Biotechnology

Earlier Definition: Technique of using live organism or their enzymes for products and process useful to humans. ex: making curd, bread, wine etc.

Today's Definition: Processes like in vitro fertilization leading to 'test-tube' baby, synthesizing gene and using it, developing a DNA vaccine or correcting a defective gene.

European Federation of Biotechnology (EFB) Definition: The integration of natural science and organisms, cells, parts thereof, and molecular analogous for products and services".

Principles of Biotechnology

• **Genetic Engineering** –The direct manipulation/alteration of genome (DNA and RNA) of an organism. It involves the transfer of new genes to improve the function or trait into host organisms and thus changes the phenotype of the host organism.

• **Bioprocess engineering:** Maintenance of sterile condition in chemical engineering process to enable growth of only desired microbes for manufacture of biotechnological products like antibiotics, vaccine, enzymes etc.

Why is sexual reproduction consider better than asexual reproduction?

Ans: Asexual Reproduction preserve gene while sexual reproduction leads to variation that leads to evolution & better adaptation of environment. Hence, Sexual Reproduction is better.

• **Traditional hybridization** used in plants and animal breeding leads to inclusion and multiplication of undesirable genes along with the desired traits.

In genetic engineering only desired genes are introduced into the target organism.

• Alien piece of DNA produces multiple copies & inherited only if it gets integrated with recipient DNA/chromosome.

Cloning : Making multiple identical copies of any template DNA.

• **First recombinant rDNA**

- **Stanley** Cohen and **Herbert** Boyer in 1972 isolated the **antibiotic resistance gene** by cutting out a piece of a **plasmid DNA of *Salmonella typhimurium***. It was linked with a plasmid vector and transferred into E.coli. As a result gene was expressed & multiplied in E.coli.
- **Molecular Scissors**– **restriction enzyme** cut the DNA at specific locations.
- **Vector** A **plasmid DNA** (self-replicating circular extra-chromosomal DNA) is used to deliver an alien piece of DNA (desired Gene) into the host organism.
- **Molecular Glue-** enzyme **DNA Ligase** helps to join the DNA fragments.

• The cut piece of DNA (Gene) linked with the **vectors** (plasmid DNA) forms the **recombinant DNA** (autonomously replicating) in vitro.

• Recombinant DNA is transferred into the Host (E.coli).

- Gene Cloning: Replication using the new host DNA polymerase enzyme and make multiple copies.

Recombinant DNA technology" or "**Genetic Engineering**" : The technique of production of new recombinant of genetic material (artificially) in the laboratory & introducing it into the host cells, where they can be propagated and multiplied.

Steps of Recombinant DNA Technology –

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.

Tools of Recombinant DNA Technology includes

- **Restriction Enzymes-** to cut DNA at specific site.
- **Polymerase enzymes**- Enzyme used in amplification/producing multiple copies of DNA
- **Ligases-** Enzyme used to join DNA fragments
- **Vectors-** Self replicating plasmid DNA that transfer the desired gene into the Host
- **Host organisms-** Organism in which desired changes needs to be introduced.

1) **Restriction Enzymes (Molecular Scissors):** larger class of enzymes called **Nucleases** that cuts the DNA from a specific site of restriction sequence.

Discovery: In 1963 two enzymes responsible for restricting the growth of bacteriophage in E-coli were isolated. One enzyme add methyl group another cut DNA (Restriction Endonuclease).

First restriction endonuclease – **Hind II**

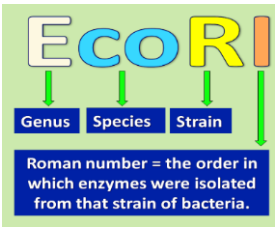
More than 900 restriction enzymes isolated from over 230 strains of bacteria. Types:

Exonucleases. remove nucleotides from the ends of the DNA

Endonucleases: make cuts at specific position within the DNA.

RESTRICTION Enzyme & Sites				
Enzyme	**Source**	**Recognition Sequence**	**Cut Between Nucleotide**	**Cut Type**
EcoR I	Escherichia Coli	5′ GAATTC 3′ 3′ CTTAAG 5′	G A	Sticky Ends
Hind III	Haemophilus influenzae Rd	5′ AAGGTT 3′ 3′ TTCGAA 5′	A A	Sticky Ends
BamH I	Bacillus amyloliquefaciens	5′ GGATTC 3′ 3′ CCTAGG 5′	G G	Sticky Ends
Sal I	Streptomyces albus	5′ GTCGAC 3′ 3′ CAGCTG 5′	G T	Sticky Ends
Pst I	Providencia stuartii	5′ GTCGAC 3′ 3′ CAGCTG 5′	A G	Sticky Ends
Pvu I	Proteus vulgaris	5′ CGATCG 3′ 3′ GCTAGC 5′	T C	Sticky Ends
Taq I	Thermus aquaticus	5′ TCGA 3′ 3′ AGCT 5′	T C	Sticky Ends
Cla I	Caryophanon latum L	5′ ATCGAT 3′ 3′ TAGCTA 5′	T C	Sticky Ends
Alu I	Arthrobacter luteus	5′ AGCT 3′ 3′ TCGA 5′	G C	Blunt ends

Naming of Restriction Enzyme



First Letter: Comes from first letter of Genus name.

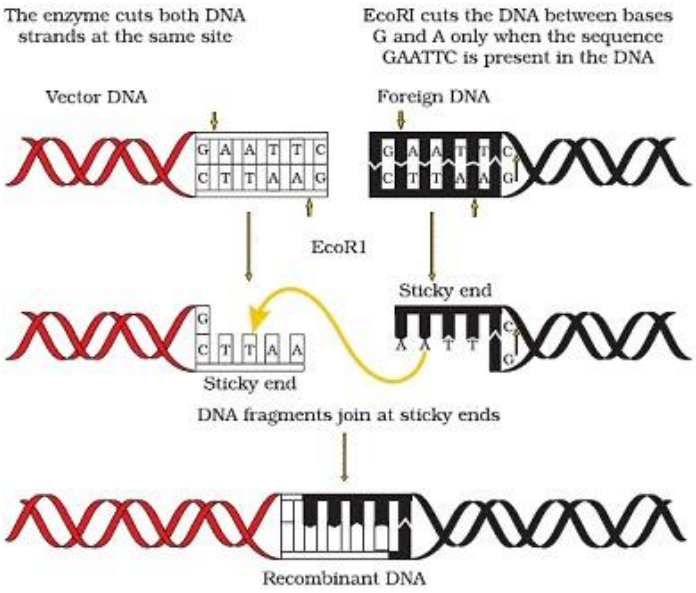
Second Two Letters: Comes from first two letters of Species name.

Third Letter: Indicates Strain

Last number: Order no. at which enzyme was isolated.

Ex: EcoRI comes from E. coli RY 13

Action of Restriction enzyme



Recognition Sequence specific sequence of base pairs from where Restriction Enzyme cut DNA molecules (at a particular point.

• Each restriction endonuclease recognises a specific palindromic nucleotide sequence in the DNA.

Palindromes are group of letters that form the same words when read both forward and backward. Eg: “MALAYALAM”.

Palindromic Sequence: Group of nucleotides reads same both from forward and backward.

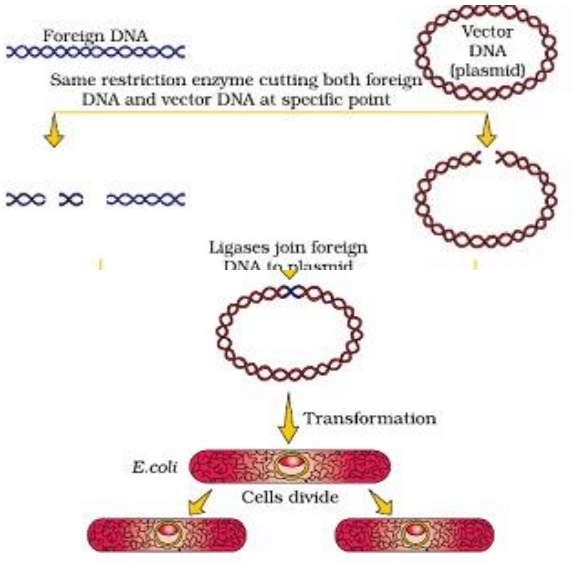
Eg: **5′ — GAATTC — 3′**

3′ — CTTAAG — 5′

Sticky Ends: The hanging ends of DNA double helix at which a few unpaired nucleotides of one strand extend beyond the other.

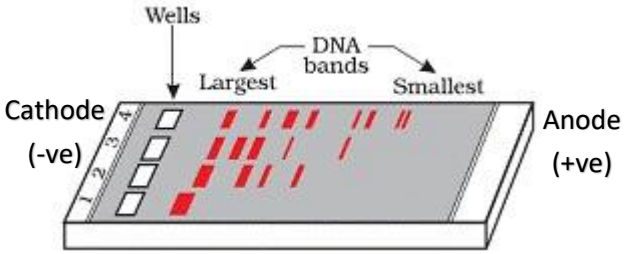
Formed when Restriction enzymes cut the strand of DNA a little away from the centre of the palindrome site between the same two bases on the opposite strands.

When cut the same restriction enzyme the resultant DNA fragments have the same kind of Sticky-ends and can be joined together using DNA ligases forming Hydrogen Bond to their complementary cut counter part.



Diagrammatic representation of Recombinant DNA technology

Separation and isolation of DNA fragments



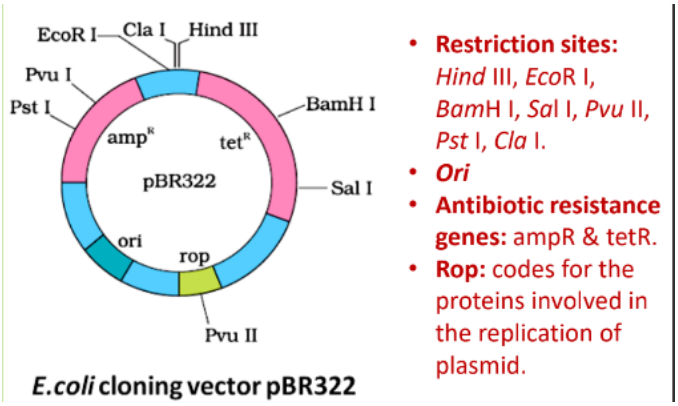
- The fragment of DNA obtained by cutting DNA using restriction enzyme is separated by technique called **Gel Electrophoresis**.
- The well contains the sample of DNA. Lane 1 consist of undigested DNA & lane 2-4 consist of digested DNA Fragments.
- Negatively charged DNA fragments move towards the anode (+ve) under an electric field through medium.
- DNA fragments separate according to their size through sieving effect provided by agarose gel (natural polymer obtained from sea weed). Bigger fragments moves slow & smaller moves faster.
- The separated DNA fragment can be visualized after staining the DNA with ethidium bromide followed by exposure to UV light. The fragments appears to be of Bright Orange colour.
- **Elution:** Separated bands of DNA are separated and extracted from agarose gel.
- The DNA fragment purified this way is used for recombination.

2) Cloning Vector

- Plasmids and Bacteriophages is commonly used vector for cloning.
- They have 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.

	pBR322		pSC101
p	Denotes Plasmid	P	Plasmid
BR	Name of scientists B- Bolivar, R- Rodriguez	SC	S- Stanley C-Cohen
322	Signifies its number of formation in laboratory	101	Numerical designation

Following features are required to facilitate cloning into a vector-



E.coli cloning vector pBR322

- **Restriction sites:** *Hind* III, *Eco*R I, *Bam*H I, *Sal* I, *Pvu* II, *Pst* I, *Cla* I.
- ***Ori***
- **Antibiotic resistance genes:** ampR & tetR.
- **Rop:** codes for the proteins involved in the replication of plasmid.

a. **Origin of replication (ori)** – Vector must have ORI.

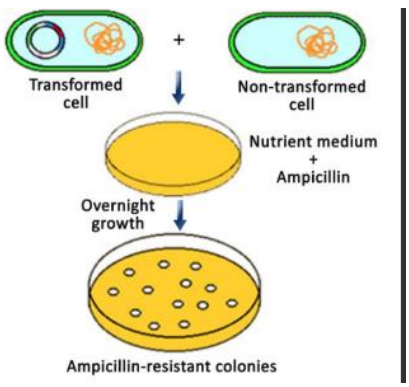
- The Specific sequence from where replication starts. (Generally contains sequence that have double bond between of A & T)
- Any piece of DNA when linked to this sequence can be made to replicate within the host cells.
- This sequence is responsible for controlling the copy number of the linked DNA.
- To get many copies of target DNA, it should be cloned in a vector whose origin support high no. of copies.

b. **Selectable marker**-

- It is the gene that help in the identifying & selecting transformants and eliminating non transformants.
- **Transformation** is a procedure through which a piece of DNA is introduced in a host bacterium. Such bacterium is called **transformants.**
- If transformation does not take place, it is **non-transformant**.
- Selectable markers for E. coli: The genes encoding resistance to antibiotics such as **ampicillin, chloramphenicol, tetracycline or kanamycin,** etc.
- Normal E.coli have no resistance against antibodies.

c. Cloning Sites

- To link the alien DNA, the vector needs a single or very few recognition sites for Restriction enzymes.
- More than one recognition sites generate several fragments. It complicates the gene cloning.
- Ligation of foreign DNA is carried out at a restriction site present in one of the two **antibiotic resistance genes**.



Insertional inactivation of antibiotic resistance gene

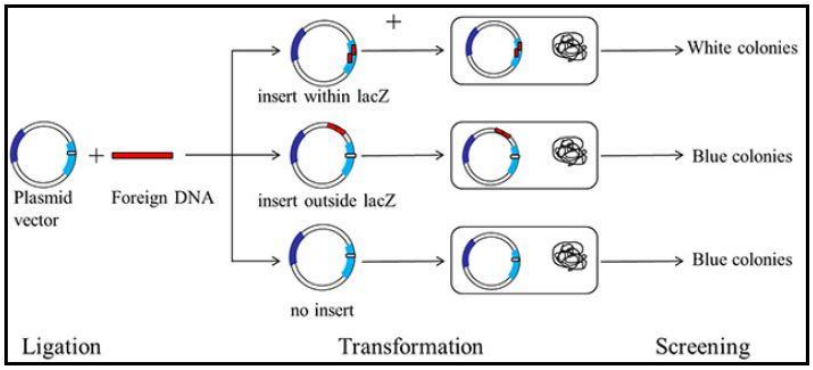
- Eg: Ligation of alien DNA at BamH I site of tetracycline resistant gene (tet^R) deactivates the gene tet^R in vector pBR322 forming Recombinant Plasmid that lose tetracycline resistance due to insertion of foreign DNA.
- E coli do not contain any antibiotic resistance gene. Hence, can not grow on culture medium containing any antibiotic. (Antibiotic kills E coli bacteria)

Cultural medium containing			
	Both Antibiotics	**Tetracycline Antibiotic**	**Ampicillin Antibiotic**
Non-Transformant	Do not grow	Do not grow	Do not grow
Transformant with Non-Recombinant DNA	Grows	Grow	Grow
Transformant with Recombinant DNA	Do not grow	Do not grow	Grow

- Transformant Host (E coli in which vector plasmid is inserted) containing both type recombinant & non-recombinant can be separated by plating the transformants on ampicillin containing medium.
- Transformant with recombinant will only grow on cultural medium containing only ampicillin antibiotic. Since, tet^R gene gets inactivated, hence E-coli cannot grow on medium containing tetracycline antibiotic.
- Transformant with non-recombinants will grow on the medium containing both the antibiotics (ampicillin & tetracycline). Since, both the gene amp^R & tet^R are functional.

Insertional inactivation : Alternative selectable marker that differentiates recombinants from non-recombinants on the basis of colour in presence of chromogenic substrate.

Desired DNA fragment inserted into the vectors gene disrupts the coding sequence of a gene and inactivate the gene. Eg:



- A recombinant DNA is inserted within lac Z gene that codes for enzyme β-galactosidase hence inactivate the lac Z gene present on the vector.
- The lac Z gene encodes the enzyme beta-galactosidase (cleaves the disaccharide lactose to produce galactose and glucose**)** which can cleave a chromogenic substrate into a blue coloured product.
- If this lac Z gene is inactivated by insertion of a target DNA fragment into it, the development of the blue colour will be prevented and it gives white coloured colonies.
- By this way, we can differentiate recombinant (white colour) and non-recombinant (blue colour) colonies.

d. Vectors for cloning genes:

In plants– *Agrobacterium tumefaction* (pathogen of dicot plant) is able to deliver a piece of DNA known as Transferred DNA (T-DNA). The tumor inducing (Ti) Plasmid transform normal plant cells into a tumor and direct these tumor cells to produce the chemicals required by the pathogen.

In animals :Retroviruses in animals have the ability to transform normal cells into cancerous cells. The tumor inducing cells containing cloning vector of Retro virus are no more pathogenic to animal. They direct these tumor cells to produce the chemicals required by the pathogen.

Competent host (For Transformation with **Recombinant DNA**)

DNA is a hydrophilic molecule & can not pass through the cell membrane of host.

i) Indirect Method: The desired gene is transferred into disarmed pathogen/vector like plasmid & viruses. The vector is allowed to infect the host cell & provides multiple copies by cloning of rDNA.

Natural Genetic Engineering (Disarmed Pathogen)

In Plants : Agrobacterium tumefaciens in host plant (any dicot plant like tomato, potato, tobacco etc) it produces crown gall (plant tumours)

In Animals : Retrovirus

ii) Direct Method or Vector less Method

a) Electroporation: Temporary holes are produced in the plasma membrane of host (bacteria) cell to facilitate entry of foreign DNA by treating host cell with divalent cations like $CaCl_2$ (calcium ions) which increases the efficiency of host cells (through cell wall pores) to take up the rDNA plasmids.

Heat Shock Treatment: rDNA can also be transformed into host cell by incubating both 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 rDNA is directly injected into the nucleus of plant/animal cells by using a glass micropipette.

c) Biolistics / Gene gun: Microscopic particles of gold / tungsten are coated with the rDNA (Gene of interest) are bombarded onto host cells.

Processes of Recombinant DNA Technology involves several steps in specific sequence-

1) Isolation of Genetic material: Genetic material is enclosed within cell membrane along with macromolecules like RNA, Protein, Polysaccharides & Lipids. So, to isolated DNA following enzymes are used :

Lysozyme -Bacteria Cell **Cellulase** -Plant cells **Chitinase** -Fungus

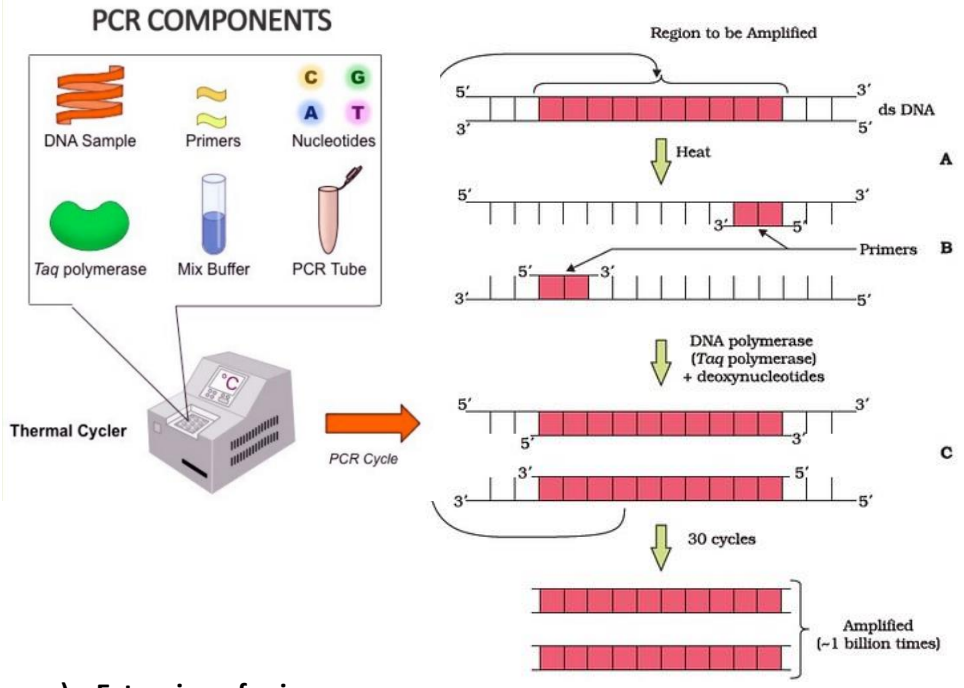
These genes on DNA are interwined with Histone protein.

RNA removed by **Ribonuclease** , Protein by **Protease &** other molecules by appropriate treatment. Purify DNA ultimately precipitate after addition of chilled ethanol that can be separate out by **spooling**. (collection of fine threads from suspension on glass rod)

2) Fragmentation of DNA by restriction endonucleases -Cutting of DNA at specific location is performed by using restriction enzyme and Agarose gel electrophoresis (to check the progression of a restriction enzyme digestion).

- **Isolation of a desired DNA fragment** by gel electrophoresis-After cutting fragments by same restriction enzyme.

3) Amplification of Gene of Interest using PCR (Polymerase Chain Reaction) to get multiple copies of the DNA or gene of interest in vitro by using set of primers and enzyme DNA polymerase.



a) Denaturation

DNA fragments are mixed with two sets of **primers** (small chemically synthesised oligonucleotides that are complimentary to the region of DNA) and then this mixture is heated to 95°C – 98°C and double stranded DNA fragments are dissociated into single strand.

b) Annealing of Primer

The solution is allowed to cool down at 50 °C to 60 °C.

The complimentary primers (Nucleotides) added gets attached to the Base pairs making it double strand and leaving rest of DNA single stranded.

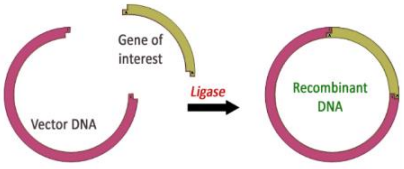
c) Extension of primers

- A Heat stable enzyme DNA/**Taq Polymerase** *(remain active during high temperature)* isolated from bacteria ***Thermus aquaticus*** is added with a large supply of all four nucleotides at approximately 70 °C.
- DNA polymerase using primer copies gene template and add complementary nucleotides to the extending strand in 5' to 3' direction to make both the strands double stranded.

Multiple Copies or Amplification : Steps a to c are repeated again and again to get multiple copies.

- In each cycle product gets double. One cycle takes approximately 1-2 mins.
- In 30 mins approximately a billion copies of desired DNA are produced.

Ligation of the DNA fragment into vector for further cloning- The Gene of Interest & cut Vector DNA are mixed and Ligase is added to form Recombinant DNA. **Vector DNA with Gene of interest** is obtain.



4) Insertion of Recombinant DNA into the Host Cell/Organism –Several methods to introduce ligated DNA into Host cell.

- Makes the recipient cells competent to receive DNA present in its surrounding etc.
- The recombinant DNA (bearing gene for resistance to an antibiotic eg- ampicillin resistant gene) is transferred into Host (like E.coli) cells, the host cell become transformed.

- Transformed will grown on cultural medium (agar) containing antibody (eg- ampicillin) could be identify with the help of selectable markers (eg-ampicillin resistant gene).

5) Obtaining the foreign gene product –

- The foreign DNA multiplies in plant or animal cell to produce desirable protein.
- Expression of foreign genes in host cells involve, optimized condition to obtain **recombinant protein** (protein encoding gene expressed in heterologous host).
- Cells harbouring cloned genes of interest may be grown on a small scale in the laboratory & in large scale in bioreactors. So that, cultures may be used for extracting the desired protein.

Bioreactors/fermenters

- These are the vessels in which raw material are biologically converted into higher yield of desired/specific products like protein, enzymes etc using microbial, plant, animal or human cells.
- Produce multiple copies of desired gene product in continuous cultural system in which used medium is drained out from one side while fresh medium is added from the other to maintain the cells in their physiological active log/exponential phase.
- These Vessels are of 100-1000 Liters capacity use to produce large quantity.
- Provide optimal growth condition (Temp, pH, Substrate, salts, vitamins, oxygen etc) to the desired product.
- A bio reactor have
  - An Agitator
  - An oxygen delivery system
  - Temp control system
  - pH control system
  - Sampling ports- withdraw the sample for testing
- A stirred tank reactor is usually cylindrical with curve base to facilitate mixing oxygen availability. It breaks the foam on the surface.

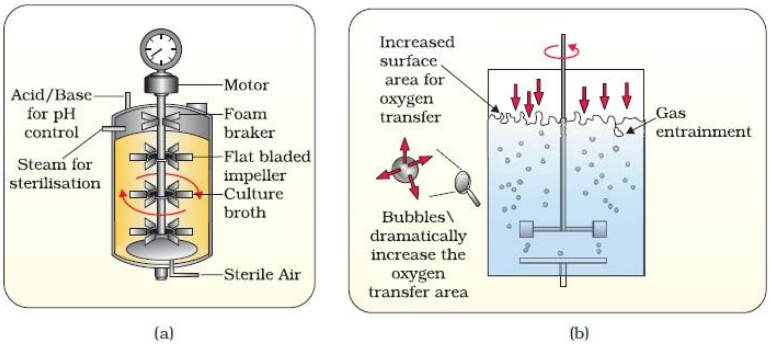


Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

- The Sparged stirred tank has sterile air bubbles sparged inside to increase oxygen transfer area.

• **Downstream Processing** The processes that make the product obtain ready for marketing.
Process of separation and purification after biosynthesis.
Adding suitable preservatives to it and send for clinical trial & strict quality control testing in case of drugs before releasing to market for public use.