UNIT 4

Biotechnology and Its Applications

BIOTECHNOLOGY : PRINCIPLES AND PROCESSES

- The term **biotechnology** is derived from a fusion of two words biology and technology. It is concerned with the exploitation of biological agents or their components for generating **useful products/services**.
- Biotechnology use living organisms in systems or processes for the manufacture of useful products; it may involve algae, bacteria, fungi, yeast, cells of higher plants and animals.
- **The European Federation of Biotechnology (EFB)** has given a definition of biotechnology as "The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services."
- **Research areas of biotechnology** – Following are three research areas of biotechnology:
  - Providing the best catalyst in the form of **improved organism**; generally a microbe or pure enzyme.
  - Creating optimal conditions through engineering for a catalyst to act, and
  - **Downstream processing** technologies to purify the protein/organic compound.

PRINCIPLES OF BIOTECHNOLOGY

- Among many, the two core techniques that enabled birth of modern biotechnology are :
  - **Genetic engineering :** 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.
  - **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.
- Genetic engineering was started by **Paul Berg** (1972), who introduced a gene of **SV-40** into a bacterium with the help of **lambda phage**. Berg was awarded **Nobel Prize** in 1980 and considered as **"father of genetic engineering".**
- As compared to traditional hybridisation method, the technique of genetic engineering is preferred. It permits to isolate only the **desirable genes** and introduce it into the target organism. This new technique excludes the introduction of **undesirable genes** into the target organisms.
- Genetic engineering is alternately called **recombinant DNA technology** or **gene cloning**. The first recombinant DNA was constructed by **Stanley Cohen** and **Herbert Boyer** in 1972. They cut the piece of DNA from a plasmid carrying antibiotic-resistance gene in the bacterium *Salmonella typhimurium*. 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 of *Escherichia coli*.

TOOLS OF RECOMBINANT DNA TECHNOLOGY

- Three types of biological tools are used in the synthesis of recombinant DNA:
  - Enzymes
  - Cloning vectors (vehicle DNA)
  - Competent host (for transformation with recombinant DNA).

Enzymes

- Different kinds of specific enzymes are used in genetic engineering (recombinant DNA technology). These include lysing enzymes, restriction (cleaving) enzymes, synthesizing enzymes, joining enzymes and alkaline phosphatase.

Lysing enzymes

- They are used to open up the cells to get DNA for genetic experiments. **Lysozyme** is usually used to dissolve the bacterial cell wall.

Restriction enzymes

- **Restriction enzymes (cleaving enzymes)** are used to break DNA molecules. They belong to a larger class of enzymes called **nucleases**.
- Restriction enzymes are of three types – exonucleases, endonucleases and restriction endonucleases.
- **Exonucleases** remove nucleotides from the terminal ends (either 5' or 3') of DNA in **one strand** of duplex.
- **Endonucleases** make cuts at specific position within the DNA. These enzymes do not cleave the ends and involve only **one strand** of the DNA duplex.
- **Restriction endonucleases** were found by **Arher in 1962 in** bacteria. They act as "**molecular scissors**" or **chemical scalpels**.
- They recognize the specific base sequence at palindrome sites in DNA duplex and cut its strands.

- The palindromes in DNA are base pair sequences that are the same when read forward (left to right) or backward (right to left) from a central axis of symmetry.
- For example, the following sequence read the same on the two strands in 5′ ---→ 3′ direction and in the 3′ ⟶ 5′ direction.

  5′— GAATTC — 3′ Palindromic
  3′— CTTAAG — 5′ sequence
- Restriction endonuclease *Eco* R I found in the colon bacteria *E.coli*, recognizes the base sequence GAATTC in DNA duplex and cuts its strands between G and A as shown below:

  5′ — G ↓ AATTC — 3′
  3′ — CTTAA ↑ G — 5′
- Three main types of restriction endonucleases are type I, type II and type III.
- **Type I restriction endonucleases** consist of 3 different subunits. They require **ATP, Mg^{2+} and S-adenosyl methionine** for restriction. Type I restriction endonucleases recognize specific sites within the DNA but do not cut these sites.
- **Type II restriction endonucleases** are simple and require Mg^{2+} ions for restriction. Only type II restriction enzymes are used in recombinant DNA technology because they can be used *in vitro* to recognize and cut within specific DNA sequence typically consisting of 4 to 8 nucleotides and cause cleavage at unmethylated sites within their recognition.
- **Type III restriction endonucleases** are intermediate between type I and type II. They possess both the activities of restriction as well as methylation. Therefore, they are not used in recombinant DNA technology.
- The **first restriction endonuclease** was *Hin* d II (hin-dee-two). Its functioning depends on a specific DNA nucleotide sequence. It was isolated from *Haemophilus influenzae*.

Table :Some common examples of restriction enzymes type II, their source, recognition sequence, site of cleavage and product

	Restriction enzyme	Source	Recognition sequence and site of cleavage	Product
1.	*Alu* I	*Arthrobacter luteus*	5′ - A - G ↓ C - T - 3′ 3′ - T - C ↑ G - A - 5′	A - G | C - T T - C | G - A Blunt ends
2.	*Bam* H I	*Bacillus amyloliquefacien* H	5′ - G ↓ G - A - T - C - C - 3′ 3′ - C - C - T - A - G ↑ G - 5′	G | G - A - T - C - C C - C - T - A - G | G Sticky ends
3.	*Eco* R I	*Escherichia coli* RY 13	5′ - G ↓ A - A - T - T - C - 3′ 3′ - C - T - T - A - A ↑ G - 5′	G | A - A - T - T - C C - T - T - A - A | G Sticky ends
4.	*Hae* III	*Haemophilus aegyptius*	5′ - G - G ↓ C - C - 3′ 3′ - C - C ↑ G - G - 5′	G - G | C - C C - C | G - G Blunt ends
5.	*Hin* d III	*Haemophilus influenzae* Rd	5′ - A ↓ A - G - C - T - T - 3′ 3′ - T - T - C - G - A ↑ A - 5′	A | A - G - C - T - T T - T - C - G - A | A Sticky ends
6.	*Hin* d II (first discovered restriction endonuclease)	*Haemophilus influenzae* Rd	5′ - G - T - C ↓ G - A - C - 3′ 3′ - C - A - G ↑ C - T - G - 5′	G - T - C | G - A - C C - A - G | C - T - G Blunt ends
7.	*Sal* I	*Streptomyces albus*	5′ - G ↓ T - C - G - A - C - 3′ 3′ - C - A - G - C - T ↑ G - 5′	G | T - C - G - A - C C - A - G - C - T | G Sticky ends
8.	*Sca* I	*Streptomyces caespitosus*	5′ - A - G - T ↓ A - C - T - 3′ 3′ - T - C - A ↑ T - G - A - 5′	A - G - T | A - C - T T - C - A | T - G - A Blunt ends
9.	*Sma* I	*Serratia marcescens*	5′ - C - C - C ↓ G - G - G - 3′ 3′ - G - G - G ↑ C - C - C - 5′	C - C - C | G - G - G G - G - G | C - C - C Blunt ends

- *Hin* d II always cut DNA molecules at a particular point by recognising a specific sequence of six base pairs. It produces **blunt ends**.
- 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. These ends with unpaired bases are called **sticky ends** or **cohesive ends**. They are named so because they form hydrogen bonds with their complementary cut counter parts.
- The sticky ends facilitate the action of the enzyme **DNA** ligase.
- Some restriction enzymes cut both the strands of a DNA molecule at the same site so that the resulting ends have **blunt or flush ends** in which the two strands end at the same point.
- **Specific name (nomenclature)** of enzyme is derived from the name of prokaryotic cell from which the enzyme is isolated.
- The first letter of the genus becomes the first letter of the name of enzyme which is written in capital letter.
- The first two letters of species make second and third letters of the name of enzyme, which is written in small letters.
- All these three letters are written in italics.
- The fourth letter of the name of enzyme is the first letter of **strain**, so written in capital.
- The Roman number written at the end of the name indicates the order in which the enzyme was isolated from that strain of the prokaryotic cell.

Synthesizing enzymes

- They play a role in the synthesis of DNA strands on suitable templates.
- They are further of 2 types; **reverse transcriptases**, which help in the synthesis of complementary DNA strands on RNA templates and **DNA polymerases** which aid in the synthesis of complementary DNA strands on DNA templates.

Joining enzymes

- **Joining enzymes (DNA ligases or sealing enzymes)** help in sealing gaps in DNA fragments which are otherwise joined by complementary base pairing. Tu ligases are examples.
- These act as a **molecular glue**. They join DNA fragments by forming phosphodiester bonds.

Alkaline phosphatases

- These cut off phosphate group from the 5′ end of linearised circular DNA to check its recircularization.

Cloning vectors (Vehicle DNA)

- The vectors are DNA molecules that can carry a foreign DNA segment and replicate inside the host cell.
- Vectors may be plasmids, bacteriophage, cosmids, phagemids, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), transposons, and virus.
- Out of these vectors, **plasmid vectors** and **bacteriophage vectors** are commonly used.

Plasmid

- Plasmid vectors are extra-chromosomal, self-replicating, usually circular, double-stranded DNA molecules, found naturally in many bacteria and also in some yeast.
- It was discovered by **William Hays and Joshua Lederberg** in 1952.
- Plasmids are usually not essential for normal cell growth and division, they often confer some traits on the host organism.
- The plasmid molecules may be present as 1 or 2 copies or in multiple copies (500–700) inside the host organism.
- The naturally occurring plasmids have been modified to serve as vectors in the laboratory. The most widely used, versatile, easily manipulated vector pBR322 is an ideal plasmid vector.

pBR322 vector

- This was the first artificial cloning vector constructed in 1977 by **Boliver** and **Rodriguez**.
- In the name pBR, p signifies plasmid, B is Boliver, and R is from Rodriguez, thus B and R represent the initials of the scientists who developed pBR322. The numeral '322' distinguishes, this plasmid from the other plasmids developed in the same laboratory, *e.g.*, pBR325, pBR327, pBR328, etc.
- pBR322 plasmid contains the following regions:
  - **Origin of replication (Ori)**: It allows production of multiple copies per cell.
  - **Antibiotic resistance genes** such as ampicillin resistance (*amp*^{r}) gene and tetracycline resistance (*tet*^{r}) gene.
  - **Unique recognition sites** for restriction endonucleases.

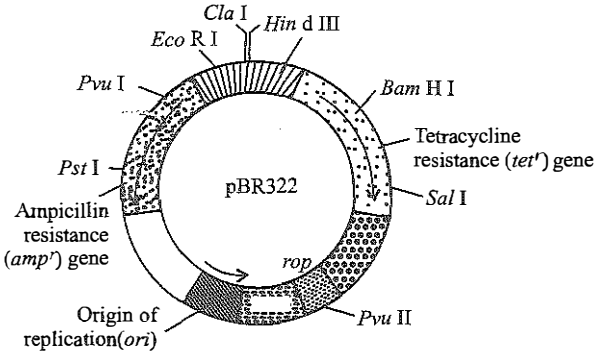

Fig.: Diagram showing essential features of plasmid pBR322

- Two unique sites, *Pst* I and *Pvu* I are located within the *amp*^{r} gene and *Bam* H I, *Sal* I, etc. are within tet^r gene.
- Some other unique restriction sites are *Eco* R I, *Cla* I, *Hin* d III, *Pvu* II. *Rop* codes for the proteins involved in the replication of the plasmid.

Bacteriophage vector

- Bacteriophages are viruses that infect bacterial cells by injecting their DNA into these cells.
- The injected DNA is selectively replicated and expressed in the host bacterial cell resulting in a number of phages which burst out of the cell (lytic pathway) and reinfect neighbouring cells.

- Two phages that have been extensively modified for development of cloning vectors are **lambda (λ) phage** and **M13 phage.**
- **Lambda phage vector** has a double-stranded, **linear DNA genome** of 48, 514bp, in which the 12 bases at each end are unpaired but complementary.
- These ends are, sticky or cohesive and are referred to as the cos sites (cohesive end sites). These sites are important for **packaging DNA into phage heads.**
- An important feature of lambda (λ) genome is that a large fragment in the central region of its genome is not essential for lytic infection of *E.coli* cells.
- Therefore, vectors have been designed such that this region can be substituted or replaced by a foreign DNA.
- These vectors allow cloning of DNA fragments upto 23 K_b in size.
- **M13 phage vector** is filamentous phage which infects *E. coli* having **F-pili.**
- Its genome is a single stranded, **circular DNA of 6407 bp.** Foreign DNA can be inserted into it without disrupting any of the essential genes.
- After the M13 phage DNA enters the bacterial cell, it is converted to a double-stranded molecule known as the **replicative form or RF**, which replicates until there are 100 copies in the cell.

Cosmid vector

- Cosmids have been constructed by combining certain features of plasmid and the 'cos' sites of phage lambda (λ).
- The simplest cosmid vector contains a plasmid origin of replication, a selectable marker, suitable restriction enzyme sites and the lamba **'cos' site.**
- Cosmids can be used to clone DNA fragments upto 45 kbp in length.
- They can be packaged into λ-particles. This is more efficient than plasmid transformation.

Bacterial artificial chromosome (BAC) vector

- The vector is based on natural, extra-chromosomal plasmid of *E. coli*.
- A BAC vector contains genes for **replication and maintenance of the F-factor**, a selectable marker and cloning site.
- These vectors can accommodate upto 300–350 kbp (kilo base pairs) of foreign DNA and are also being used in genome sequencing projects.

Yeast artificial chromosome (YAC) vector

- These are used to clone DNA fragments of more than **1 Mb** in size, therefore, they have been exploited extensively in mapping the large genomes, *e.g* , in the Human Genome Project.
- These vectors contain the **telomeric sequence**, the **centromere and the autonomously replicating sequence from yeast chromosomes.**
- They also contain restriction enzyme sites and genes which act as selectable markers in yeast.

Phagemid vectors

- Phagemid vectors is a composite structure made of bacteriophage and plasmid. They are used for carrying larger DNA sequences.

Animal and plant viral vectors

- Viruses that infect plant and animal cells can be used to introduce foreign DNA into plant and animal cells in culture. This is known as **plant and animal viral vectors.**
- A vector based on Simian Virus 40 (SV40) was used in the first cloning experiment involving mammalian cells in 1979. Since 1979, a number of vectors based on other types of viruses like adenovirus and papillomavirus have been used to clone genes in mammals. At present, retroviral vectors are the most commonly used vectors for cloning genes in mammalian cells in case of plants, plant viruses like cauliflower mosaic viruses, tobacco mosaic virus and gemini viruses were used but with limited success.

Transposon as vector

- DNA sequences which change their location in the genome and hence are said to be mobile are called transposons.
- They are able to be excised from one locus and become inserted at separate locus. They are used as vectors.
- The activator (Ac) and dissociation (Ds) are popular transposons of maize which are also called **Ac-Ds Elements.** The transposons of *Drosophila* are known as **P-Elements.**

Shuttle vectors

- The plasmid vectors can replicate only in *E coli*. Many of the vectors for use in eukaryotic cells are constructed such that they can exist in both the eukaryotic cells and *E.coli*. Such vectors are known as shuttle vectors.
- In case of plants, a naturally occurring plasmid of the bacterium *Agrobacterium tumefaciens* called Ti plasmid has been suitably modified to function as a vector. Most of the eukaryotic vectors are infact, **shuttle vectors.**

Characteristics of a cloning vector

Origin of replication

- This is a sequence from where **replication starts** and any piece of foreign DNA is linked to this sequence.
- The replication occurs inside the host cells. This sequence is also responsible for controlling copy number of linked DNA.
- Therefore, if any person wants to produce many copies of the target DNA he/she should clone in a vector **whose origin gives support to high copy number.**

Selectable marker

- The vector also requires a selectable marker (antibiotic resistance gene) to identify and eliminate non transformants and selectively permit the growth of the transformants.
- Transformation is a process through which a piece of DNA is introduced in a host bacterium.
- Generally, the genes encoding resistance to antibiotics such as **tetracycline, ampicillin, kanamycin** or **chloramphenicol**, etc. are useful in selectable markers for *E. coli*.

Recognition sites (cloning sites)

- The vector must also have one unique restriction endonuclease **recognition site** to enable foreign DNA to be inserted into the vector during the generation of a recombinant DNA molecule.
- Presence of a unique restriction site allows the particular enzyme to cut the vector only once.
- Most of the commonly used vectors contain unique recognition sites for several restriction enzymes in a small region of DNA which is referred to as a **polylinker** or **multiple cloning** site (MCS).
- A polylinker provides flexibility in the choice of restriction enzyme(s) that can be used for **cloning**.
- The ligation of alien DNA is carried out at a restriction site present in one of the two antibiotic resistance genes.

Competent host
(For transformation with recombinant DNA)

- Competent host bacterium is essential for transformation with recombinant DNA. **Transformation** is a process by which a cell takes up naked DNA fragment from the environment, incorporates it into its own chromosomal DNA and finally expresses the trait controlled by the incoming DNA.
- Since DNA is a hydrophilic molecule, it cannot pass through membranes, so the bacterial cells must be made capable 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.
- Efficient transformation takes only a few minutes and the cells are plated on a suitable medium for the selection of transformed clones.

Selection of the recombinant clones

- When recombinant DNA is constructed and used for transformation of bacterium, the following types of bacterial cells are obtained :
  - **Non-transformed cells** (majority of the cells).
  - **Transformed cells containing unaltered vector**.
  - **Transformed cells having recombinant DNA**.
- Desired gene is isolated by a two-step indirect selection procedure.
- The first step of this procedure consists of **identification** and **isolation**, following the transformation of host cells, of the small number of cells that contain recombinant DNA from a large number of **nontransformed** cells and those cells that are transformed by the unaltered vector molecules.
- The next step, is to **identify the clone** having the desired DNA insert from among the large number of clones containing recombinant DNA molecules.
- This is generally achieved by inserting a **selectable marker** gene into the vector used for producing the recombinant DNAs.
- A **selectable marker gene** or **reporter gene** produces a phenotype, which permits either an easy selection of quick identification of the cells in which it is present.
- A selectable marker governs a feature, which enables only those cells that possess them to survive under the selective conditions *e.g.*, an antibiotic like kanamycin are good selectable markers. When a population of bacterial cells is plated on a kanamycin containing medium, only those cells that have the kanamycin resistance gene (kan^r) survive and form colonies.
- The **non-transformed bacterial cells** are eliminated by plating them on a medium containing the selection agent. All the colonies that develop on the selective medium are transformed either by the unaltered vector or the recombinant DNA.
- The second step consists of identification and isolation of those clones that are transformed by the recombinant DNAs from among those that contain the unaltered vector.
- In case the vector has two selectable markers, *e.g.*, pBR322, the DNA insert may be placed within one of these markers, say amp^r gene.
- The other marker, tet^r, is used for elimination of the **non-recombinant cells**. The transformed clones are then replica-plated on ampicillin containing medium. The clones containing the recombinant DNA will be sensitive to ampicillin due to the inactivation of ampr by the insertion of the DNA fragment. Such clones are identified and isolated from the **master plate**.
- 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, a recombinant DNA is inserted within the coding sequence of an enzyme, **β-galactosidase**. This results into inactivation of the enzyme, which is referred to as **insertional inactivation**.
- The presence of a chromogenic substrate 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 and the colonies do not produce any colour, these are identified as **recombinant colonies**.
- In animals the term transformation is replaced by the term **transfection**.

Vectorless gene transfer

- Different alternative methods have been used to introduce the recombinant DNA into recipient cells of animals without involving carrier molecules.

Microinjection

- In this method foreign DNA is directly injected into the nucleus of animal cell or plant cell by using micro needles or micro pipettes. It is used in oocytes, eggs and embryo.

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.

Gene gun or biolistic

- DNA coated onto microscopic pellets of gold or tungsten of size 1-2 μm is literally shot with high velocity into target cells.
- Although it is developed for plants yet this technique is also used to insert genes into animal cells.

Direct DNA injection

- Direct DNA injection on DNA into skeletal muscle led to the possibility of using gene as vaccines. Due to low level of expression therapeutic benefits for the treatment of genetic disorder could not be derived. This method gave birth to the concept of DNA vaccine or genetic immunization.

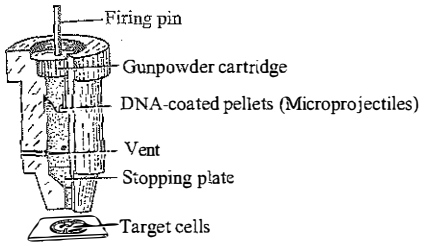

Fig.: Gene gun

Gel electrophoresis

Separation and isolation of DNA fragments

- After the cutting of DNA by restriction enzymes, fragments of DNA are formed. These fragments can be separated by a technique called **gel electrophoresis**.
- Electrophoresis is a technique of separation of charged molecules under the influence of an electrical field so that they migrate in the direction of electrode bearing the opposite charge, through a medium/matrix.
- The most commonly used matrix is **agarose** which is a polysaccharide extracted from sea weeds.
- DNA fragments separate according to size through the pores of agarose gel.

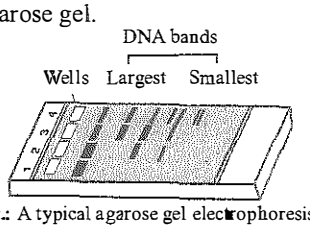

Fig.: A typical agarose gel electrophoresis

- The separated DNA fragments can be seen only after staining the DNA with a compound known as **ethidium bromide** (EtBr) followed by exposure to UV radiation as bright orange coloured bands.
- The separated bands of DNA are cut out from the agarose gel and extracted from the gel piece. This step is called **as elution**.

PROCESSES OF RECOMBINANT DNA TECHNOLOGY

Isolation of the genetic material (DNA)

- In order to cut the DNA with restriction enzymes, it needs to be in pure form, free from other macromolecules.
- The DNA is enclosed within the membranes so it has to break the cell open to release DNA along with other macromolecules such as RNA, proteins, polysaccharides and also lipids.
- This can be achieved by treating the bacterial cells/plant or animal tissue with enzymes such as **lysozyme (bacteria), cellulase (plant cells), chitinase (fungus).**

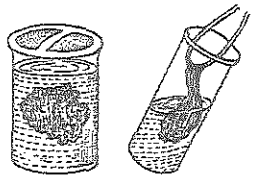

Fig.: DNA that separates out can be removed by spooling (spool = reel)

- The RNA can be removed by treatment with ribonuclease whereas proteins can be removed by treatment with protease.
- Other molecules are removed by proper treatments. The purified DNA finally precipitates out after the addition of chilled ethanol. This is seen as collection of the threads in suspension.

Cutting of DNA at specific locations

- 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.
- The joining of DNA involves several processes. After having cut the source DNA as well as the vector DNA with a specific restriction enzyme, the cut out **'gene of interest'** from the source DNA and the **cut vector** with space are mixed and **ligase** is added. This results in the preparation of **recombinant DNA**.

Amplification of gene of interest using PCR

- Polymerase chain reaction (PCR) is a technique of synthesizing multiple copies of the desired gene (or DNA) *in vitro*. This technique was developed by **Kary Mullis in 1985**.
- It is based on the principle that a DNA molecule, when subjected to high temperature, splits into two strands due to denaturation.
- These single stranded molecules are then converted to original double stranded molecules by synthesizing new strands in presence of enzyme **DNA polymerase**.
- A double stranded molecule of DNA is duplicated and multiple copies of the original DNA sequence can be generated by **repeating the process several times**.
- The basic requirements of PCR are :
  - **DNA template**. The desired segment of the target DNA molecule that is to be amplified.
  - **Two nucleotide primers**. Two nucleotide primers, usually 10-18 nucleotides long and complementary to the sequences present at the 3′ borders of the target DNA segment.
  - **Enzyme**. High temperature (more than 90°C) stable DNA polymerase (usually *Taq* polymerase), for synthesis of new DNA molecules.

Procedure of PCR

- At the start of PCR, the DNA from which a segment is to be amplified, an excess of the two primer molecules, the four **deoxynucleoside triphosphates** and the DNA

polymerase are mixed together in the reaction mixture that has appropriate quantities of Mg^{2+}.

- The PCR operation is followed in a sequence where denaturation, primer annealing and primer extention occurs.

Denaturation

- The reaction mixture is first **heated** to a temperature between **90 - 98°C** (commonly 94°C) that ensures DNA denaturation *i.e.*, the separation of the two strands.
- Each single strand of the target DNA then acts as a template for DNA synthesis.

Primer annealing

- The mixture is now cooled to a temperature (generally **40-60°C**) that permits annealing of the primer to the complementary sequences in the DNA; these sequences are located at the **3′-ends** of the two strands of the desired segment.
- Since the primer concentration is kept very high relative to that of the template DNA, **primer-template hybrid** formation is greatly favoured over reannealing of the template strands.

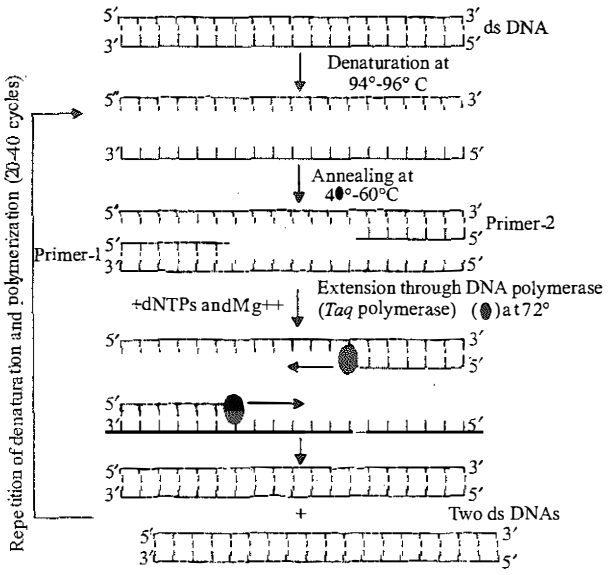

Fig.: A schematic representation of polymerase chain reaction (PCR) showing (i)denaturation, (ii) annealing, (iii) extension and (iv) resulting into two double stranded DNA fragments that enter the next PCR cycle to be duplicated. dsDNA= double stranded DNA, dNTPs = deoxynucleotide triphosphates.

Primer extension

- The temperature is now so adjusted that the DNA polymerase synthesizes the complementary strands by utilizing 3′– OH of the primers.
- This reaction is the same as that occurs in *vivo* during replication of the leading strand of a DNA duplex.
- The primers are extended towards each other so that the DNA segment lying between the two primers is copied.
- This is ensured by employing primers complementary to the 3′- ends of the segment to be amplified. The duration of primer extension is usually **2 min** at **72°C**.
- It has been shown that in case of longer target sequences, best results are obtained when the period of extension is kept at the rate of **1 min per Kb** of the target sequence and the extension is carried out at **68°C** in the place of usual 72°C.
- *Taq* polymerase (isolated from a bacterium *Thermus aquaticus*) which remains active during the high temperature, usually amplifies DNA segments of upto 2 Kb.
- To begin the second cycle, the DNA is again heated to convert all the newly synthesized DNA into single strands, each of which can now serve as a template for synthesis of more new DNA.
- Thus, the extension product of one cycle can serve as a template for subsequent cycles and each cycle essentially doubles the amount of DNA from the previous cycle.
- As a result, from a single template molecule, it is possible to generate 2^n molecules after n number of cycles.

Applications of PCR

- Some of the areas of application of PCR are mentioned here:
  - Diagnosis of pathogen.
  - Diagnosis of specific mutation.
  - DNA fingerprinting.
  - Detection of specific micro-organisms.
  - In prenatal diagnosis.
  - Diagnosis of plant pathogens.
  - In palaeontology.
  - Gene therapy.

Insertion of recombinant DNA into the host cell/ organism

- The vector DNA (*e.g.*, plasmid DNA) and alien (foreign) DNA carrying gene of interest are cut by the same restriction endonuclease to produce complementary sticky ends. This process of cutting DNA by restriction enzymes is called **restriction digestion**.
- With the help of DNA **ligase enzyme**, the complementary sticky ends of the two DNAs are joined (**annealing**) to produce a recombinant (**chimaera**) DNA (rDNA).
- The ligase forms new sugar-phosphate bonds to join two DNAs.
- Eukaryotic genes do not function properly when cloned into bacterial cell, because of their inability to excise introns of eukaryotic genes and their destruction by bacterial restriction enzymes.
- In such cases, DNA is made from *m*RNA by reverse transcription or synthesised artificially. This rDNA is inserted into host bacterium by **transformation** using cold $CaCl_2$ solution.
- The bacterial cell containing the desired rDNA is selected using selective antibiotic in the culture medium.

Obtaining desirable gene product

- When recombinant DNA is transferred into a bacterial, plant or animal cell, the foreign DNA is multiplied. Most of the recombinant technologies are aimed to produce a **desirable protein**.

- If any protein encoding gene is expressed in a heterologous host it is known as a **"recombinant protein"**.
- After the cloning of the gene of interest one has to maintain the optimum conditions to induce the expression of the target gene and consider producing it on a large scale.
- The cells having cloned genes of interest can be grown on a small scale in the laboratory. The cultures may be used for extracting and purifying the desired protein.
- The cells can also be multiplied in a continuous system where the used medium is passed out from one side and fresh medium is added from the other side to maintain the cells in their physiologically most **active lag** or **exponential phase**.
- This type of culturing method produces a **larger biomass** to get higher yield of desired protein.

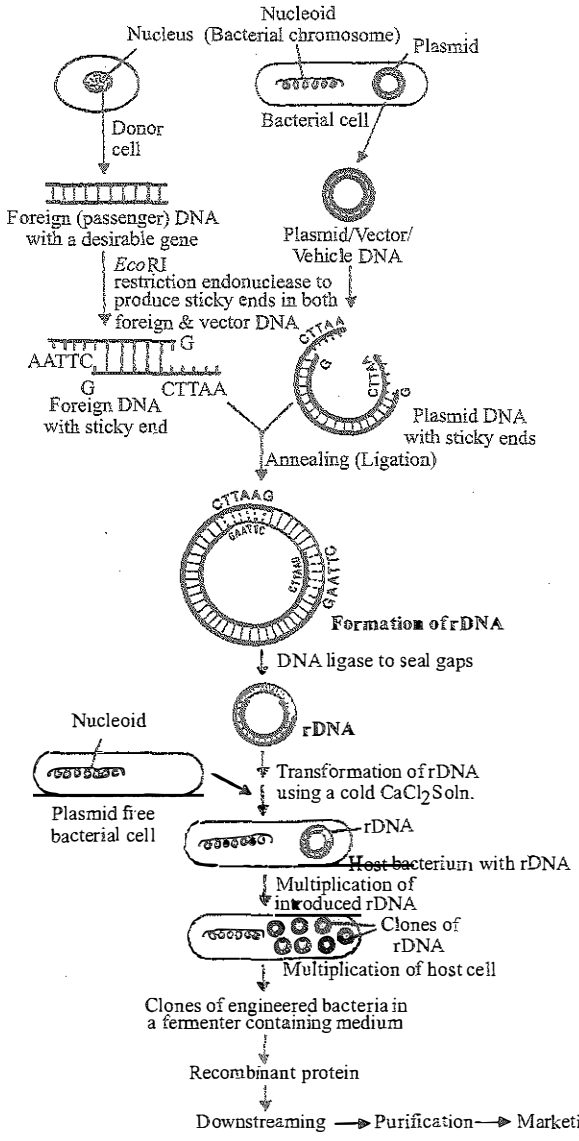

Fig.:Diagram showing various steps involved in recombinant DNA technology

Bioreactors (Fermenters)

- Bioreactors are considered as vessels in which raw materials are biologically converted into specific products by microbes, plant and animal cells or their enzymes.
- Small volume cultures cannot give large quantities of the products. To **produce large quantities** of these products, development of bioreactors was required where large volume (100 - 1000 litres) of culture can be processed.
- Bioreactor provides the optimal conditions for obtaining the desired product by **providing optimum growth conditions** such as temperature, pH, substrate, vitamins, oxygen and salts.
- The most commonly used bioreactors are of **stirring type**. A bioreactor (fermenter) has a provision for **batch culture** or **continuous culture**. In continuous culture, the culture medium is added and the product is taken out.

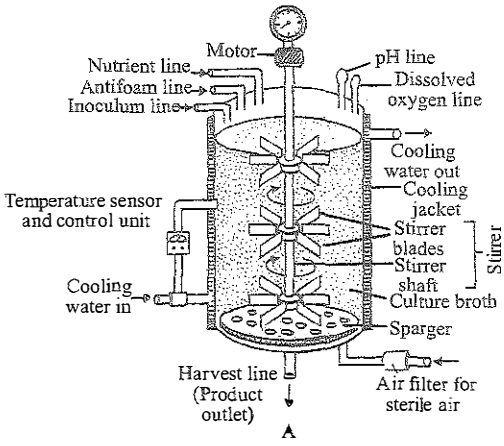

Fig. : Simple stirred tank bioreactor for continuous culture

- A simple **stirred tank bioreactor** is a large stainless steel vessel which has the main parts as cooling jacket, air inlet and filter, stirrer, sparger, lines, temperature sensor and control unit.
- **Cooling jacket** reduces temperature because the culture generates heat during growth. Most fermentations are aerobic and require a large volume of sterile air through **air inlet and filter**. **Stirrer** consists of a vertical rotating **stirrer shaft** and flat and vertical **stirrer blades**. **Sparger** is a porous ring (ring with small holes) at the bottom of the tank for proper aeration. The top of the tank has a number of inlet tubes called **lines** through which materials can be introduced or withdrawn. It consists of :
  - **Inoculum line (sample line)** is to add inoculum (genetically modified microorganisms).
  - **Antifoam line** is for introducing antifoaming agents.
  - **Nutrient line** is for introducing more nutrients.
  - **pH line** is meant for introducing acid or alkali to maintain optimum pH.
  - **Dissolved oxygen** line is for introducing dissolved oxygen.
- At the base of the tank, there is a **harvest line (product outlet)** to extract culture medium and microbial products.
- **Temperature sensor and control unit** records temperature and maintains the control of the system.

Downstream processing

- After completion of the biosynthetic stage, 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 through 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.

CONCEPT MAP

Biotechnology

Traditional view
- Biotechnology (Erey, 1917) is the technological employment of microorganisms, plant cells, animal cells, their components or biological processes to generate products and services useful to human beings.

Modern view
- Modern biotechnology is the use of genetically modified organisms (GMOs) or genetically engineered plant and animal cells in developing production technologies that not only enhance their productivity but also give rise to new products.

Principles of Biotechnology → **Chemical engineering**

Genetic engineering
- 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.
- The techniques of genetic engineering include **creation of recombinant DNA (rDNA).**

Tools of recombinant DNA technology

Restriction enzymes
- Restriction enzymes belong to a larger class of enzymes called **nucleases**. These are of three kinds: **exonucleases, endonucleases and restriction endonucleases.**. Exonucleases remove nucleotides from the ends of the DNA, whereas endonucleases make cuts at specific positions within the DNA.
- Each restriction endonuclease recognises a specific **palindromic nucleotide sequences** in the DNA.
- *Eco* R I, *Bam* H I, *Hin* d III are some of the examples of restriction endonucleases.

Gel electrophoresis
- It is a technique to **separate** and **isolate DNA fragments**.

Cloning vectors
- They are carriers or vehicles of desired DNA fragments (passenger DNA) which can undergo independent replication to increase copies of desired genes.
- Cloning vectors are of four types – **plasmids, viruses, cosmids** and **artificial chromosomes**.

Competent host
(For transformation with recombinant DNA)
- It involves **vector transfer** and direct or **vectorless transfer.**
- Vector transfer involves transformation with **cloning vectors**.
- Vectorless transfer involves
  (i) **Microinjection** (ii) **Particle** or **gene gun** or **biolistic**
  (iii) **Electroporation** (iv) **Direct DNA injection.**

Processes of rDNA technology

Isolation of genetic material
- To isolate the DNA from the bacterial cells/plant or animal tissue it is treated with enzymes such as **lysozyme** (bacteria), **cellulase** and **pectinase** (plant cells), **chitinase** (fungus).
- The treated cells are homogenised and centrifuged.
- Homogenised product is then treated with **proteases** (for digesting histones and other proteins), **ribonucleases** (digestion of RNAs), **amylases** and **lipases**.
- DNA remains intact. It is precipitated by the addition of chilled ethyl alcohol.

Amplification of gene of interest using PCR
- PCR or **polymerase chain reaction** is the *in vitro* synthesis of multiple copies of a gene or DNA segment.
- Amplified DNA segment can be used to ligate with the vector for formation of rDNA and its **cloning**.

Obtaining the desirable gene product
- When rDNA is transferred into a bacterial, plant or animal cell, the foreign DNA gets multiplied.
- The foreign gene gets expressed under appropriate conditions.
- If any protein encoding gene is expressed in a heterologous host, 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.
- 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 is used.

Cutting of DNA at specific locations
- Restriction enzyme digestions are performed by incubating purified DNA molecules with the restriction enzyme.
- The process is repeated with the vector DNA also.
- After having cut the source DNA as well as the vector DNA with a specific restriction enzyme, the cut out **gene of interest** from the source DNA and the cut vector with space are mixed and **ligase** is added.
- This results in the preparation of recombinant DNA.

Insertion of recombinant DNA into the host cell/organism
- The rDNA is inserted into host bacterium by **transformation** using cold $CaCl_2$ solution.
- The bacterial cell containing the desired rDNA is **selected** using selective antibiotic in the culture medium.

Downstream processing
- Downstream processing is the recovery of product from the fully grown genetically modified cells, its **purification and preservation**.
- The procedure and vigour of downstream processing and quality control varies from product to product.