasmid is called **disarmed plasmid**.
- Same as Ri plasmid of ***A. rhizogenes*** (causing hairy root disease) are also used as vector for gene transfer into plant cell.



(c) Gene Transfer in animal :

Retroviruses have also been disarmed and are now used to deliver desirable genes into animal cells.

(ii) Direct Method :

Foreign genes can also be transferred directly by the following methods:

(a) Electroporation: It creates transients (temporary pores) in the plasma membrane to facilitate entry of foreign DNA.

(b) Chemical mediated genetic transformation: It involves certain chemicals such as **polyethylene glycol** (PEG), that help in the uptake of foreign DNA into host cells.

(c) Microinjection: It is the introduction of foreign genes mainly into **animal cells** using micropipettes or glass needles.

(d) Particle gun/Biolistic method / Gene gun: It is a technique in which **tungsten or gold particles** coated with foreign DNA are bombarded into target cells to facilitate entry of the foreign genes.

(e) Liposome mediated gene transfer: In this method, DNA is enclosed within lipid bags. These lipid bags are fused with protoplast.

(F) Selection of Transformant with Recombinant DNA :

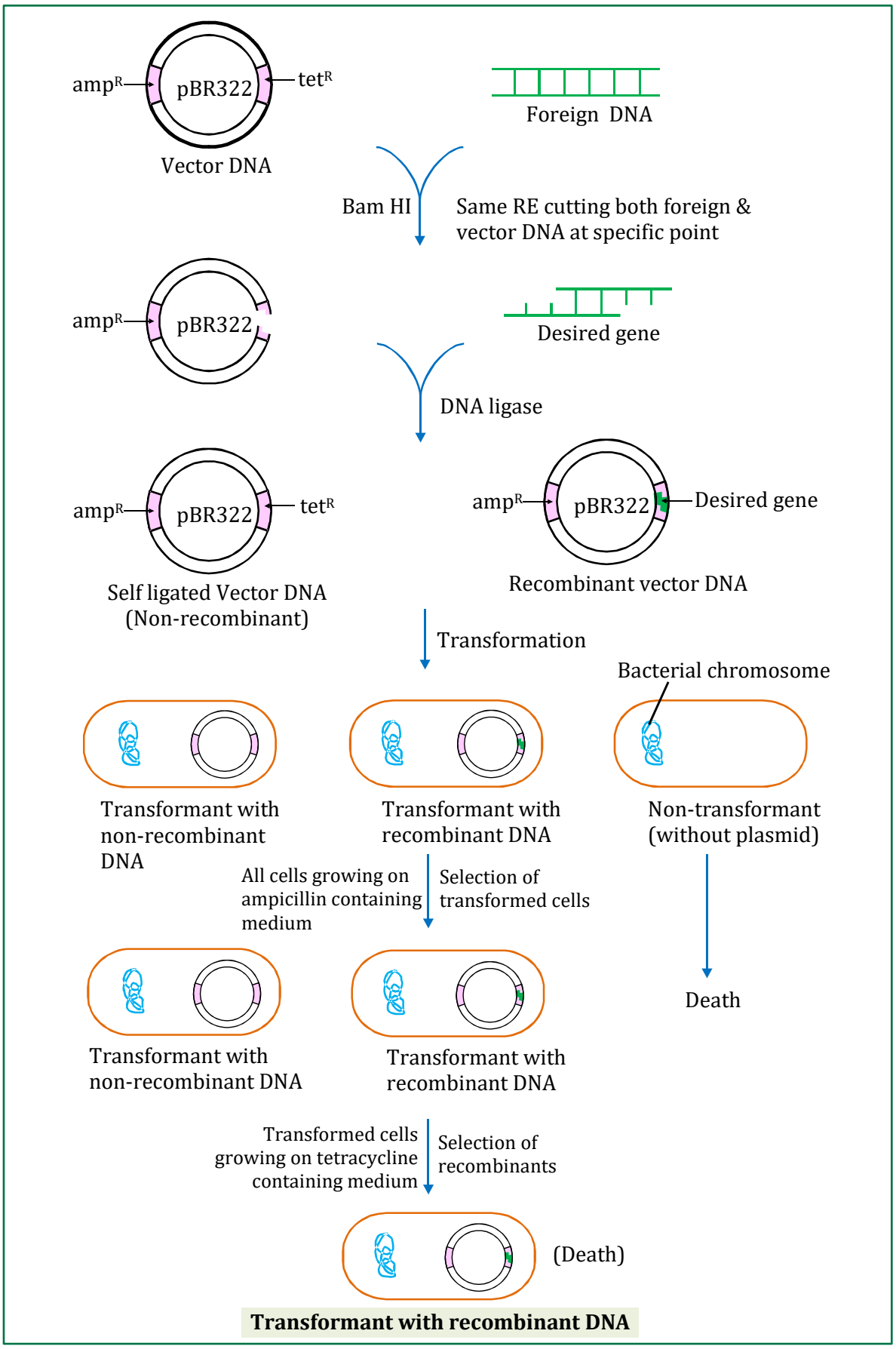
(i) Selection by Two Antibiotic Resistance Gene:

- You can ligate a foreign DNA at the Bam HI site of tetracycline resistance gene in the vector pBR322. The recombinant plasmids will lose tetracycline resistance due to insertion of foreign DNA (insertional inactivation) now, it can be selected out from non-recombinant ones by following bases.
- 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.

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.

(ii) Selection by one Lac Z gene and one antibiotic resistance gene:

- Selection of recombinants due to inactivation of antibiotic resistance gene is a **cumbersome** (troublesome) procedure because it requires simultaneous plating on two plates 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, desired gene 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.

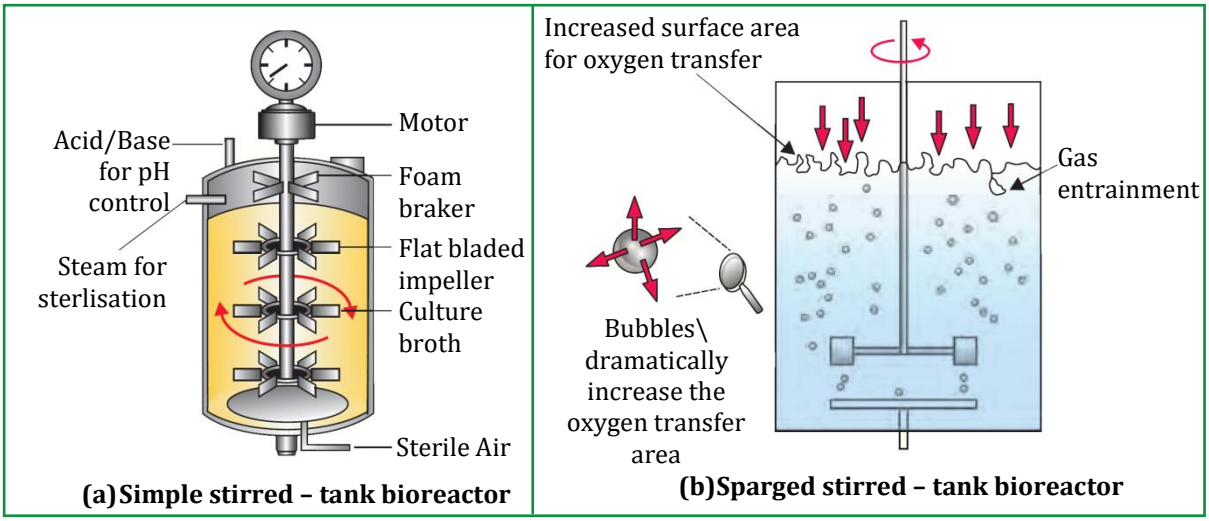

amp^R
pBR322
tet^R
Vector DNA
Foreign DNA
Bam HI
Same RE cutting both foreign & vector DNA at specific point
amp^R
pBR322
Desired gene
DNA ligase
amp^R
pBR322
tet^R
Self ligated Vector DNA (Non-recombinant)
amp^R
pBR322
Desired gene
Recombinant vector DNA
Transformation
Bacterial chromosome
Transformant with non-recombinant DNA
Transformant with recombinant DNA
Non-transformant (without plasmid)
All cells growing on ampicillin containing medium
Selection of transformed cells
Death
Transformant with non-recombinant DNA
Transformant with recombinant DNA
Transformed cells growing on tetracycline containing medium
Selection of recombinants
(Death)
Transformant with recombinant DNA

(G) Obtaining the Foreign Gene Product :

- When you insert a piece of alien DNA into a cloning vector and transfer it into a bacterial, plant or animal cell, the alien DNA gets multiplied.
- In almost all recombinant technologies, the ultimate aim is to produce a **desirable protein**. Hence, there is a need for the recombinant DNA to be expressed. The foreign gene gets expressed under appropriate conditions. The expression of foreign genes in host cells involve understanding many technical details.
- After having cloned the gene of interest and having optimised the conditions to induce the expression of the target protein, one has to consider producing it on a **large scale**.
- If any protein encoding gene is expressed in a heterologous host **(both are different species or organism)**, it is called a **recombinant protein**. The cells harbouring cloned genes of interest may be grown on a small scale in the laboratory. The cultures may be used for extracting the desired protein and then purifying it by using different separation techniques.
- The cells can also be multiplied in a 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. This type of culturing method produces a larger biomass leading to higher yields of desired protein.
- 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.
- **Bioreactor** 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. A bioreactor provides the optimal conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).
- 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.

Bioreactor has :

(i) An agitator system
(ii) An oxygen delivery system
(iii) A foam control system
(iv) A temperature control system
(v) pH control system
(vi) Sampling ports, so that small volumes of the culture can be withdrawn periodically.



(a) Simple stirred – tank bioreactor

(b) Sparged stirred – tank bioreactor

(H) Downstream Processing :

- After completion of the biosynthetic stage **(multi-step, enzyme-catalyzed process where substrates are converted into more complex products in living organisms),** 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**.
- The product has to be formulated with suitable preservatives. Such formulation has to undergo thorough clinical trials as in case of drugs. Strict **quality control** testing for each product is also required.
- The downstream processing and quality control testing vary from product to product.

BEGINNER'S BOX-1 — PRINCIPLE TO PROCESS OF RECOMBINANT DNA THECHNOLOGY

1. Transfer of any gene into a completely different organism can be done through :-
(1) Genetic engineering (2) Tissue culture
(3) Transformation (4) RNA interference

2. DNA probe is used in :-
(1) Gel electrophoresis (2) Northern blotting
(3) DNA fingerprinting (4) Interferon synthesis

3. First artificial gene synthesized by Khorana was a gene of :-
(1) Arginine (2) Lysine
(3) Alanine t-RNA of yeast (4) Valine t-RNA

4. pBR322 is an artificial gene vector which does not have:-
(1) Ampicillin marker gene (2) Cos site
(3) Restriction site for cla I enzyme (4) Ori

5. The thermostable enzyme Taq & Pfu isolated from thermophilic bacteria are :-
(1) RNA polymerase (2) DNA primers
(3) DNA polymerases (4) DNA ligase

6. Which of the following technique is used for the separation of DNA fragments ?
(1) Gel electrophoresis (2) Chromatography
(3) Transformation (4) Transduction

7. Which of the following enzyme is used in case of fungus to cause release of DNA along with other macromolecules?
(1) Lysozyme (2) Cellulase (3) Chitinase (4) Amylase

8. Which of the following are used in gene cloning?
(1) Nucleoids (2) Lomasomes (3) Mesosomes (4) Plasmids

9. The linking of antibiotic resistance gene with the plasmid vector became possible with:-
(1) DNA polymerase (2) exonucleases (3) DNA ligase (4) endonucleases

10. An antibiotic resistance gene in a vector usually helps in the selection of :-
(1) Competent cells (2) Transformed cells
(3) Recombinant cells (4) None of the above

BEGINNER'S BOX — ANSWERS KEY

BEGINNER'S BOX-1	Que.	1	2	3	4	5	6	7	8	9	10
	Ans.	1	3	3	2	3	1	3	4	3	3

The two very significant discoveries made Biotechnology successful are :
(i) Plasmid
(ii) Restriction Endonuclease
Note : First discovered R.E. – Hind II

During nomenclature of R.E., Roman numerical signifies the order in which the enzyme was isolated from the strain of bacteria.

Tools of Recombinant DNA Technology

- Enzyme
  - Lysing
    - Lysozyme
    - Cellulase & Pectinase
    - Chitinase
  - Cleaving
    - Exonuclease
    - Endonuclease
  - Synthesizing
    - Reverse transcriptase
    - DNA polymerase
  - Joining
    - DNA ligase
- Vehicle or Vector DNA
  - Plasmid
  - Bacteriophage
- Desired DNA
  - c-DNA Complementary DNA
  - s-DNA Synthetic DNA
- Host
  - Plant
  - Animal
  - Bacteria *(E. coli)*
  - Fungi (Yeast)

Gene Transfer Method

- Indirect
  - Vector or carrier mediated
    - (i) *Agrobacterium tumefaciens*
    - (ii) *Agrobacterium rhizogenes*

    } For plant
  - (iii) *Retro virus-for animal cell*
- Direct
  - Vector less
    - Electroporation
    - Chemical mediated
      - Polyethylene glycol (PEG)
    - Microinjection
      - Best for Animal cell
    - Particle gun/Biolistic
      - For plant cell
    - Liposome mediated