NEET- 2020- 45 Days Crash Course

Date : 26th AUG 2020

Chapter Name : BIOTECHNOLOGY PRINCIPLES AND PROCESSES

REVISION

Biotechnology - Introduction

- It is a technique using live organisms or their cellular components or enzymes to produce useful products and processes for man.

❖ Principles of Biotechnology

R-DNA technology

I. **Genetic engineering :** In this Technique, chemistry of genetic material (DNA & RNA) is altered and introduced these in to host organisms and thus alter the phenotype of the host organism is called **genetic engineering (= Recombinant DNA technology)**.

II. **Maintenance of Microbial contamination free (sterile) surrounding in chemical engineering :** It helps in the growth of only the desired microorganism/eukaryotic in large quantities for the manufacture of biotechnological products such as antibiotics, vaccines, enzymes, medicines, hormones etc.

- Genetic engineering involves formation of **'recombinant DNA (rDNA)**, use of gene cloning and gene transfer.

- **Alien DNA** is linked with the **'ori'** site or origin of replication (it is a site of initiation of replication) can replicate and multiply itself in the host organism that is called for **cloning**.

1st r RNA – By Boyer and Cohen

- It is helpful in forming multiple identical copies of any template DNA.

Recombinant DNA Technology – 1

- **Stanley Cohen and Herbert Boyer (1972)** firstly constructed **recombinant DNA**.
- They isolated piece of DNA from a plasmid carrying **antibiotic-resistance gene** of the bacterium **Salmonella typhimurium** and fused it to the **plasmid of E. coli.**

- The linking of these two is performed by **DNA ligase** resulting recombinant DNA is created in vitro. Now it is transferred into E. coli where it can replicate in the presence of the new host 's by **DNA polymerase enzyme**

Genetically modified organism (GMO) or transgenic organism

- Identification of DNA with desirable genes.
- Introduction of the identified DNA into the host
- Maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

❖ Tools of Recombinant DNA Technology

Restriction Enzymes

Exonucleases

Separate nucleotides from the terminal ends of DNA in one strand of duplex.

Endonucleases

Cuts at specific position within the DNA.

Restriction Enzymes Mechanism

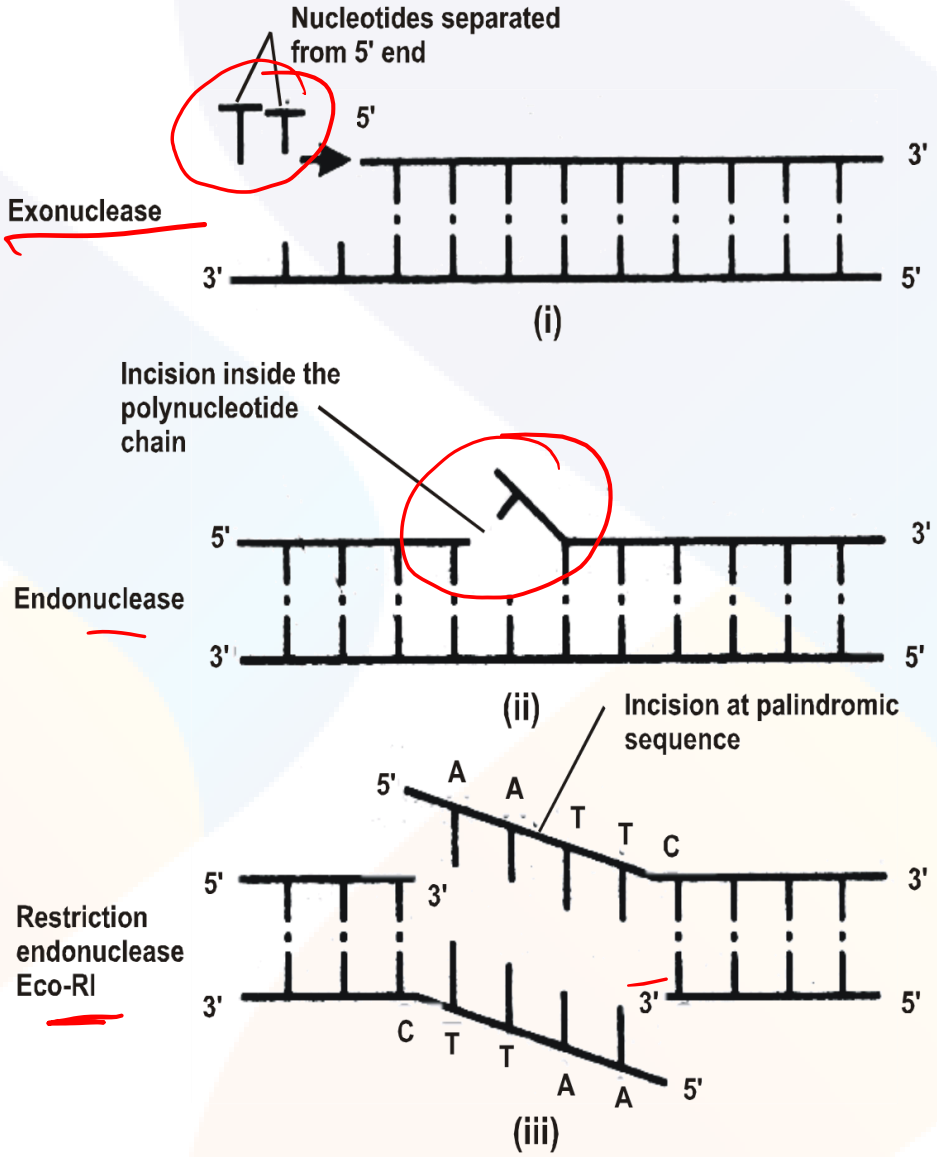


Fig:- (i), Action of exonuclease (ii), Action of enodnuclease.
(iii), Action of restriction enzyme.

Restriction Endonucleases

- Cut the DNA strands at specific base sequence in palindrome site. Thus they function as **'molecular scissors' or chemical scalpels**.
- **W. Arber, H. Smith and D. Nathans** discovered these enzymes.
- The first restriction endonuclease was **Hind II** isolated from **Haemophilus influenzae Rd** produces blunt ends.
- Naming these enzymes is the first letter of the name comes from the bacterium's genus names and the second two letters come from the species of the prokaryotic cell from which they were isolated
- Ex: **EcoRI** comes from Escherichia coli RY 13. In EcoRI, 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.
- DNA recognised by restriction endonuclease is called **palindromic nucleotide sequence**.

Ex:

MALAYALAM

5' —— G A A T T C —— 3'
3' —— C T T A A G —— 5'

Restriction Enzyme Action

Restriction enzymes cut the strand of DNA a little away from the centre of the palindrome sites but between the same two bases of the opposite strands. This leaves single stranded unpaired bases at cut ends are called **sticky ends or cohesive ends.**

Sticky ends facilitate the action of DNA ligase.

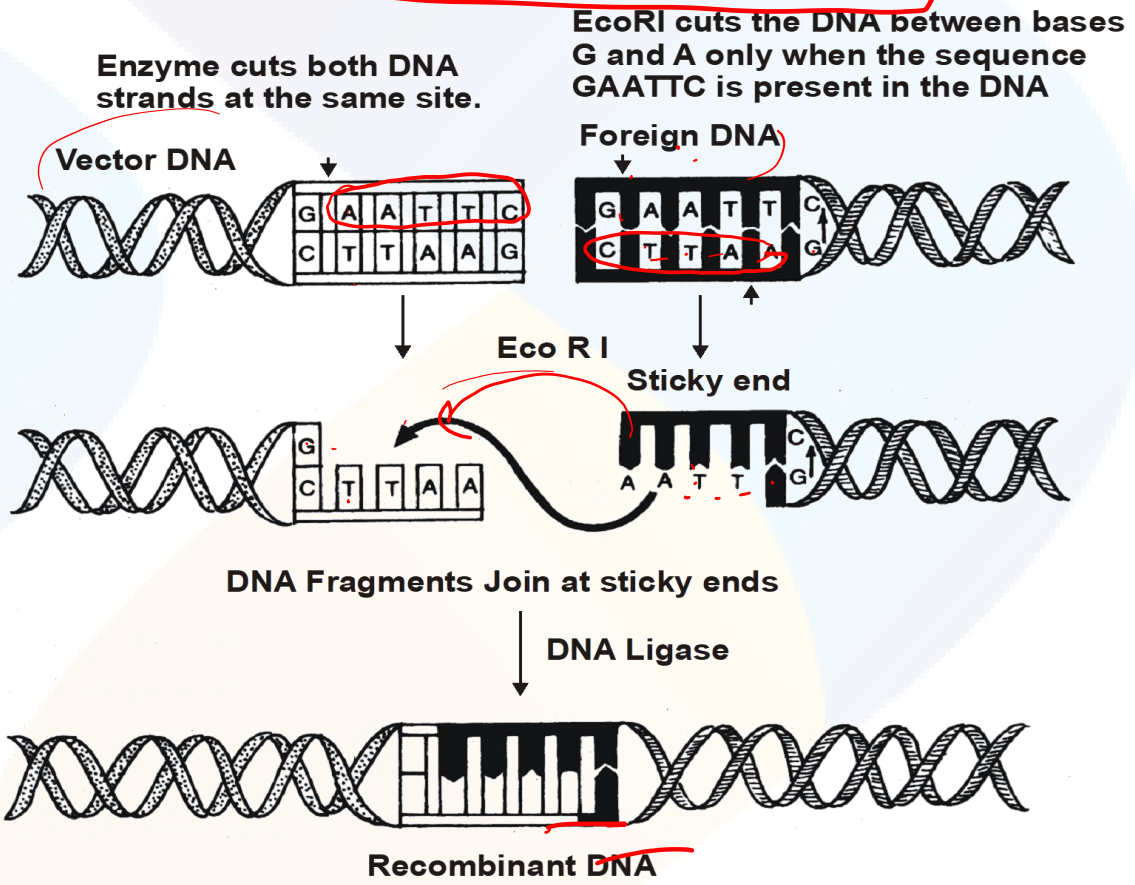


Fig:- Formation of recombinant DNA by action of restriction endonuclease enzyme- Eco RI

Example of Restriction Enzymes

Some restriction enzymes, type II, their source, recognition sequence and site of cleavage.			
S.No.	**Restriction Enzyme**	**Source**	**Recognition sequence and site of Cleavage**
1.	Alu I	Arthrobacter luteus	5'-A-G↓C-T-3' 3'-T-C↑G-A-5'
2.	Bam H I	Bacillus amyloliquefaciens H	5'-G↓G-A-T-C-C-5' 3'-C-C-T-A-G↑G-5'
3.	Eco R I	Escherichia coli RY 13	5'-G↓A-A-T-T-C-3' 3'-C-T-T-A-A↑G-5
4.	Eco R II	Escherichia Coli R245	5'↓C-C-T-G-G-3' 3'-G-G-A-C-C↑5'
5.	Hae III	Haemophilus aegyptius	5'-G-G↓C-C-3' 3'-C-C↑G-G-5'
6.	Hin d III	Haemophilus influenzae Rd	5'-A↓A-G-C-T-T3' 3'-T-T-C-G↑A-A-5'
7.	Hin d II	Haemophilus influenzae Red	5'G-T-C↓G-A-C-3' 3'-C-A-G↑C-T-G-5'
8.	Sal I	Streptomyces albus	5'-G↓T-C-G-A-C-3' 3'-C-A-G-C↑T-G-5'
9.	Sca I	Streptomyces caespitosus	5'-A-G-T↓A-C-T-3' 3-T-C-A↑T-G-A-5'
10.	Sma I	Serratia marcescens	5'-C-C-C↓G-G-G-3' 3'-G-G-G↑C-C-C-5'

Tools of rDNA Technology - Enzymes

- **DNA Ligases** -They form **phosphodiester bonds** between adjacent nucleosides and covalently link two individual fragments of double-stranded DNA. They help in sealing gaps in DNA fragments. Thus they act as a **molecular glue**. **T4 DNA ligase**

- **Alkaline Phosphatase** - It is helpful to remove the phosphate group from the 5' end of DNA molecule, leaving a free 5' hydroxyl group. It can be isolated from calf intestine or bacteria. It is used to prevent unwanted self -ligation of vector DNA molecules

- **DNA Polymerase** - It is employed to polymerize replication of DNA on DNA template or complementary DNA (cDNA).

- It catalyses in 5'-3' and 3'-5' exonucleolytic degradation of DNA.

- DNA polymerase firstly investigated by **A. Kornberg in E.coli** and is now known as **DNA polymerase I**.

- **DNA polymerase II and DNA polymerase III**. It produces a parallel strand in the presence of ATP on DNA template.

Cloning Vectors

- Vector DNA is able to carry a foreign DNA segment and replicate inside the host cell.
- **Plasmids, bacteriophage, cosmids, phagemids, Yeast artificial chromosomes (YACs), Bacterial artificial chromosomes (BACs), transposons etc**. are used as vectors
- **Plasmid :** It is extra chromosomal, self–replicating, usually circular, double -stranded DNA molecules, found in many bacteria and some yeast.
- **pBR322 Vector :** In pBR322, p- denotes plasmid, BR- stands for Bolivar and Rodriguez who constructed this plasmid, 322 - is a number given to distinguish this plasmid
- **Origin of replication or (Ori) :** It is a site of replication.
- **Antibiotic resistance genes : amp-r** gene **(ampicillin resistance gene)** and **tet-r (tetracycline resistance gene)** gene.
- **Unique recognition sites for restriction endonucleases :** Two unique sites, Pst I and Pvu I are located within the ampr gene and Bam HI, sal I, etc, are within tetr gene
- Some other unique restriction sites are **Eco RI Cla I, Hind III, Pvu II, rop** codes for the proteins involved in the replication of the plasmid.

Structure of Plasmid pBR 322

- **Bacteriophage :** These viruses infect bacterial cells by injecting their DNA into these cells.
- Lambda (l) phage and M 13 phage are used as clonning vectors.

- **Cosmid :** It is constructed by combining certain features of plasmid and the 'cos' sites of phage lambda.

- **Bacterial Artificial chromosome (BAC) :** They can accommodate upto 300–350 kb of foreign DNA and are also being used in genome sequencing projects.

- **Yeast artificial Chromosome (YAC) :**These vectors are employed to clone DNA fragements of more than 1 Mb in size

- **Shuttle vectors :** Several vectors are constructed for the use in both eukaryotic cell and E.coli. These are called shuttle vectors **Ex: yeast episomal plasmid or YEp**.

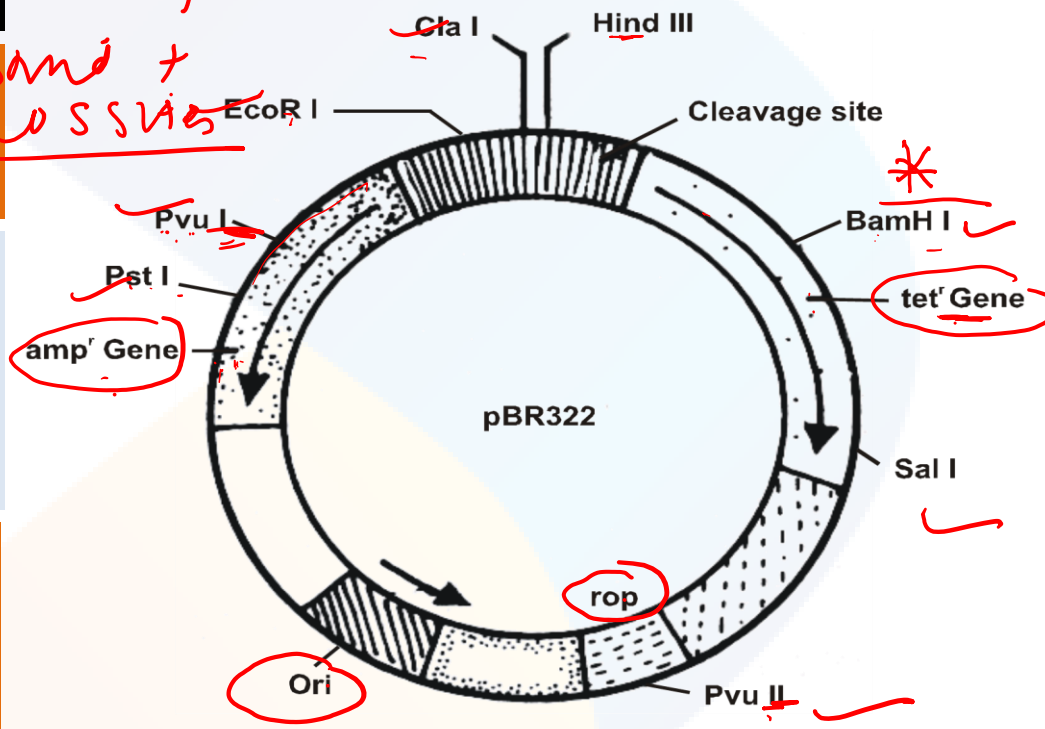


Fig:- Structure of Plasmid pBR 322

Characteristics of a Cloning vector

- **Origin of replication (ori)** : from where replication starts.

- **Selectable marker :** To identify and eliminate non-transformants and selectively permit the growth of the transformants. Genes encoding resistance to antibiotics such as tetracycline, ampicillin, kanamycin or chloramphenicol are useful in selectable markers for E.coli.

- **Recognition Sites (Cloning sites) :** Vector bears one unique restriction endonuclease recognition site which enables foreign DNA to be inserted into the vector during the formation of recombinant DNA molecule.
- Vectors contain unique recognition sites for several restriction enzymes in a small region of DNA which is referred to as a poly linker or multiple cloning site (MCS).

- **Pbr322** bears genes for resistance against two antibiotics (ampicillin and tetracycline). A foreign DNA is linked at the bam HI site of tetracycline resistance gene in the vector pbr322.
- The recombinants grow in ampicillin containing medium but not on that containing tetracycline.
- Non-recombinants grow on the medium containing both the antibiotics.

- **Colour reaction :** selectable marker is developed to differentiate recombinants and non-recombinants on the basis of their ability to produce colour in the presence of a chromogenic substance.
- Recombinant DNA is inserted in the coding sequence of an enzyme **b-galactosidase** this causes which is called **insertional inactivation** and, therefore, the colonies do not produce any colour, these colonies are marked as recombinant colonies.

Competent host

- **Agrobacterium tumefaciens**, a pathogen (disease causing agent) of saveral dicot plants is able to transfer a piece of DNA known as **'T-DNA'** to convert normal plant cells into tumour

- Retroviruses change normal cells into cancerous cells, similarly **retroviruses** are used to carry desirable genes into animal cells.

- Divalent cation, such as calcium which increases the efficiency with which DNA enters the bacterium through pores in its cell wall.

- Recombinant DNA (rDNA) can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 42ºC (heat shock), and then putting them back on ice

Direct Gene Transfer

- **Microinjection :** Foreign DNA is directly injected into the nucleus of animal cell or plant cell by using micro needles or micro pipettes.
- **Jeffey S. chamberlain et al (1993)** of Human genome Centre, Michigan university U.S.A have cured mice that inherited a neuro-muscular disease which is like muscular dystrophy of humans.
- **Gene gun or Particle gun or Biolistic:** In this process, DNA coated onto **microscopic pellets of gold or tungsten** is literally shot with high velocity into target cells
- **Direct DNA Injection**: Direct injection of DNA into skeletal muscle led to the possibility of using Gene as vaccines.
- **Electroporation:** In this method the electrical impulses induce transient (temporary) pores in the plant cell membrane through which the DNA molecules are incorporated into the plant cells.

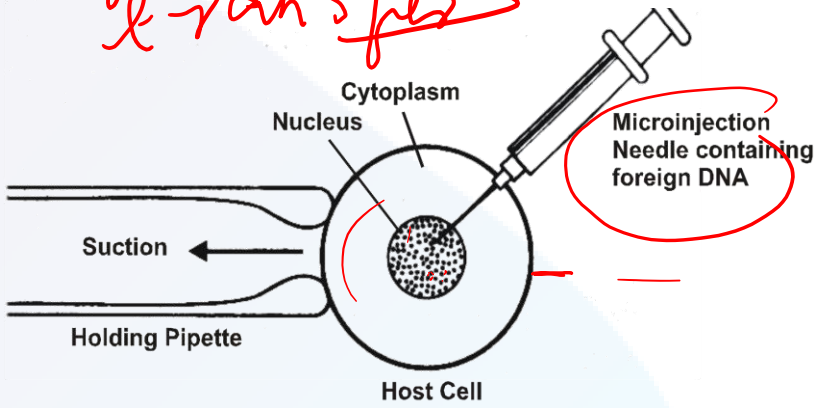


Fig:- Introduction of foreign DNA in a host cell with a microinjection Needle.

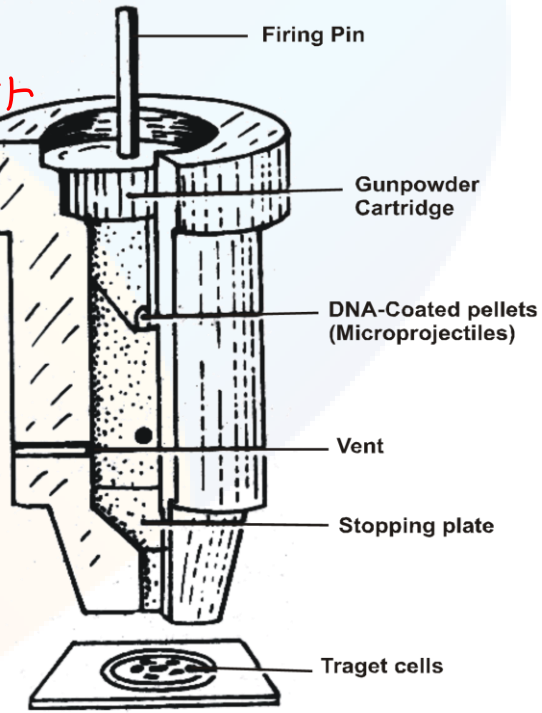


Fig :- Gene gun.

Gel Electrophoresis

- This method is employed to separate fragments of DNA after the cutting of DNA by restriction enzymes.
- **Electrophoresis** technique involves separation of charged molecules under the influence of an electrical field
- The separation of DNA fragments depend upon size of pores of agarose gel.
- Pore size depends on agarose concentration.
- Separated DNA fragments are stained with **ethidium bromide** followed by exposure to UV radiation resulting bright orange coloured bands
- The separated bands of DNA are cut out from the **agaroses gel** and extracted from the gel piece called as **elution**

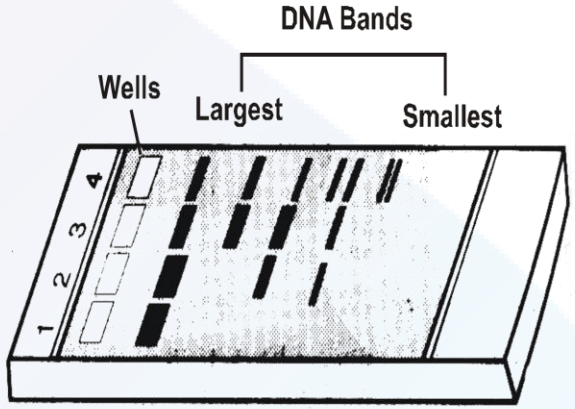


Fig:- A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested lanes of DNA fragments (lanes 2 to 4)

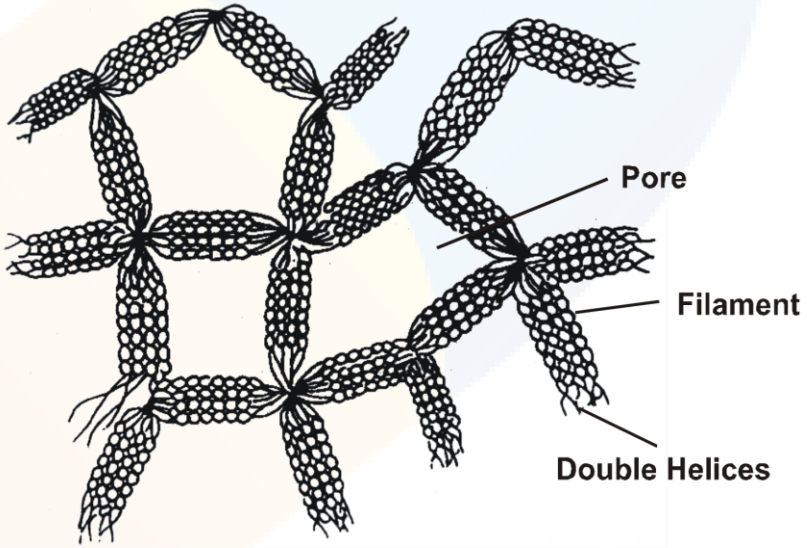
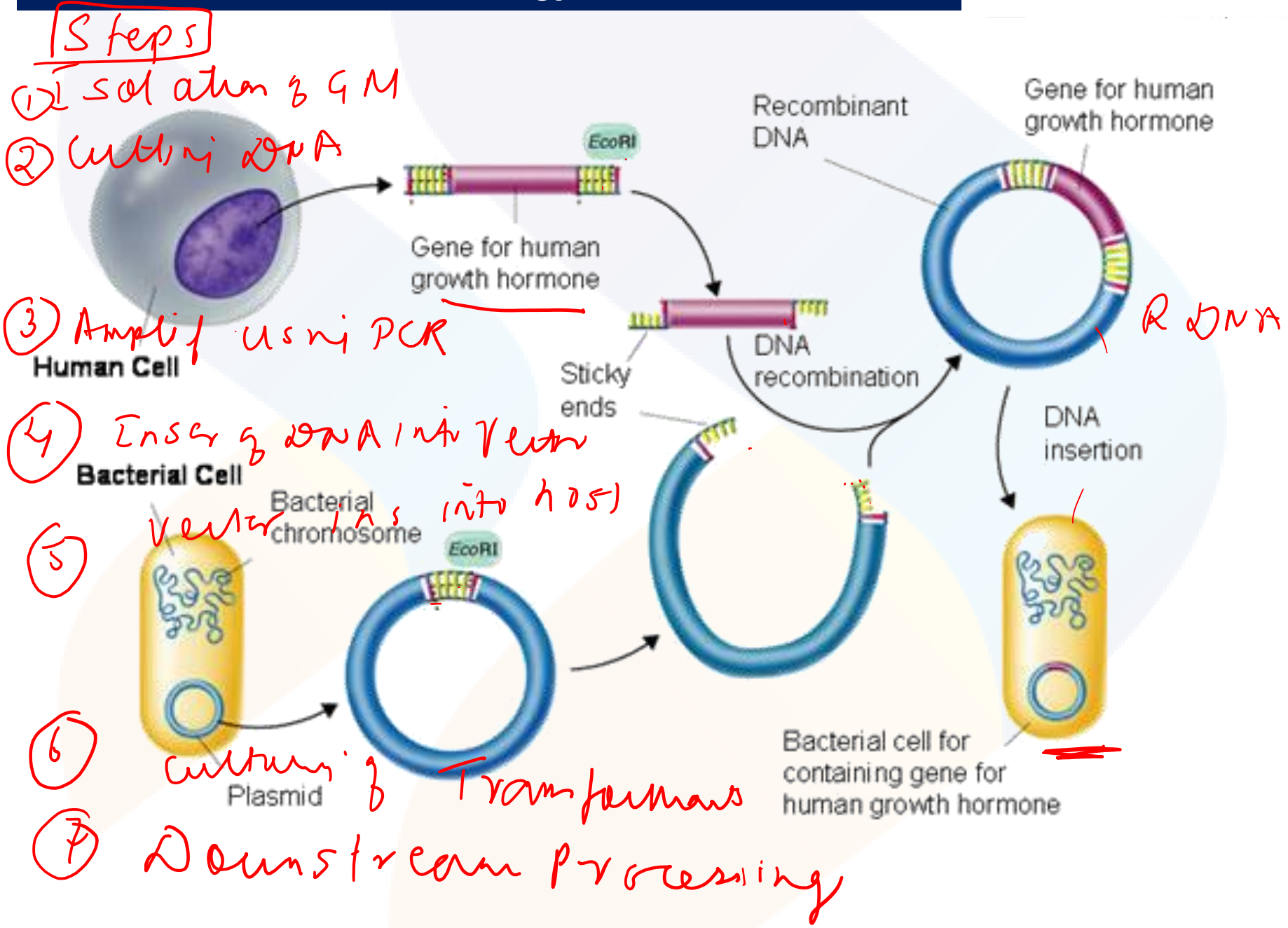


Fig :- Formation of pores in the agarose gel.

Recombinant DNA Technology



Process of rDNA Technology

- **Isolation of the Genetic Material (DNA) :** bacterial cell / plant or animal tissue with enzymes such as **lysozyme (bacteria), cellulase (plant cells), chitinase (fungus).**

- **RNA** can be removed by treating with **ribonuclease** while proteins can be removed by treating with protease.

- Purified DNA finally precipitates out after the addition of **chilled ethanol**. This is seen as collection of fine threads in suspension.

- **Cutting of DNA : Restriction enzyme** is used to cut the purified DNA at specific sites.

Agarose gel electrophoresis is employed to check the progress of a restriction enzyme digestion. DNA is negatively charged molecule, it moves towards the positive electrode. This same process is also repeated with the vector.

- Source DNA and the cut vector are added in the medium containing **ligase enzyme**. The latter is responsible to the fusion of them resulting rDNA is formation

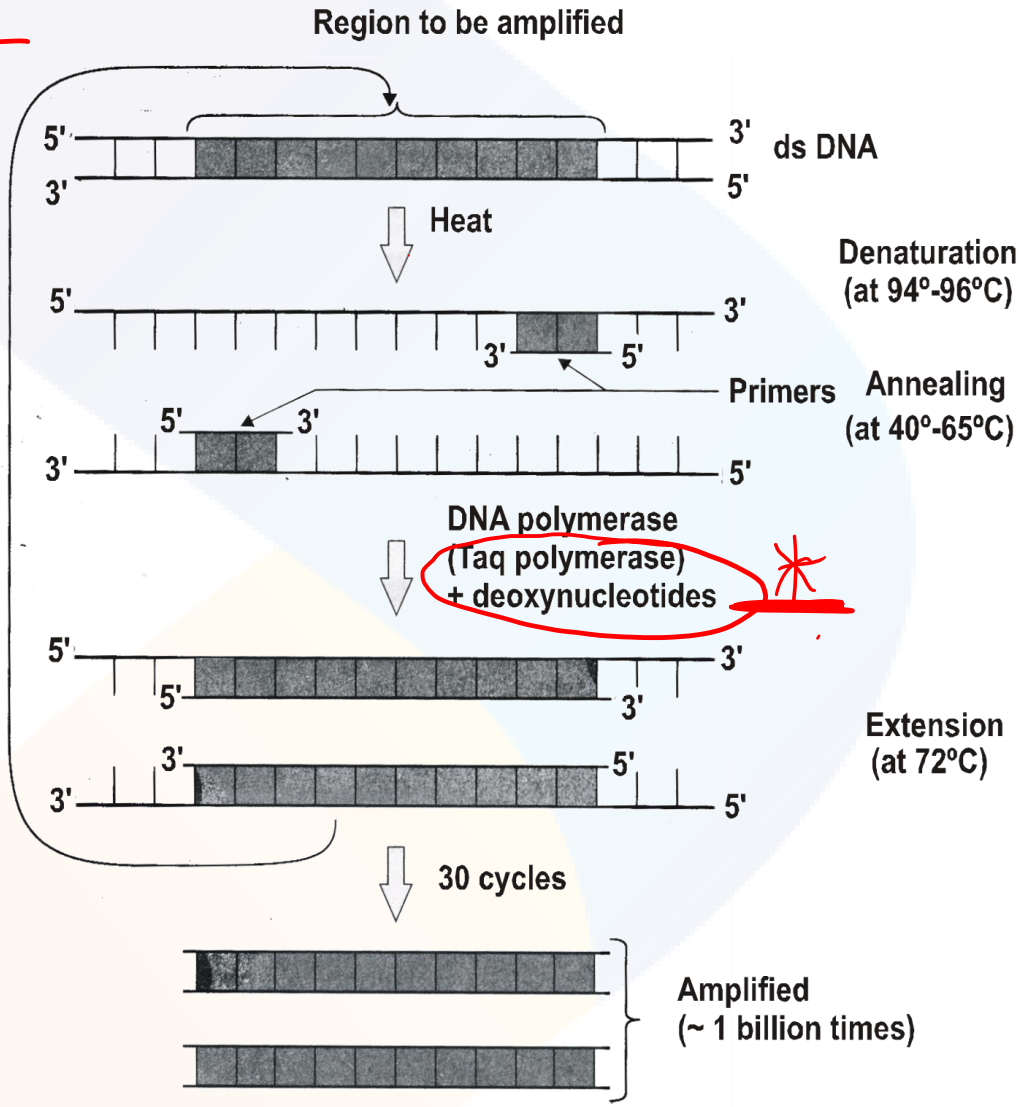
Gene amplification - PCR

- Discovered by **kary mullis in 1985**.

- **Denaturation:** In this process, the target DNA is heated to a high temperature (usually 94º
- to 96ºC)

- **Primer annealing :** The two sets of primers (small chemical synthesized oligonucleotides that are complementary to the regions of DNA) undergo biochemical process of annealing at an optimum temperature of **40-65ºC.**

- **Extension :** The final step is extension, where in **Taq DNA polymerase (of a thermophile bacterium Thurmus aquaticus)** synthesizes the DNA region between the primers, using DNTPs (deoxynucleoside triphosphates) and Mg_2+ at 72°C.



Application of Polymerase Chain Reaction (PCR)

- **Diagnosis of Pathogens** : PCR-based assays have been developed that detect the presence of gene sequences of the infectious agents.
- **Diagnosis of Specific Mutation :** Sickle cell anaemia, muscular dystrophy, phenylketonuria, AIDS, hepatitis, tuberculosis and chlamydia can be diagnosed.
- **DNA Fingerprinting :** for generating abundant amount of DNA for analysis in the DNA fingerprinting technique. used in forensic science
- **Detection of Specific Microorganisms**
- **In prenatal Diagnosis :** It is useful to detect genetic disease in foetus before birth.
- **Diagnosis of Plant Pathogens :** PCR can be used to detect many diseases of plants **Ex: viroids (associated with apple, grape, citrus, pear, etc.) viruses (like TMV, bean yellow mosaic virus etc).**
- **In palaeontology :** DNA fragements of the mummified remains of humans and extinct animals like wooly mammoth and dinosaurs can be cloned by the use of PCR.
- **Gene Therapy :** PCR proves to be of immense help in monitoring a gene in gene therapy experiments.

Comparison between PCR and Gene Cloning

Comparison between PCR and Gene Cloning			
S.No.	Parameter	PCR	Gene Cloning
1	Efficient	More	Less
2	Requirement	DNA	Restriction Enzyme, ligase, Vector, bacterial cell.
3	Manipulation	In vitro	in vitro and in vivo
4	Cost	Less	More
5	Automation	Yes	No
6	Error probability	Less	More
7	Labour intensive	No	Yes
8	Time for a typical experiment	4 hours	2-4 days
9	User's skill	Less required	More required
10	Application	More	Less

Preparation & Insertion of rDNA into the Host Cell

- **Vector DNA like plasmid DNA and allien or foreign DNA (carrying desired gene)** are cut by the same restriction endonuclease to form complementary sticky ends that is called **restriction digestion**. Both of them are joined by the use of **DNA ligase enzyme** resulting **recombinant DNA or chimera DNA (rDNA) is formed.**

- Recipient cells after making them **'competent'** to receive, take up DNA present in its surrounding.

- **Obtaining the Foreign Gene Product:** any protein encoding gene is expressed in a heterologous host, is called a **recombinant protein**.

- Cultures may be used for extracting the desired protein and then purifying it by using different separation techniques.

- **Bioreactors** can be thought of as vessels in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbes, plant, animal or human cells.

Bioreactors

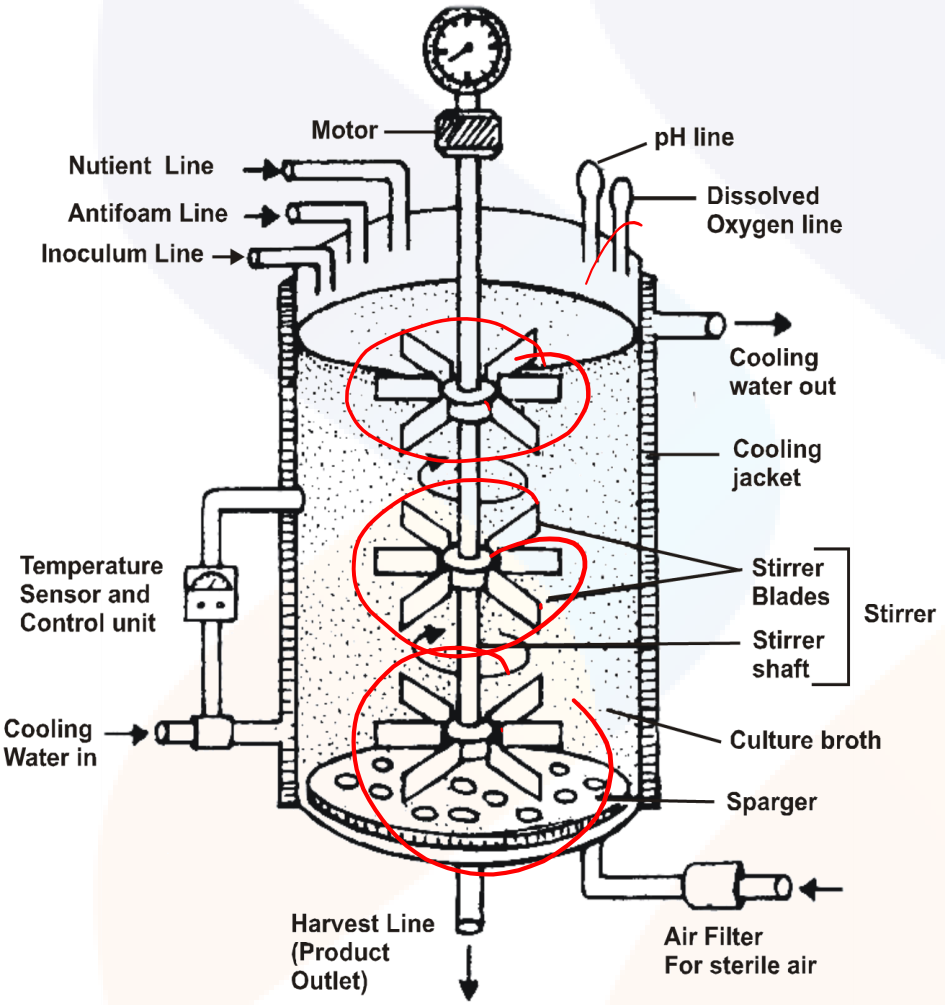


Fig :- Simple stirred tank bioreactor for continuous culture.

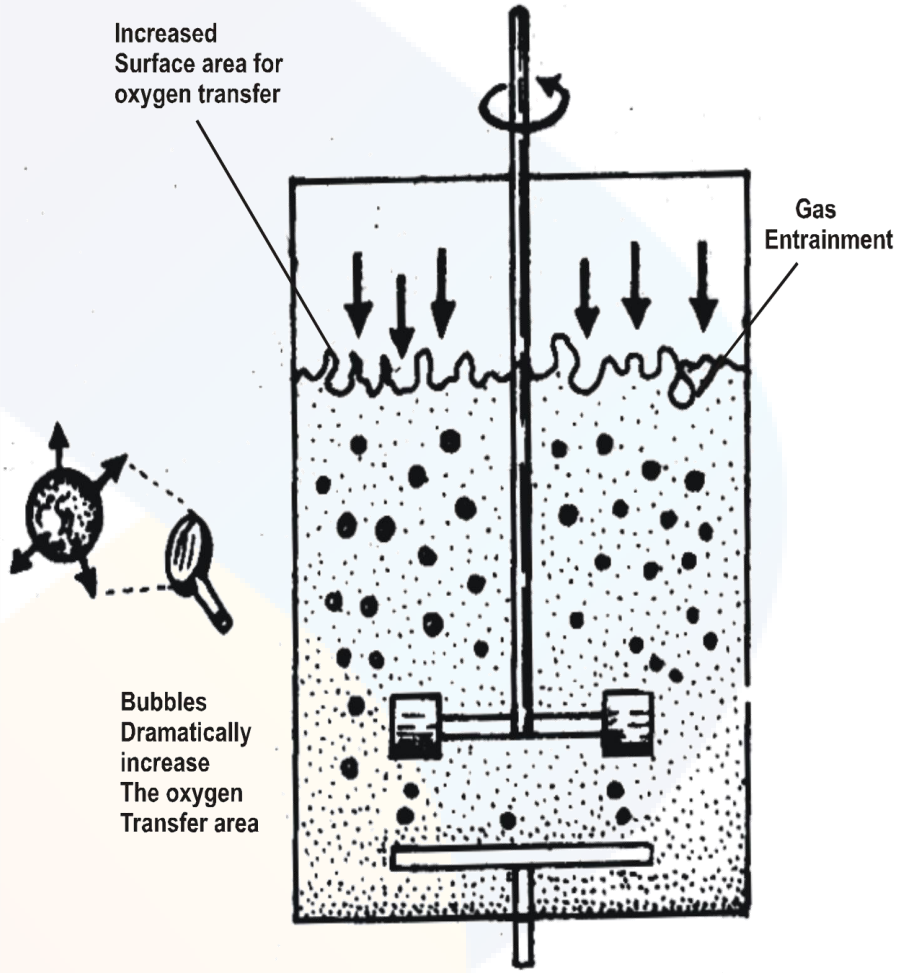


Fig:- Sparged- stirred tank bioreactor through which sterile (free from any germs) air bubbles are sparged.

Downstream Processing

- The down steam processes include separation and purification,
- **RFLP (Restriction Fragment length polymorphism)** : produced by single base alterations in the recognition sequence
- **RFLP are used:**
  (i)To determine relationship between different strains & species.
  (ii) To utilize in DNA Fingerprinting. (AIPMT Pre. 2012)
  (iii) To select desired organisms containing suitable features.
  (iv) To utilize as a marker for the identification of varieties.
- **RAPD (Random Amplified Polymorphic DNA)** : PCR is used in the study of polymorphism of genomic DNA this method is called RAPD.
- **AFLP (Amplified fragement Length polymorphism)** : It uses restriction enzyme digested genomic DNA as template for PCR amplification
- The amplified products are then resolved by denaturing polyacrylamide gel electrophoresis (PAGE)
- **Reporter gene** : Reporter or marker genes present in cells produce a specific phenotype by which they can be easily detected
- Ex: **npt II (Neomycin phosphotransferase II gene).**