[TOPIC 1] Principles of Biotechnology and Tools of Recombination DNA Technology

1.1 Biotechnology

Biotechnology can be defined as the use of microorganisms, plants or animal cells or their components to produce products and processes useful to humans. According to the **European Federation of Biotechnology** (EFB), biotechnology is the integration of natural science and organisms, cells, parts thereof and molecular analogues for products and services. The term 'Biotechnology' was coined by **Karl Ereky** in 1919.

Principle of Biotechnology

Principles of biotechnology are based on the concept of the following techniques:

(*i*) **Genetic engineering** is the technique to alter the chemistry of genetic material (DNA/RNA), to introduce these into another organisms to change the phenotype of the host organism.

(*ii*) **Sterlisation techniques** Adequate maintenance of sterile conditions to support growth of only the desired microbes/eukaryotic cells in large quantities for the manufacture of biotechnological products like antibiotics, vaccines, enzymes, etc.

Techniques of Genetic Engineering

The techniques of genetic engineering include the following:

(*i*) Creation of **recombinant DNA** by combining desired genes.

(*ii*) Gene transfer.

(*iii*) Maintenance of DNA in host and gene cloning.

The basic steps in genetic engineering can be summarised as:

(*i*) Identification of DNA with desirable genes.

(*ii*) Introduction of the identified DNA into a suitable host to form recombinant DNA (*r*DNA).

(*iii*) Maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

At last, recombinant protein is obtained from host through a process known as **Down Stream Processing** (DSP).

Construction of First Artificial Recombinant DNA

(*i*) It was achieved by linking a gene encoding antibiotic resistance with a native **plasmid** (an autonomously replicating circular extrachromosomal DNA) of *Salmonella typhimurium*.

(*ii*) **Stanley Cohen** and **Herbert Boyer** accomplished this in 1972. They isolated the antibiotic resistance gene by cutting out a piece of DNA from a plasmid of *typhimurium*.

(*iii*) The cutting of DNA at specific locations was carried out by **molecular scissors**, i.e. **restriction enzymes**.

(*iv*) The cut piece of DNA was then linked to the plasmid DNA with the enzyme DNA **ligase**. The plasmid DNA acts as **vector** to transfer the piece of DNA attached to it.

(*v*) When this DNA is transferred into *E. coli*, it could replicate using the new host's DNA polymerase enzyme and make multiple copies.

(*vi*) This ability to multiply copies of antibiotic resistance gene in *E. coli* was called **cloning** of antibiotic resistance gene in *E. coli*.

Key Tools of Genetic Engineering

Genetic engineering or recombinant DNA technology can be accomplished only with the usage of key tools like

(*i*) Restriction enzymes

(*ii*) Cloning vectors

(*iii*) Competent host organism

Restriction Enzymes

Restriction enzymes or 'molecular scissors' are used for cutting DNA.

(*i*) Two enzymes from *E. coli* that were responsible for restricting the growth of bacteriophage were isolated in 1963, one of them added methyl group to DNA and the other cut DNA into segments. The later was called **restriction endonuclease**.

(*ii*) The first restriction endonuclease *Hin*dII was isolated by **Smith Wilcox** and **Kelley** (1968). They found that it always cut DNA molecules at a particular point by recognising a specific sequence of six base pairs known as **recognition sequence**.

(*iii*) Besides *Hin*dII, more than 900 restriction enzymes have been isolated now, from over 230 strains of bacteria, each of which recognises different **recognition sequences**.

(*iv*) **Mechanism of Action of Restriction Enzymes**

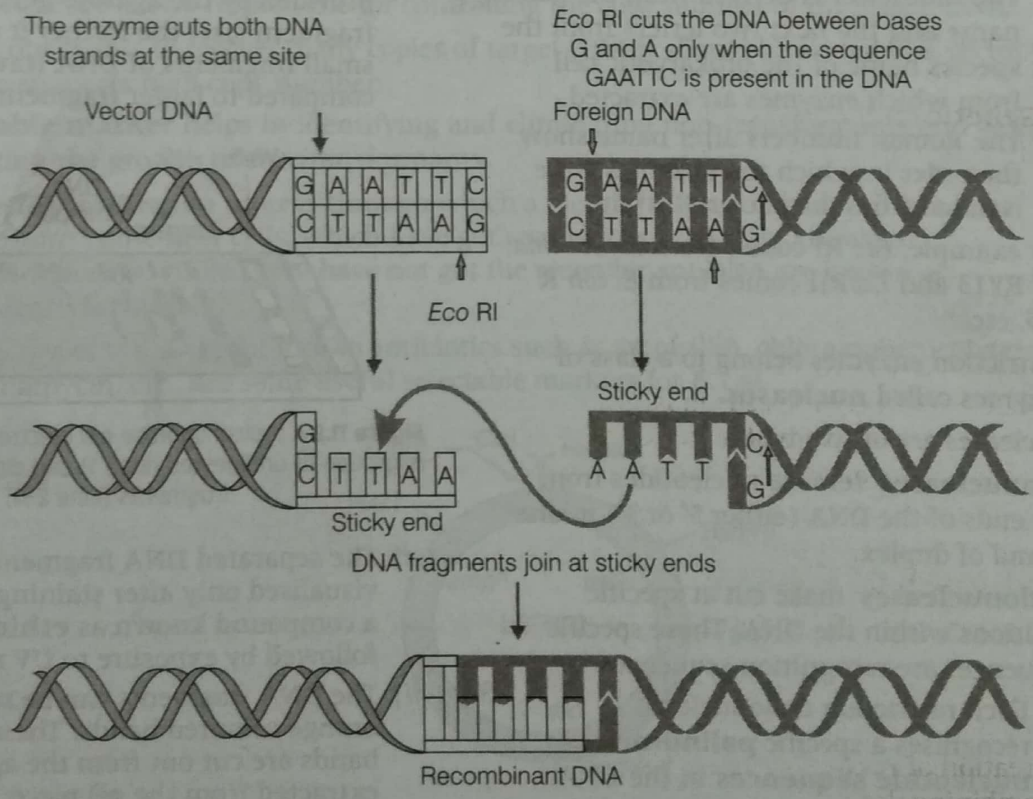

Figure 11.1 Steps in formation of recombinant DNA by the action of restriction endonuclease enzyme-*Eco*RI

(a) 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.

(b) This leaves single-stranded portions at the ends.

(c) There are overhanging stretches called **sticky ends** on each strands as given in above figure. These are named so, because they form hydrogen bonds with their complementary cut counterparts.

(d) The stickiness of the ends facilitates the action of the enzyme DNA ligase.

(e) Restriction endonucleases are used in genetic engineering to form recombinant molecules of DNA, which are composed of DNA from different sources/genomes.

(f) These sticky ends are complementary to each other when cut by same restriction enzyme, therefore can be joined together (end-to-end) using DNA ligases.

(*v*) **Naming of Restriction Enzymes**

(a) The first letter is derived from the genus name and the next two letters from the species name of the prokaryotic cell from which enzymes are extracted.

(b) The Roman numbers after name show the order in which the enzymes were isolated from the bacterial strain.

For example, *Eco* RI comes from *Escherichia coli* RY13 and *Eco*RII comes from *E. coli* R 245, etc.

(*vi*) Restriction enzymes belong to a class of enzymes called **nucleases**.

Nucleases are of two types:

Exonucleases remove nucleotides from the ends of the DNA (either 5′ or 3′) in one strand of duplex.

Endonucleases make cut at specific positions within the DNA. These specific sequences are recognition sequences.

(a) Each restriction endonuclease recognises a specific **palindromic nucleotide sequences** in the DNA.

(b) **Palindrome** in DNA is a sequence of base pairs that reads same on the two strands when orientation of reading is kept same.

For example, the following sequences read the same on the two strands in 5′ → 3′ direction as well as 3′ → 5′ direction.

5′— GAATTC — 3′

3′ — CTTAAG — 5′

Separation and Isolation of DNA Fragments

(*i*) The cutting of DNA by restriction endonucleases results in the fragments of DNA.

(*ii*) The technique, which separates DNA fragments based on their size is called **gel electrophoresis.**

(*iii*) 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.

(*iv*) The most common medium used is **agarose**, a natural polymer extracted from sea weeds.

(*v*) The DNA fragments separate (resolve) according to their size through sieving effect provided by the agarose gel. The smaller the fragment size, the farther it moves. So, small fragments of DNA travel more as compared to larger fragments.

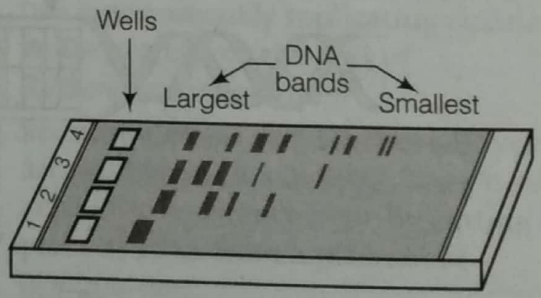

Figure 11.2 A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested set of DNA fragments (lane 2-4)

(*vi*) The separated DNA fragments can be visualised only after staining the DNA with a compound known as **ethidium bromide** followed by exposure to UV radiation.

(*vii*) The DNA fragments can be seen as bright orange coloured bands. These separated bands are cut out from the agarose gel and extracted from the gel piece. This is called **elution**.

(*viii*) The purified DNA fragments can be used in constructing recombinant DNA by joining them with cloning vectors.

Cloning Vectors

Cloning vectors are the DNA molecules that can carry a foreign DNA segment and replicate inside the host cell is called as a **vector** the host cell.

(*i*) The vectors used in recombinant DNA technology can be:

(a) **Plasmids** Autonomously replicating circular, extrachromosomal DNA.

(b) **Bacteriophages** Viruses infecting bacteria.

(c) **Cosmids** Hybrid vectors derived from plasmids which contain *cos* site of λ phage.

(*ii*) **Copy number** can be defined as the number of copies of vectors present in a cell. So, in R-DNA technique those vectors are selected which have high copy number.

(*iii*) **Bacteriophages** have high number per cell, so their copy number is also high in genome.

(*iv*) **Plasmids** have only one or two copies per cell.

(*v*) Copy number can vary from 1-100 or more than 100 copies per cell.

(*vi*) If an alien piece of DNA is linked with bacteriophage or plasmid DNA, its number can be multiplied equal to the copy number of the plasmid or bacteriophage.

(*vii*) **Features Required to Facilitate Cloning into Vector**

(a) **Origin of replication** (*Ori*) is a sequence from where replication starts.

- Any piece of DNA when linked to this sequence can be made to replicate within the host cells.
- The sequence is also responsible for controlling the copy number of the linked DNA.
- So, if one wants to recover many copies of target DNA it should be cloned in a vector whose origin supports high copy number.

(b) **Selectable marker** helps in identifying and eliminating non-transformants and selectively permitting the growth of the transformants.

- **Transformation** is a process through which a piece of DNA is introduced in a host bacterium. Those host cells, which have got recombinant DNA are known as **transformants**, while those have not got the recombinant DNA are known as **non-transformants**.
- The genes encoding resistance to antibiotics such as ampicillin, chloramphenicol, tetracycline or kanamycin, etc., are some useful selectable markers for *E. coli*.

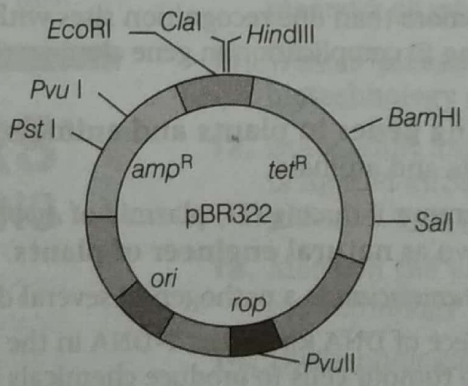

Figure 11.3 *E. coli* cloning vector pBR322 showing restriction sites (*Hin*dIII, *Eco*RI *Bam* HI, *Sal* I, *Pvu* II, *Pst* I, *Cla* I), *ori* and antibiotic resistance genes (*amp*^{R} and *tet*^{R}). *rop* codes for the proteins involved in the replication of the plasmid.

- **Ligation of alien DNA** is carried out at a restriction site present in one of the two antibiotic resistance genes. Example, ligating 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. But, it still can be selected out from non-recombinant ones by plating the transformants on ampicillin containing medium.
  - The transformants 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. Because in recombinants, tetracycline antibiotic resistance gene is disturbed.
  - The non-recombinants will grow on the medium containing both the antibiotics. Because both antibiotic resistance genes are functional.
  - In this example, one antibiotic resistance gene helps in selecting the transformants whereas, the other antibiotic resistance gene gets 'inactivated due to insertion' of alien DNA and helps in selection of recombinants.
- **Selection of recombinants** due to inactivation of antibiotics is a cumbersome procedure, because it requires simultaneous plating on two plates having different antibiotics. So, alternative selectable markers are 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 method, a recombinant DNA is inserted within the coding sequence of an enzyme β-galactosidase. So, if gene of interest gets incorporated, β-galactosidase gene will be disturbed.
  - This results in inactivation of the enzyme β-galactosidase (insertional inactivation) and it is not produced anymore.
  - The bacterial colonies whose plasmids do not have an insert, produce blue colour, because β-galactosidase enzyme produce a colour, but others do not produce any colour, when grown on a chromogenic substrate, because here β-galactosidase enzyme is not produced. This happens because of inactivation of gene producing this enzyme, which is referred to as insertional inactivation.

(c) **Cloning sites** are required to link the alien DNA with the vector.
- The vector requires very few or single recognition sites for the commonly used restriction enzymes.
- The presence of more than one recognition sites within the vector will generate several fragments leading to complication in gene cloning. So one recognition site for an enzyme is preferred.

(d) **Vectors for cloning genes in plants and animals** are many which are used to clone genes of interest in plants and animals.
- In plants, the Tumour inducing (Ti) plasmid of *Agrobacterium tumefaciens* is used as a cloning vector. It is known as **natural engineer of plants**.
  - *Agrobacterium tumefaciens* is a pathogen of several dicot plants.
  - It delivers a piece of DNA known as T-DNA in the Ti plasmid which transforms normal plant cells into tumour cells to produce chemicals required by pathogens. The tumour inducing plasmid of this bacteria has now been modified into a cloning vector which is no more pathogenic to the plants.
- Retrovirus, adenovirus, papillomavirus are also now used as cloning vectors in animals because of their ability to transform normal cells into cancerous cells.

Competent Host Organism (For transformation with recombinant DNA)

Competent host organism is required because DNA being a hydrophilic molecule, cannot pass through cell membranes. Hence, the bacteria should be made **competent** to accept the DNA molecules.

(*i*) **Competency** is the ability of a cell to take up foreign DNA.

(*ii*) Methods to make a cell competent are as follows:

(a) **Chemical method** In this method, the cell is treated with a specific concentration of a divalent cation such as calcium to increase pore size in cell wall.

- The cells are then incubated with recombinant DNA on ice, followed by placing them briefly at 42°C and then putting it back on ice. This is called **heat shock treatment**. Because of this permeability of bacterial cell increases.
- This enables the bacteria to take up the recombinant DNA.

(b) **Physical methods** In this method, a recombinant DNA is directly injected into the nucleus of an animal cell by **microinjection** method.

- In plants, cells are bombarded with high velocity microparticles of gold or tungsten coated with DNA called as **biolistics** or **gene gun method**.

(c) **Disarmed pathogen vectors** when allowed to infect the cell, transfer the recombinant DNA into the host.

[TOPIC 2] Processes of Recombinant DNA Technology

2.1 Recombinant DNA Technology

Recombinant DNA technology involves the following steps in sequence:

(*i*) Isolation of the genetic material (DNA) is carried out in the following steps:

(a) DNA is enclosed within the membranes. To release DNA along with other macromolecules such as RNA, proteins, polysaccharides and lipids, bacterial cells/plant or animal tissue are treated with enzymes such as **lysozyme** (bacteria), **cellulase** (plant cells), **chitinase** (fungus).

(b) RNA can be removed by treatment with **ribonuclease** whereas proteins can be removed by treatment with **protease**.

(c) Other molecules can be removed by appropriate treatments and ultimately purified DNA precipitates out after the addition of chilled ethanol. This can be seen as collection of fine threads in the suspension which can be removed by spooling.

(*ii*) **Cutting of DNA at specific locations** is done by using restriction enzymes. The purified DNA molecules is incubated with the specific restriction enzyme at optimum conditions for the enzyme to act.

(*iii*) **Isolation of desired DNA fragment** is carried out using agarose gel electrophoresis (as discussed earlier).

(*iv*) **Amplification of gene of interest using Polymerase Chain Reaction** (PCR) is a reaction in which multiple copies of specific DNA (gene of interest) sequence are made (amplification) *in vitro*. The technique was developed by **Kary Mullis** in 1985 who received Nobel Prize for chemistry in 1993.

(a) PCR technique requires

- A **DNA template**, which is a double-stranded DNA that needs to be amplified.
- **Primers** are small chemically made synthesised oligonucleotides of about 10-18 nucleotides that are complementary to a region of template DNA.
- **Enzymes** used are DNA *Taq* polymerase (from a bacterium, *Thermus aquaticus*).
- Deoxyribonucleotides for synthesis of new strand.

(b) Steps in PCR

- **Denaturation** of double-stranded DNA is carried out by applying high temperature of 95°C for 15 seconds. Each separated single-strand acts as a template for DNA synthesis.

- **Annealing** is carried out by two sets of primers, which are added in the reaction. They anneal to the 3′ end of each separated strand. Primers act as initiator of replication.
- **Extension** is done by DNA polymerase by adding nucleotides complementary to the template in the reaction.
- **A thermostable** DNA polymerase (*Taq* polymerase) is used in the reaction, which can tolerate the high temperature of the reaction.

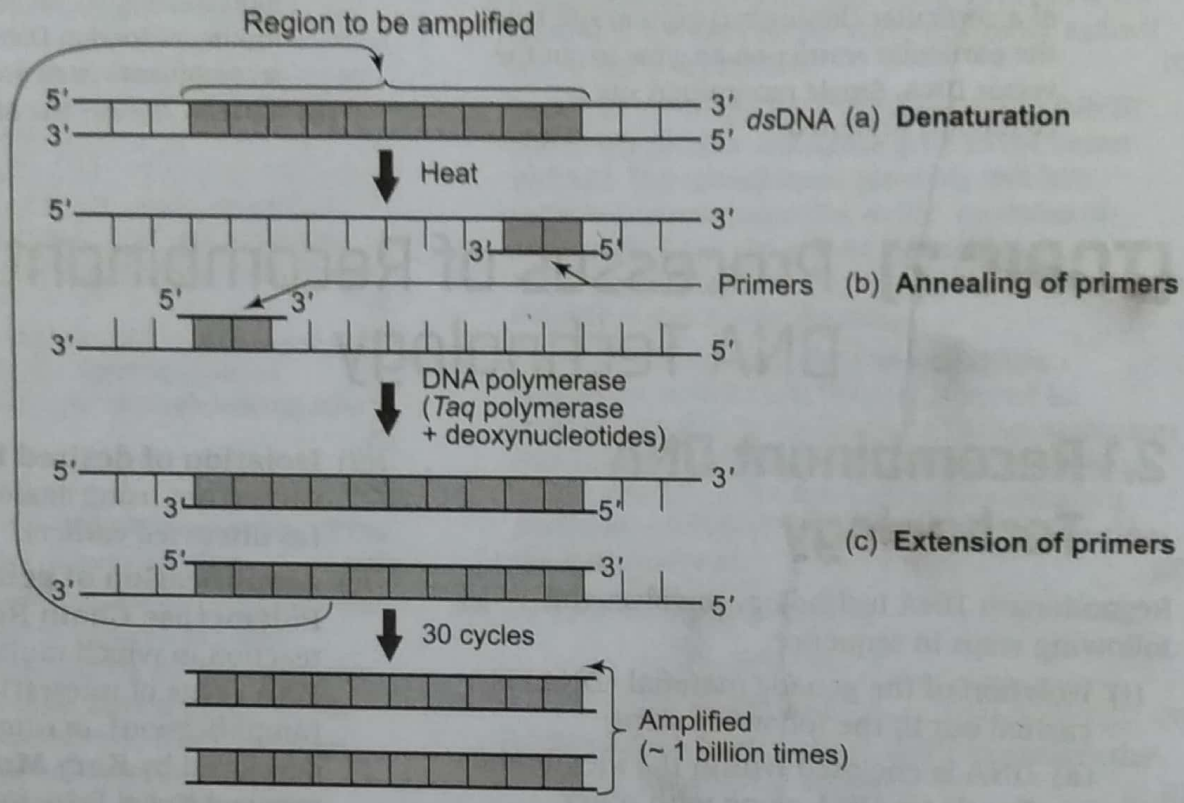

Figure 11.4 Polymerase Chain Reaction (PCR) Each cycle has three steps (a) Denaturation (b) Primer annealing and (c) Extension of primers

(c) These steps are repeated many times to obtain several copies of the desired DNA.

(*v*) **Ligation of DNA fragment into a vector** requires a vector DNA and source DNA.

(a) These are cut with the same endonuclease to obtain sticky ends.

(b) Both are then ligated by mixing vector DNA, gene of interest and enzyme DNA ligase to form recombinant DNA.

(*vi*) **Insertion of recombinant DNA into the host cell/organism** occurs by several methods, before which the recipient cells are made competent to receive the DNA.

(a) If recombinant DNA carrying antibiotic resistance gene (e.g. ampicillin), is transferred into *E. coli* cells, the host cell is transformed into ampicillin resistant cells.

(b) The ampicillin resistant gene can be called a **selectable marker**.

(c) When transformed cells are grown on agar plates containing ampicillin, only transformants will grow and others will die.

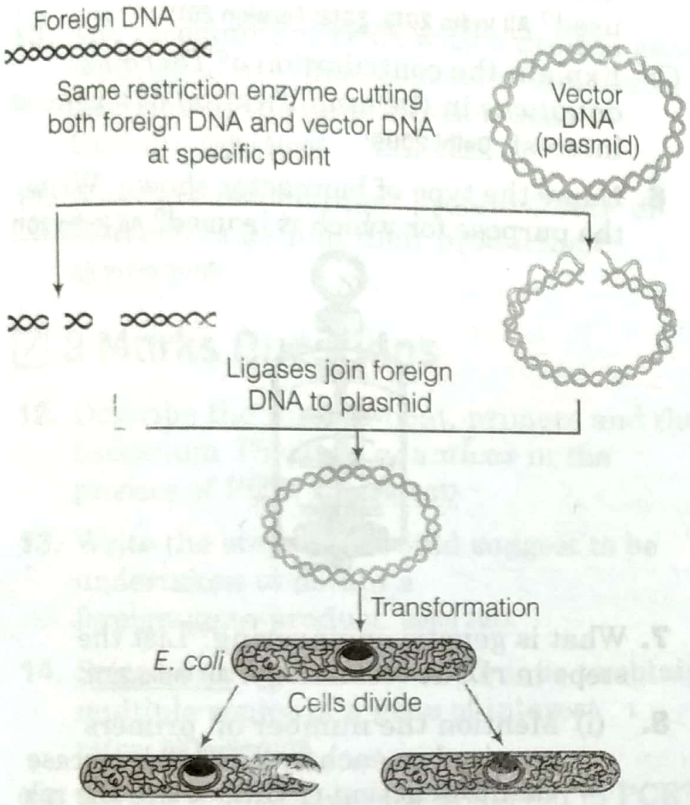

Figure 11.5 Diagrammatic representation of recombinant DNA technology

(*vii*) **Culturing the host cells** The cell containing the foreign gene is cultured on an appropriate medium at optimal conditions. The DNA gets multiplied.

(*viii*) Extraction of desired gene product is carried out in the following steps:

(a) A protein encoding gene expressed in a heterologous host is called **recombinant protein.**

(b) Cells having genes of interest can be grown on a small or on a large scale.

(c) In small scale, cells are grown on cultures and then expressed protein is extracted and purified by various separation methods.

(d) On large scale, cells are grown in a continuous culture system in which fresh medium is added from one side to maintain cells growth phase and t desired protein is collected from th other side.

2.2 Bioreactors

Bioreactors are the large volume (100-1000 L) vessels in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial plant, animal or human cells.

(*i*) It provides the optimal conditions for achieving the desired product by providing growth conditions like temperature, pH, substrate, salt, vitamins and oxygen.

(*ii*) The most commonly used bioreactors are of stirring type:

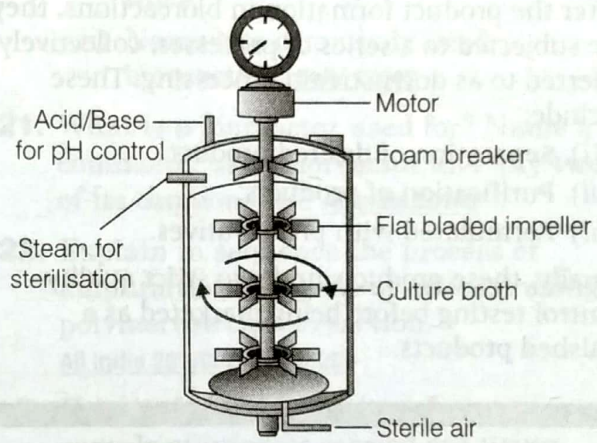

Figure 11.6 Simple stirred-tank bioreactor

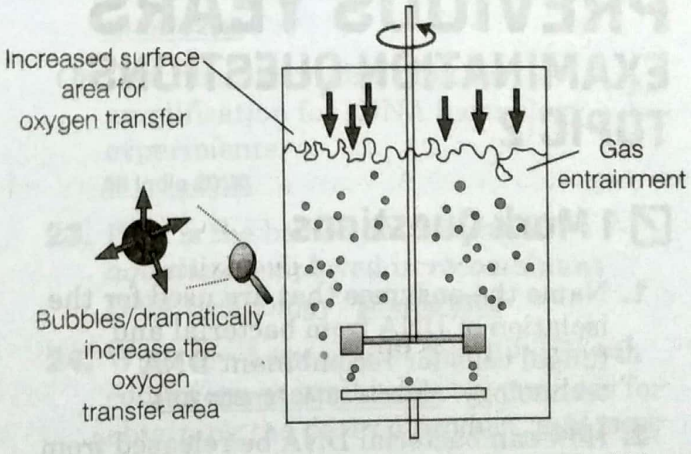

Figure 11.7 Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

(*iii*) The stirred-tank reactor is usually cylindrical or with a curved base to facilitate the mixing of the reactor contents.

(*iv*) The stirrer facilitates mixing and oxygen availability throughout the bioreactor.

(*v*) In the sparged stirred-tank bioreactor, sterile air bubbles are sparged to increase the surface area for oxygen transfer.

(*vi*) The components of a bioreactor are:

(a) An agitator system

(b) An oxygen delivery system

(c) A foam control system

(d) A temperature control system

(e) pH control system

(f) Sampling ports to withdraw culture periodically.

Downstream Processing

After the product formation in bioreactions, they are subjected to a series of processes, collectively referred to as downstream processing. These include:

(*i*) Separation of desired product.

(*ii*) Purification of products.

(*iii*) Formulated with preservatives.

Finally, these products undergo strict quality control testing before being marketed as a finished products.