BTNY-MM: XII

11. BIOTECHNOLOGY: PRINCIPLES & PROCESSES

Biotechnology is the technological exploitation of biological processes for the benefit of mankind.

Old Biotechnology:-It deals with the techniques in biotechnology based on the **natural capabilities** of microorganisms.
e.g.: Microbe-mediated processes (making curd, bread, wine etc).
Modern Biotechnology:-It deals with the techniques based on **genetic engineering.**
e.g.: - *In vitro* fertilisation ('test-tube' baby programme)
- Synthesis and using of a gene
- Preparation of a DNA vaccine
- Correcting a defective gene (***gene therapy***)

PRINCIPLES OF BIOTECHNOLOGY

2 core techniques of biotechnology are:
- **Genetic engineering/ rDNA technology:** The manipulation of gene to alter the phenotype of the host organism to suit human needs.
- **Biochemical engineering:** Process that helps the growth of desired microbe/eukaryotic cell in large quantities in sterile medium for the manufacture of biotechnological products (antibiotics, vaccines, enzymes, etc).

➔ **Merits of genetic engineering over traditional hybridisation**
Traditional hybridisation techniques lead to inclusion and multiplication of undesirable genes along with desired genes. Genetic engineering helps to isolate and introduce only desirable genes into the target organism.

- **3 basic steps in genetically modifying an organism:**
  1. Identification of DNA with desirable genes
  2. Introduction of the identified DNA into the host. A piece of DNA is not able to multiply itself in the progeny cells of the organism. But, when it gets integrated into the recipient genome, it multiplies and inherits along with the host DNA.
  3. Maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

- **Stanley Cohen & Herbert Boyer** (1972) constructed the first recombinant DNA.
  They linked a gene of antibiotic resistance with a native plasmid of *Salmonella typhimurium*.

PROCESSES of rDNA TECHNOLOGY

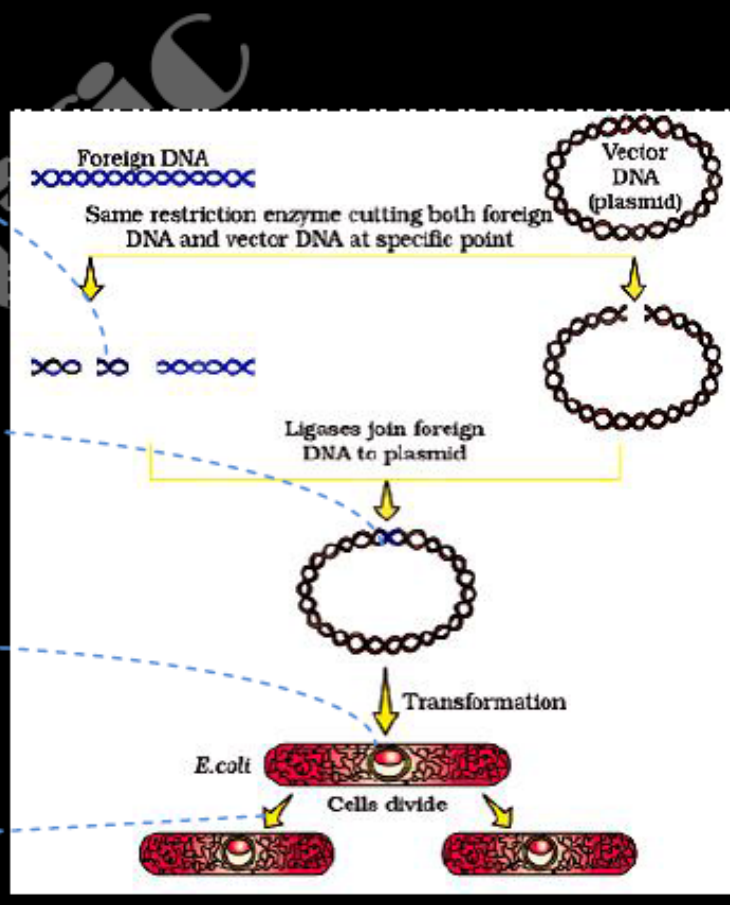
I. Isolation of a desired DNA fragment
- i. Breaking the cell wall
- ii. Removal of other substances from the DNA
- iii. Separation of purified DNA
- iv. Cutting of DNA at specific location using *Restriction Endonuclease*
- v. Desired DNA fragment is separated using *Gel electrophoresis*
- vi. Amplification of gene of interest using *PCR.*

II. Ligation of the DNA fragment into a vector *(preparing rDNA)*
DNA fragment is incorporated into the vector using *DNA ligase.*
- Since DNA fragment and vector was cut with same restriction enzymes, it has same sticky ends. When DNA fragment & vector mixed with *DNA ligase,* the 2 will anneal producing a recombinant DNA.

III. Transferring the recombinant DNA into the host (*transformation*)
- *i.* Making the host cell *competent* (ready to take up rDNA) by *heat shock treatment /micro-injection / gene gun method / disarmed pathogen vector.*
- ii. Selection of *transformants*.

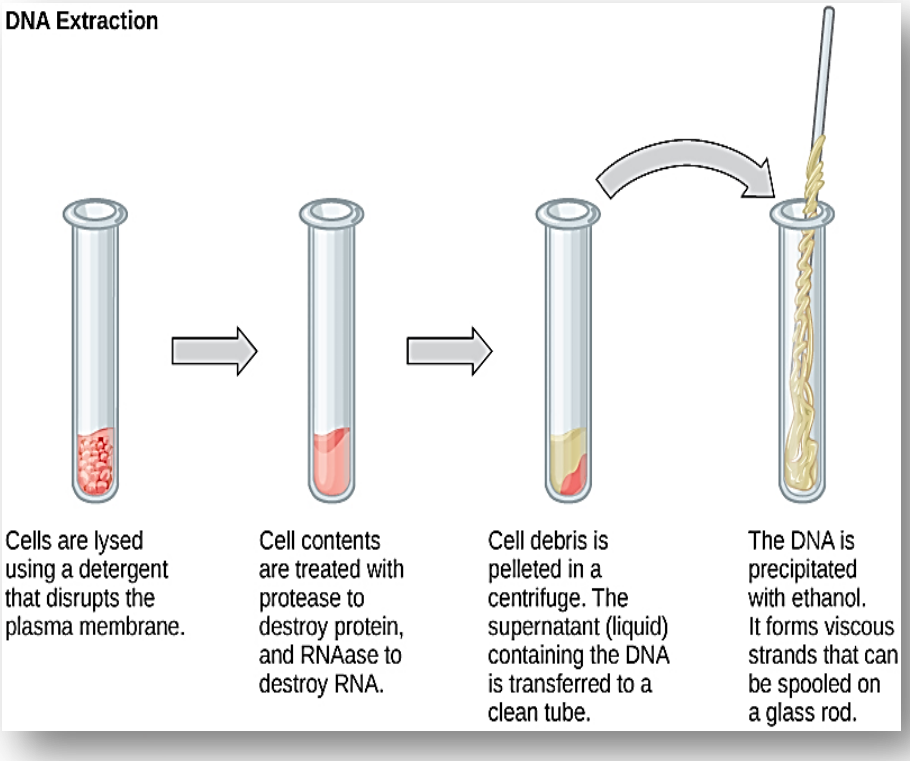
IV. Culturing the host cells in a medium
Bioreactors are used to produce in large quantities.

V. Extraction of the desired product
The extraction & purification of desired product from the culture medium is known as *Downstream processing.*



Explanation:-

I. Isolation of a desired DNA fragment



i. **Breaking the cell wall.**
Treat the cells with enzymes such as **lysozyme** (bacteria), **cellulase** (plant cells), **chitinase** (fungus) - to break the cell

ii. **Removal of other substances from the DNA**
The RNA & proteins interwined with DNA can be removed by treatment with **ribonuclease** & **protease**.
Other molecules can be removed by appropriate treatments.

iii. **Separation of purified DNA**
Add **chilled ethanol** to precipitates out the purified DNA (can be seen as collection of fine threads in the suspension). This can be separated by ***spooling***.

iv. **Cutting of DNA at specific location using *Restriction Endonuclease.***
Cut the purified source DNA as well as the vector DNA with a specific ***restriction enzyme***.

Restriction Enzymes

- These enzymes actually occur in bacteria to '*restrict'* the replication of attacking bacteriophages by identifying their introduced phage DNA and cut it into pieces.
- These are enzymes that cuts the DNA (cleave phosphodiester bond).
- Restriction enzymes belong to a class of enzymes called **nucleases**. They include **exonucleases** & **endonucleases**.

Restriction enzyme

Exonucleases	**Endonuclease**
Remove nucleotides from the ends of DNA	Cuts at specific positions within the DNA

Action:

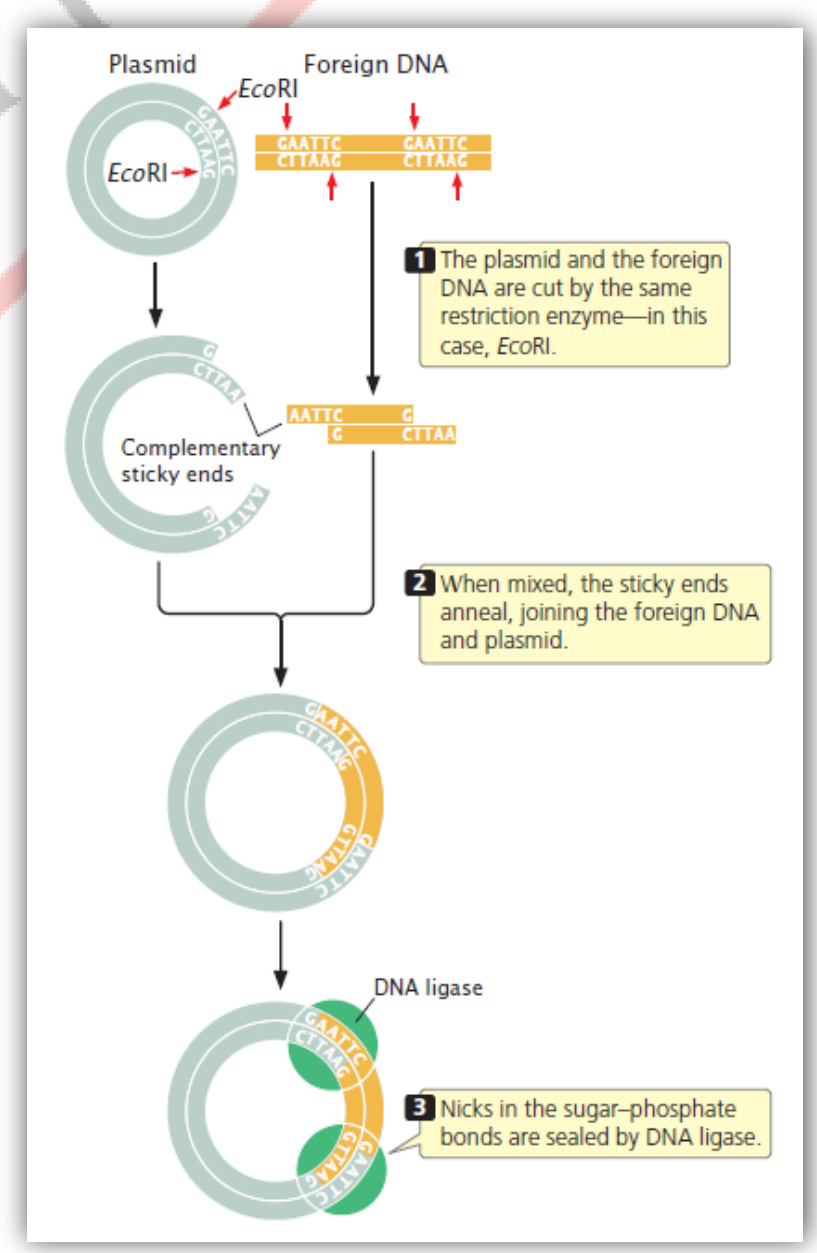
Cuts at specific **recognition sequences** (often 4/6 base pairs long) which are *palindromic*.
→Leaves single stranded overhanging stretches portions at the ends (**Sticky ends**-they form H-bonds with their complementary cut counterparts. This stickiness of the ends facilitates the action of the enzyme **DNA ligase**).

- The first restriction endonuclease–*Hind II*.
- Today more than 900 restriction enzymes have been isolated from over 230 strains of bacteria.

Nomenclature (naming) of restriction enzymes: - Eg: *Eco*RI

✓ First letter indicates **genus** of the prokaryotic cell from which enzyme were isolated. — ***E-*** *Escherichia*
✓ Second two letters indicate **species**. — ***co-*** *coli*
✓ Next letter indicates the **strain name** of bacteria. — **R**- RY 13
✓ This is followed by Roman numerals indicates the **order of isolation** from the strain. — **I-** first

Enzyme	**Prokaryote from which enzyme is extracted**	**Recognition sequence**
*Hin*d III	*Haemophilus influenzae* D-strain	5'- AAGCTT -3' 3'- TTCGAA- 5'
*Eco*RI	*Escherichia coli* RY13 -strain	5'- GAATTC -3' 3'- CTTAAG- 5'
*Bam*HI	*Bacillus amyloliquefaciens* H-strain	5'- GGATCC -3' 3'- CCTAGG- 5'
*Pvu*I	*Proteus vulgaris*	5'- CAGCTG -3' 3'- GTCGAC- 5'
Sal I	*Streptomyces albus*	5'- GTCGAC -3' 3'- CAGCTG- 5'
*Pst*I	*Providencia stuartii*	5'- CTGCAG -3' 3'- GACGTC- 5'
*Cla*I	*Caryophanon latum*	5'- ATCGAT -3' 3'- TAGCTA- 5'



v. Desired DNA fragment is separated using *Gel electrophoresis.*

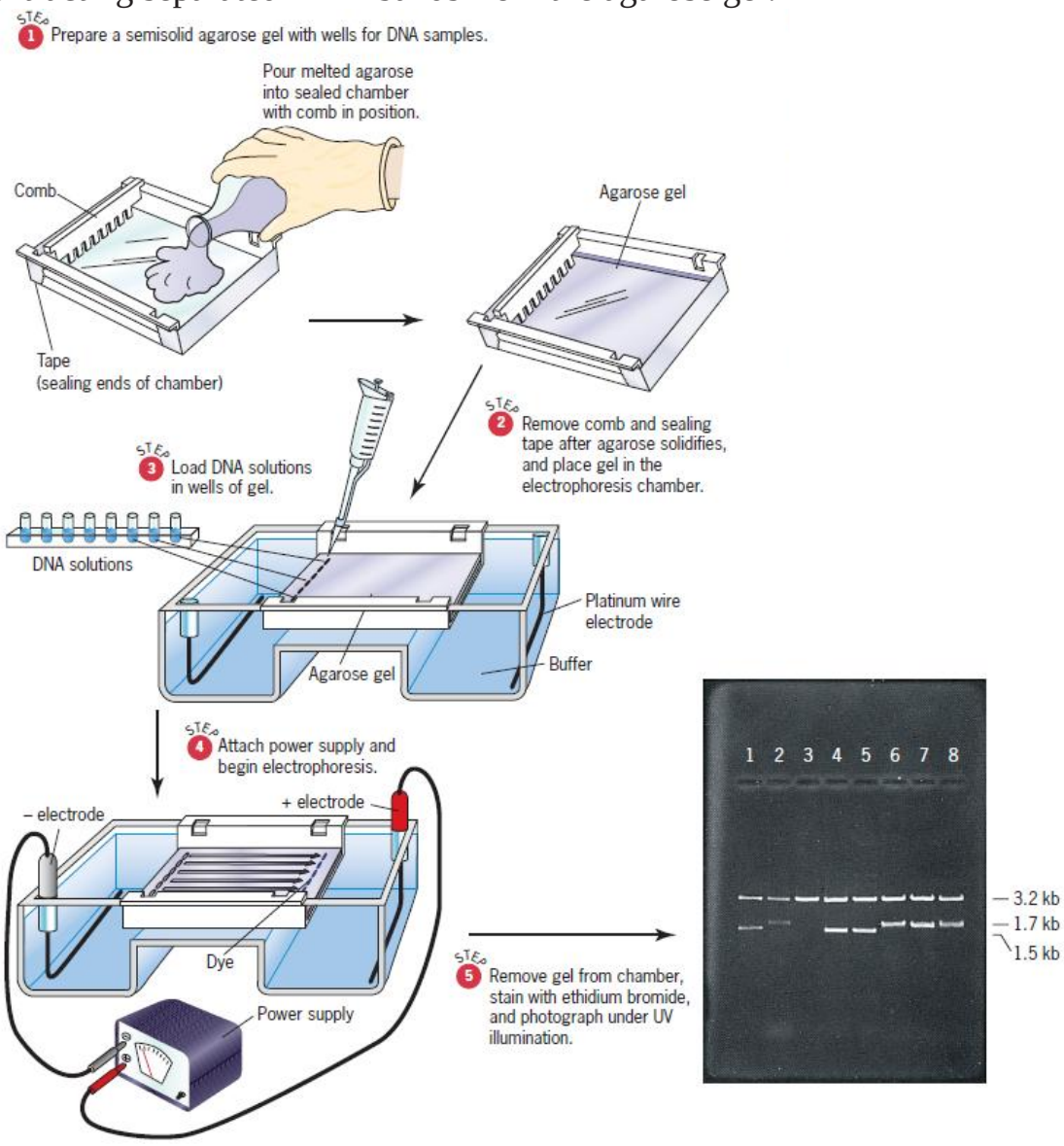
- The fragments of DNA formed by the cutting of DNA by restriction endonucleases can be separated by a technique known as **gel electrophoresis.** It separate DNA fragments based on their size, under the influence of electric field.

Steps:

a. DNA are forced to move under an electric field through the **agarose gel** (medium).Negatively charged DNA will move towards anode a/c to their size through sieving effect provided by the medium. (Hence, the smaller sized fragment move farther).

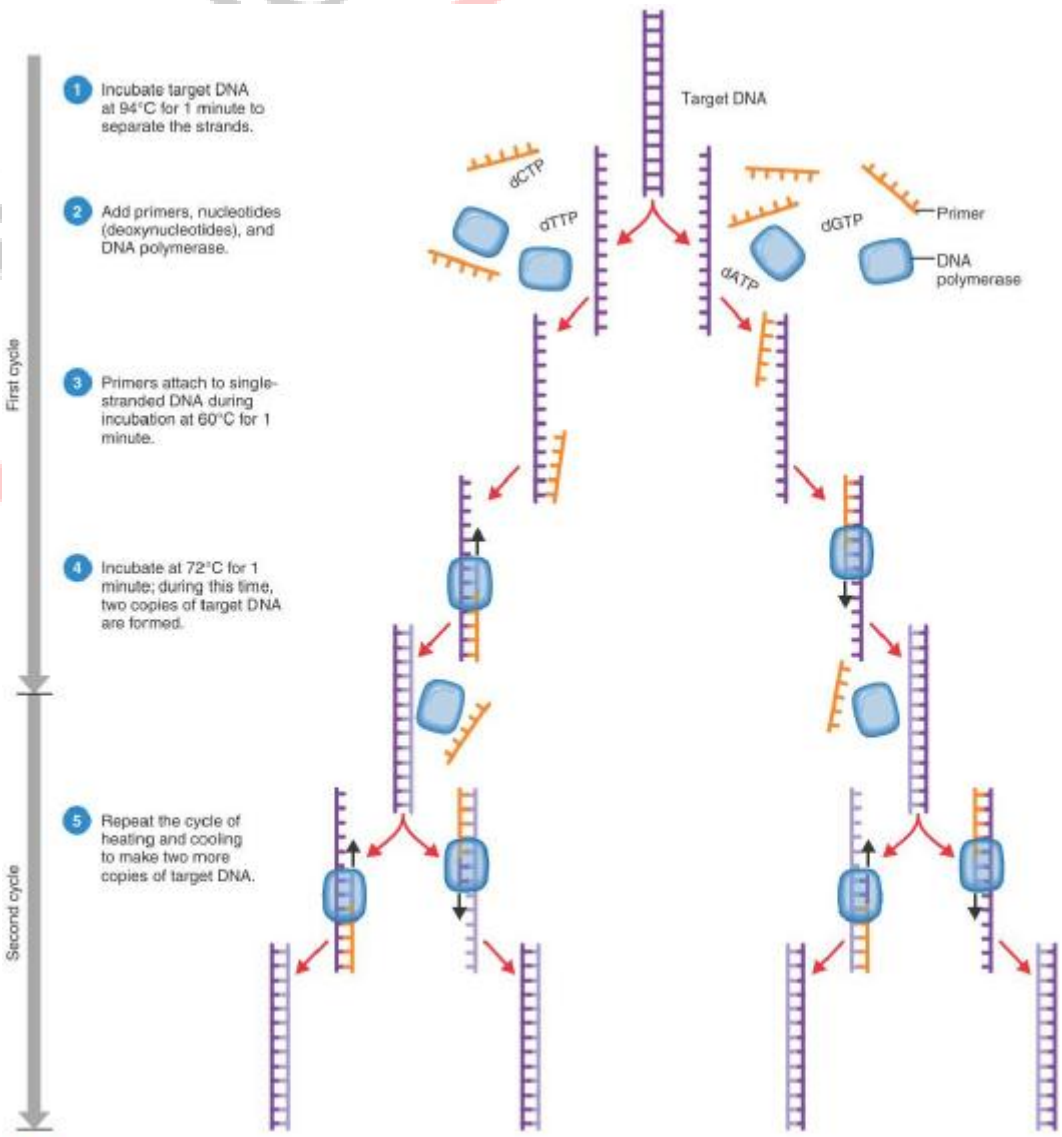
b. Stain the DNA with **ethidium bromide**
c. Exposed to UV radiation
} *Separated DNA fragments are visualised as bright* ***orange bands***

d. Elusion: The process of extracting separated DNA bands from the agarose gel.



vi. Amplification of gene of interest using *PCR.*

- **Kary Mullis** (1983) developed **Polymerase Chain Reaction (PCR)** to synthesis multiple copies of the gene of interest (here template) *in vitro* using free nucleotides, 2 sets of **primers** & the enzyme **DNA polymerase.**
- **Primers** are small chemically synthesized oligonucleotides that are complementary to the regions of DNA.
  (Primers **provide a free 3'-OH** required by DNA polymerase for the formation of the phosphodiester bond)
- For amplification a thermostable ***Taq* polymerase** (isolated from a bacterium, *Thermus aquaticus*) is used.
  It remains active in high temperature.
- Through continuous DNA replication, the DNA segment is amplified up to 1 billion copies.



The polymerase chain reaction.

Steps: -

I. **Denaturation:** The DNA fragment is heated about 94^0C results in separation of DNA strands.
II. **Annealing of primer:** The solution is allowed to cool to 60^0C. During this time, one primer **anneal** (*join*) with 3' end of one DNA strand and the other primer binds with the 3' end of its complementary strand.
III. **Primer extension**: DNA polymerase extends the primers by adding nucleotides complementary to the template.
IV. **Amplification**: The process of replication is repeated.

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

II. Ligation of the DNA fragment into a vector *(preparing rDNA)*

DNA fragment is incorporated into the vector using *DNA ligase*.

rDNA= Foreign DNA + Vector

***Vectors*:-**

- They are the DNA molecules that can carry a foreign DNA segment and replicate inside the host cells.

E.g. **Plasmids** (circular extra-chromosomal DNA of bacteria), **bacteriophages**.

Desirable properties of cloning vectors are-

(a) Presence of ***ori***.
- This is a sequence from where replication starts. A piece of DNA linked to *ori* only can replicate within the host cells.

(b) High ***copy no.***
- When the cloning vectors are multiplied in the host the linked piece of DNA is also multiplied to the numbers equal to the copy number of the vectors.

(c) Presence of ***selectable markers***.
- It helps to select the transformants and eliminate the non-transformants. Upon the introduction of foreign gene in this marker sequence, phenotype is load & that helps in screening whether the cell has been transformed or not.
  (Transformation is a procedure in which a piece of DNA is introduced in a host bacterium).

(d) Presence of unique ***recognition sequence*** in cloning sites.
- In order to link the alien DNA, the vector needs very few **recognition sites** for restriction enzymes.
  (Presence of more than one recognition sites generates several fragments, which complicates the gene cloning).

(e) Ability to sustain in host cell.

(f) Small sized.

Vectors for cloning genes in plants and animals:

Genetic tools of some pathogens can be transformed into useful vectors for delivering genes to plants & animals.

➔ E.g. *Agrobacterioum tumifaciens* (a pathogen of many dicot plants) can deliver a piece of DNA (T-transforming- DNA) to transform normal plant cells into a **tumor.** These tumor cells produce the chemicals required by the pathogen.
The **tumor inducing (Ti) plasmid** of *A. tumifaciens* is modified into a cloning vector which is not pathogenic to the plants but is able to use the mechanisms to deliver genes of interest into plants.
As gene transfer occur without human effort, the bacterium *A. tumifaciens*, is known as natural genetic engineer.

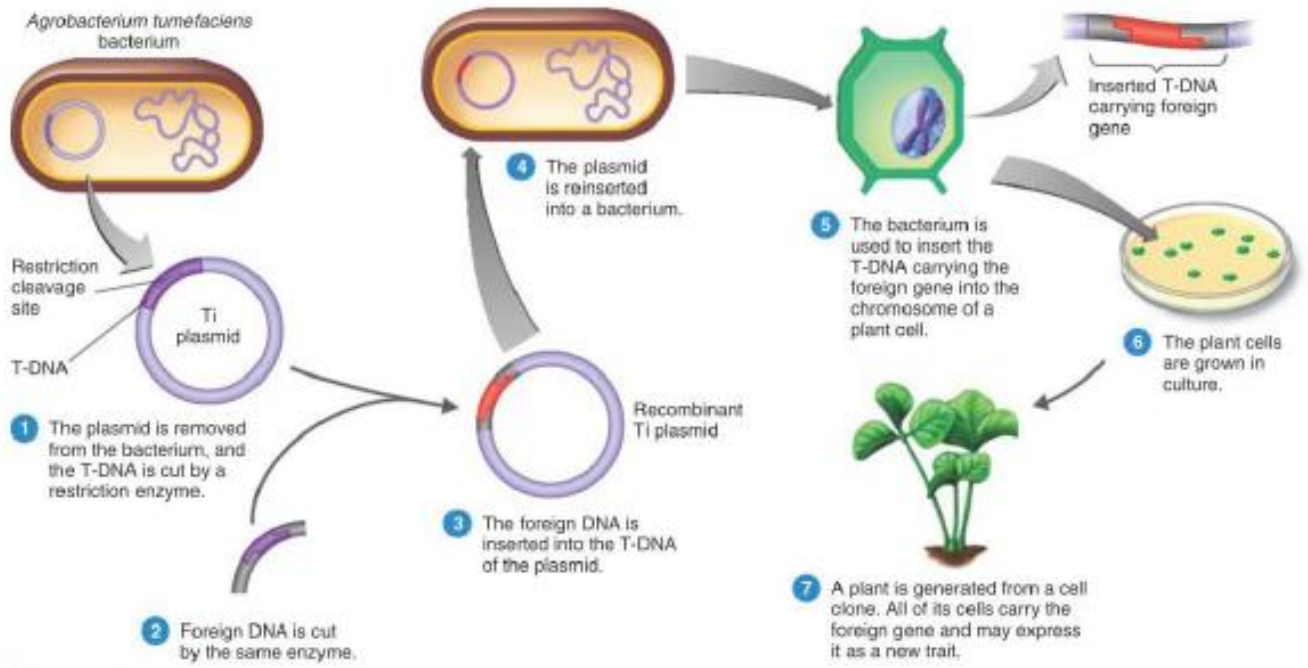


Figure 9.20 Using the Ti plasmid as a vector for genetic modification in plants.

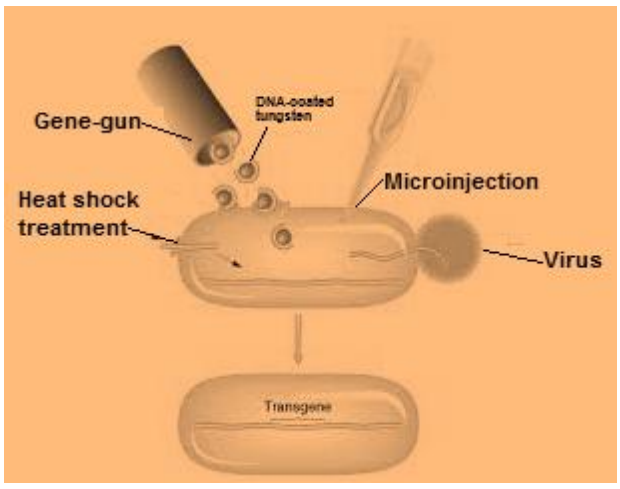
- Retroviruses (e.g., Simian Virus 40) can transform normal cells into **cancerous** cells. So they are used to deliver desirable genes into animal cells.

Since DNA fragment and vector was cut with same restriction enzymes, it has same sticky ends. When DNA fragment & vector mixed with *DNA ligase*, the 2 will anneal producing a recombinant DNA.

III. Transferring the recombinant DNA into the host (*transformation*)

i. Gene tranferring.

➔ Since DNA is a hydrophilic (water soluble) molecule, rDNA cannot pass through cell membranes (lipid soluble).
It can be done in 4 ways:-



(1st-way) ***By Electroporation***

Making the host cell *competent* (ready to take up rDNA)

Steps:-

a. Treating bacterial cell with a specific concentration of a divalent cation (such as Ca^{2+})
b. Incubate the cells with recombinant DNA on ice.
c. Placing them briefly at 42^0C (heat shock)
d. Put them back on ice.
This makes temporary holes in the cell surface enables the bacteria permeable to recombinant plasmid to enter.

(2nd-way) ***By micro-injection***

- In this, recombinant DNA is directly injected into the nucleus of an animal cell.

(3rd-way) ***By gene gun method***

- In this, cells are bombarded with high velocity micro-particles of gold or tungsten coated with DNA. This method is suitable for plants.

(4th-way) ***By disarmed pathogen vector.***

- Which when infect the cell, transfer the recombinant DNA into the host.

ii. Selection/ Screening of *transformants*.

Selection of transformed cells from non-transformed ones with the help of selectable markers & its insertional inactivation.

Selection is of 2 types:-

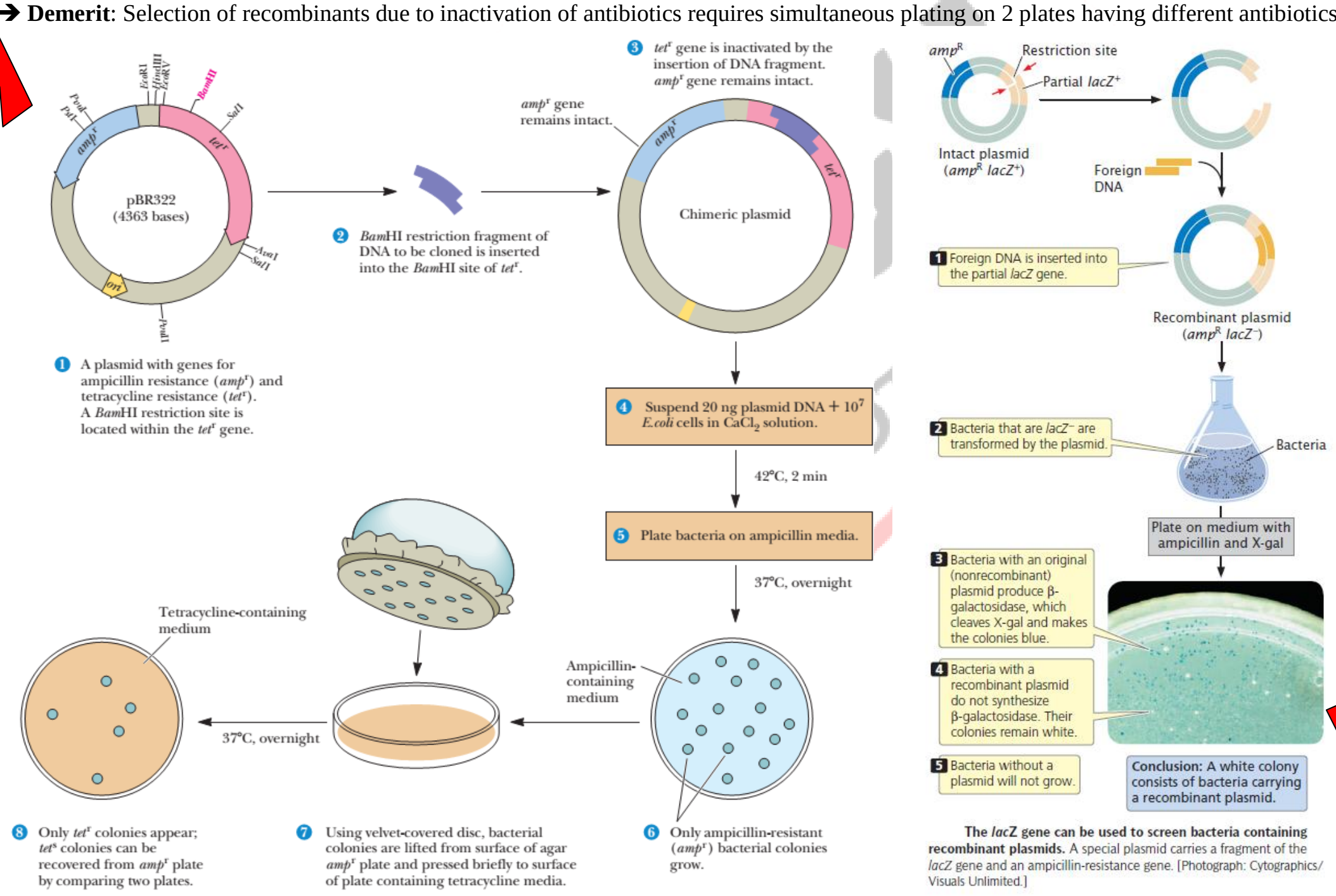
A. Inactivation of antibiotic resistance property.

- The ligation of alien DNA is carried out at a restriction site present in 1 of the 2 **antibiotic resistance** genes.

  E.g. In *E.coli*, presence of plasmid **pBR322** with genes encoding resistance to antibiotics ampicillin, tetracycline etc.
  (The normal *E. coli* cells do not carry resistance against any of these antibiotics).
  - If ligate a foreign DNA at the *Bam*H I site of **tet^R** gene, then bacteria/ **transformant** will not grow on tetracycline (as **tet^R** gene has been inactivated), but will grow on ampicillin.
  - If transformant has non recombinant plasmid (without foreign gene), it will grow on both culture media containing tetracycline & ampicillin.

  In this case, one antibiotic resistance gene helps to select the transformants, whereas the other antibiotic resistance gene gets inactivated due to insertion of alien DNA(***insertional inactivation***), and helps in selection of recombinants.

➔ **Demerit**: Selection of recombinants due to inactivation of antibiotics requires simultaneous plating on 2 plates having different antibiotics.



B. Blue-white selection method.

E.g. A recombinant DNA is inserted within the coding sequence of an enzyme, **β -galactosidase.** So the enzyme is inactivated. Such colonies do *not produce any colour*. These are identified as recombinant colonies.
If the plasmid in bacteria have no an insert it gives blue coloured colonies in presence of chromogenic substrate.

IV. Culturing the host cells

- Culturing of transformed cell in suitable medium to produce a desirable protein. The foreign gene gets expressed under appropriate conditions.

(If a protein encoding gene is expressed in a heterologous host, it is called a **recombinant protein)**.
The cells can also be multiplied in a **continuous culture system.** Here, the used medium is drained out from one side while fresh medium is added from the other. It maintains the cells more physiologically active and so produces a larger biomass leading to higher yields of desired protein.

Bioreactors

- To produce large quantities of products, the **bioreactors** are used where large volumes (100-1000 litres) of culture can be processed.
- Bioreactors are the vessels in which raw materials are biologically converted into specific products, enzymes etc., using microbial plant, animal or human cells.
- A bioreactor provides the optimal growth conditions (temperature, pH, substrate, salts, vitamins, oxygen) for achieving the desired product.

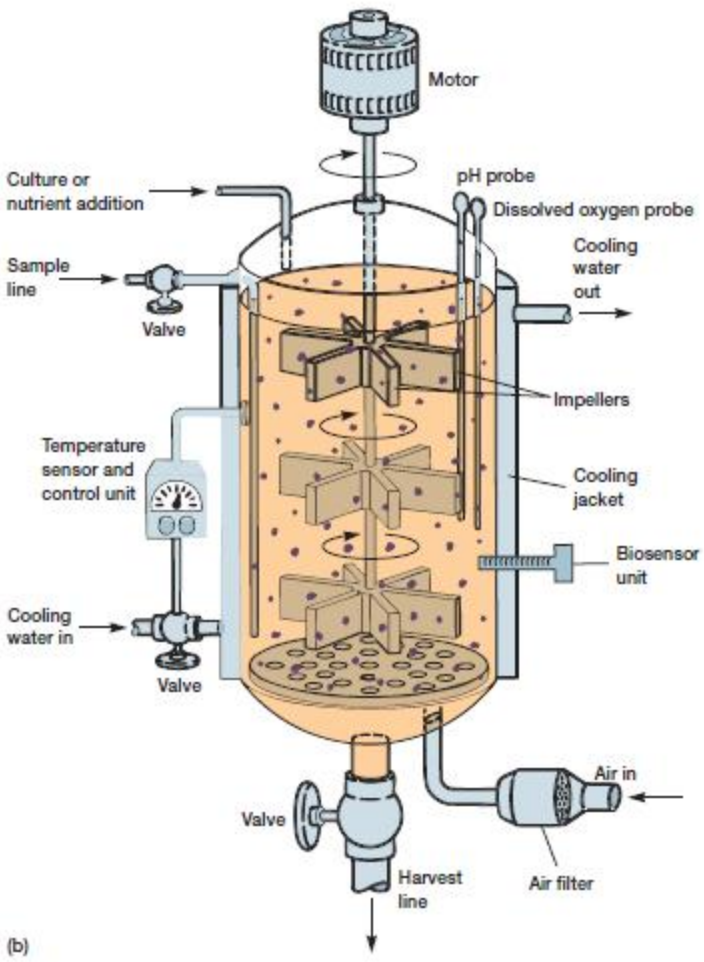
Stirred-tank reactor

- The most commonly used bioreactors are of stirring type.
  It is usually cylindrical or with a curved base to facilitate the mixing of the reactor contents. The stirrer facilitates even mixing and O_2 availability. Alternatively air can be bubbled through the reactor.

❖ The bioreactor has-

- An agitator system
- An oxygen delivery system
- A foam control system
- A temperature control system
- pH control system
- Sampling ports (for periodic withdrawal of the culture).

(a)



(b)

Industrial Stirred Fermenters. (**a**) Large fermenters used by a pharmaceutical company for the microbial production of antibiotics. (**b**) Details of a fermenter unit. This unit can be run under aerobic or anaerobic conditions, and nutrient additions, sampling, and fermentation monitoring can be carried out under aseptic conditions. Biosensors and infrared monitoring can provide real-time information on the course of the fermentation. Specific substrates, metabolic intermediates, and final products can be detected.

Sparged stirred-tank reactor

➔ It is a stirred type reactor in which air is bubbled at the bottom of the tank through a porous ring called **spranger**.

V. Extraction of the desired product

- The product obtained from bioreactor is not in pure form.
- **The extraction & purification of desired product from the culture medium is known as *Downstream processing.***

Procedure-

Step 1. **Separation** and **purification** of gene products.
Step 2. Addition of suitable **preservatives**.
Step 3. Goes thorough **clinical trials** (in case of drugs).
Step 4. **Quality control testing** (vary from product to product).