BIOTECHNOLOGY: PRINCIPLES AND PROCESSES

Introduction:

- The term 'biotechnology' was coined by **Karl Ereky**. It is the broad area which deals with the techniques of using living organisms or enzymes from organisms to produce products useful for human welfare.
- Biotechnology deals with the processes like *in vitro* fertilization leading to a 'test-tube' baby, synthesizing a gene and using it, developing a DNA vaccine or correcting a defective gene, etc.
- Biotechnology can be defined by the European Federation of Biotechnology (EFB) as- 'The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'.

Principles of Biotechnology:

- **Genetic engineering** is the technique to alter the chemistry of genetic material (DNA/RNA) and introduce these into the host organism to change its phenotype.
- **Bioprocess/ Chemical engineering** is the maintenance of sterile conditions to enable the growth of only desired microbes/eukaryotic cells in large quantities for the manufacture of biotechnological products like antibiotics, vaccines, enzymes, etc.

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Basic Steps of Genetic Engineering/Making Genetically Modified Organism:

- **Identification of DNA with desirable genes:** With the use of the techniques of genetic engineering, isolation of only one or a set of desirable genes without any interference of undesirable genes is possible.
- **Introduction of the identified DNA into the suitable host to form recombinant DNA (rDNA):** With the use of vector such as plasmid, an alien piece of DNA is introduced into the host organism where it linked with the **origin of replication** and start replicating and multiplying itself in the host organism.
- **Maintenance of introduced DNA in the host and transfer of the DNA to its progeny.**

Construction of First Artificial Recombinant DNA:

- It was accomplished in 1972 by the contribution of two scientists, **Stanley Cohen** and **Herbert Boyer**.
- They isolated the antibiotic resistance gene by cutting out a piece of DNA with the help of **restriction enzymes** from the plasmid (an autonomously replicating circular extra-chromosomal DNA) of *Salmonella typhimurium*.

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- The cut piece of DNA was then linked with the plasmid vector with the help of enzyme DNA ligase and then transferred into *E. coli* where it could replicate using the new host's DNA polymerase enzyme and make multiple copies.

Processes of Recombinant DNA Technology:

Isolation of Genetic material (DNA):

- Genetic material is isolated from other macromolecules by using enzymes such as lysozyme (bacteria), cellulase (plant cells), and chitinase (fungus). The RNA can be removed by treatment with ribonuclease whereas proteins can be removed by treatment with protease.
- Purified DNA precipitates out after the addition of chilled ethanol and the collection of fine threads in the suspension is removed by a process called **spooling.**

Cutting of DNA at specific location:

- Purified DNA is cut by using restriction enzyme and Agarose gel electrophoresis is used to check the progression of a restriction enzyme digestion.

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Amplification of Gene of Interest using PCR:

- Polymerase Chain Reaction is an *in vitro* technique of synthesizing the multiple copies of the gene of interest by using the two sets of primers (chemically synthesised oligonucleotides that are complementary to a region of DNA), enzyme DNA polymerase (*Taq* polymerase) and deoxynucleotides are added.
- Polymerase Chain Reaction involves three main steps which are:
  - **Denaturation:** Double helical DNA is denatured at high temperature of around 94°C for 15 seconds. Each DNA strands act as a template for the synthesis of a new strand.
  - **Annealing:** Two sets of oligonucleotide primers anneal at lower temperature at 54°C to the separated single strands of DNA.
  - **Extension:** *Taq* DNA Polymerase is used to extend the primers by adding dNTPs (deoxynucleoside Triphosphates) complementary to those of the template DNA. *Taq* DNA Polymerase also requires Mg^{2+} as a cofactor. This occurs at 72°C.
  - ***Taq* Polymerase** is isolated from a thermophilic bacterium, *Thermus aquaticus*. This enzyme has the ability to remain active at high temperature.

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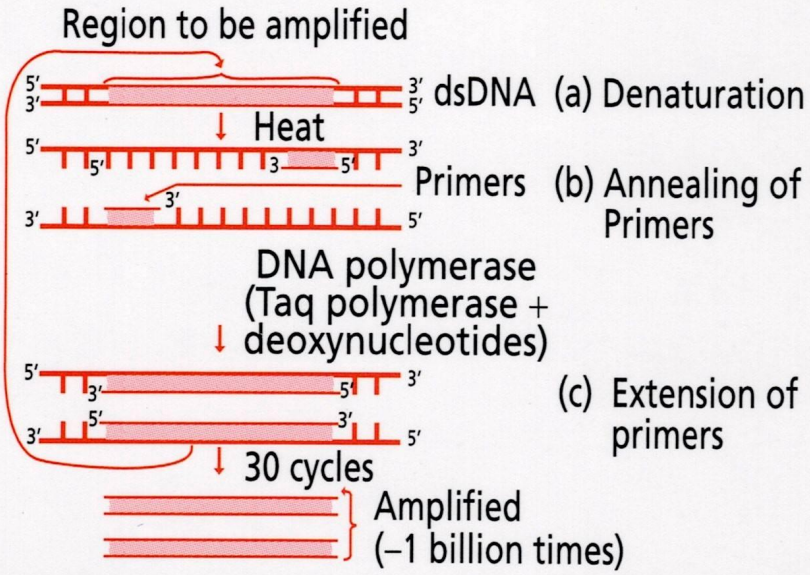


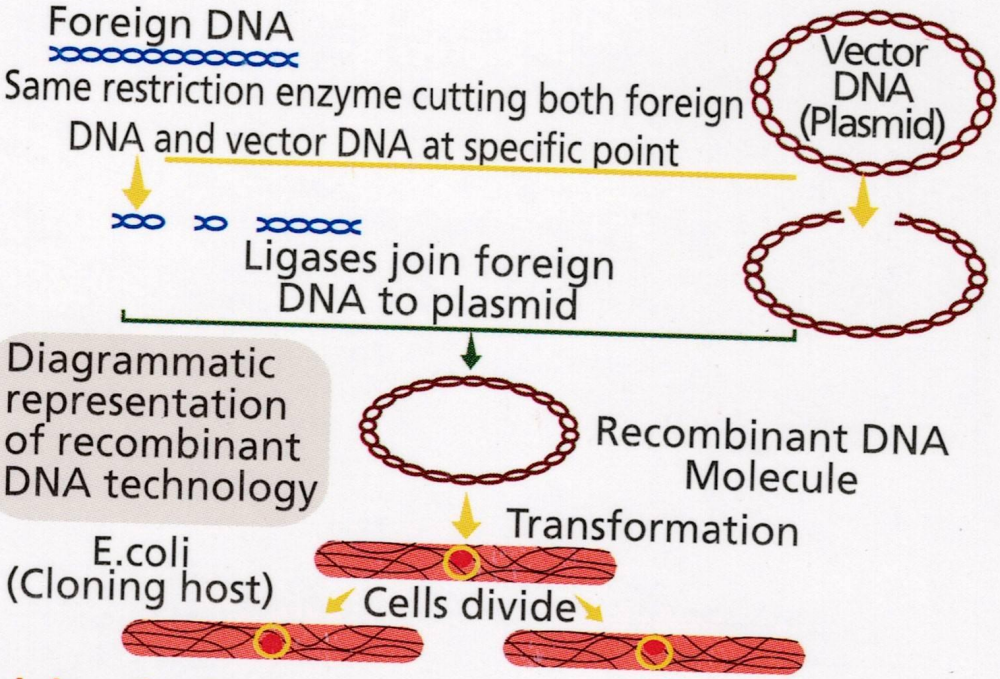
Polymerase Chain Reaction (PCR) Each cycle has three steps (a) Denaturation (b) Primer annealing and (c) Extension of primers

- The amplified fragment of DNA having gene of interest ligated with a vector for further cloning.

Insertion of Recombinant DNA into the Host Cell/Organism:

- Using several methods, ligated DNA is introduced into the competent host cell/organism.
- The recombinant DNA bearing gene for resistance to an antibiotic is transferred into *E.coli* cells, the host cell become transformed into ampicillin-resistance cells.
- When these transformed cells are grown on agar plates containing ampicillin, only transformants containing

ampicillin resistance gene will grow whereas the untransformed recipient cells will not survive.



Diagrammatic representation of recombinant DNA technology

Obtaining the foreign gene product:

- The foreign DNA multiplies in plant or animal cell to produce desirable protein.
- Expression of protein encoding foreign gene in heterologous host cells involves optimum condition to obtain **recombinant protein.**
- The cells with foreign genes grown on a small scale in the laboratory. These cell cultures can then be used for extracting the desired protein and then purifying it by using different separation techniques.

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- The recombinant cell is multiplied in a **continuous culture** system in which used medium is drained out from one side while fresh is added from the other to maintain the cells in their physiological active log/ exponential phase. It results in the production of a larger biomass leading to higher yields of desired protein.

Bioreactors:

- These are the vessels with approximate capacity of 100-1000 litres. In these, large volumes of raw materials are biologically converted into specific products, individual enzymes, etc., using microbial, plant, animal or human cells.
- These provide optimal growth conditions such as temperature, pH, substrate, salts, vitamins, etc. to get desired product.
- A bioreactor has various components such as **agitator system, oxygen delivery system, foam control system, temperature** and **pH control system, sampling ports.**
- Stirring type bioreactors are the most commonly used bioreactors. These are of two types:
  - A **Simple stirred-tank bioreactor** has a curved base that facilitates the mixing of the reactor contents and the stirrer facilitates even mixing and oxygen availability throughout the bioreactor.

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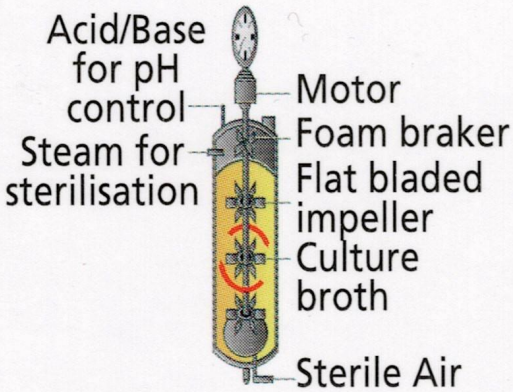
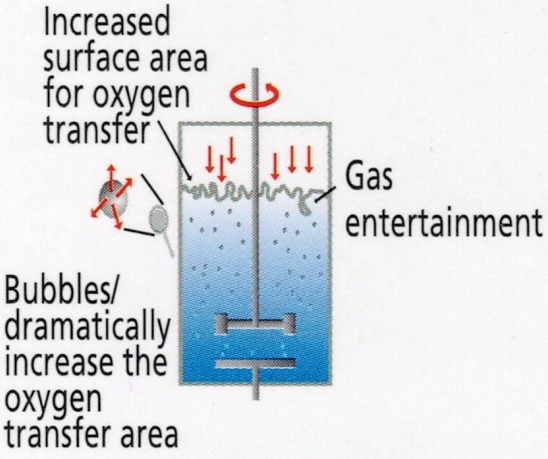
- In **Sparged stirred-tank bioreactor** sterile air is sparged through the reactor. These have large surface area for oxygen transfer.



Simple stirred-tank bioreactor



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

Downstream Processing:

- After completion of the biosynthetic stage, the product is subjected through a series of processes such as **separation and purification**, before it is ready for marketing as a finished product.
- In the product suitable preservatives are added and then it undergo thorough clinical trials, strict quality control testing before releasing to market for public use.

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Tools of Recombinant DNA Technology:

1. Restriction enzymes or 'molecular scissors':

- These are specialised enzymes that recognise and cut the specific sequence of DNA.
- These belong to a larger class of enzymes called **nucleases**, which are of two types, **Exonucleases** – Remove nucleotides from the ends of the DNA and **Endonucleases** – Cuts at specific positions within the DNA.
- The first restriction endonuclease was Hind II. It was isolated and characterized by **Smith, Aerber** and **Nathan**. This enzyme cuts the DNA at the **recognition sequence** of six base pairs.
- Restriction endonuclease recognises a specific **palindromic nucleotide sequences** in the DNA. These are the sequence of 4 to 8 base pairs that read same in 5′ → 3′ direction as well as in 3′ → 5′ direction.

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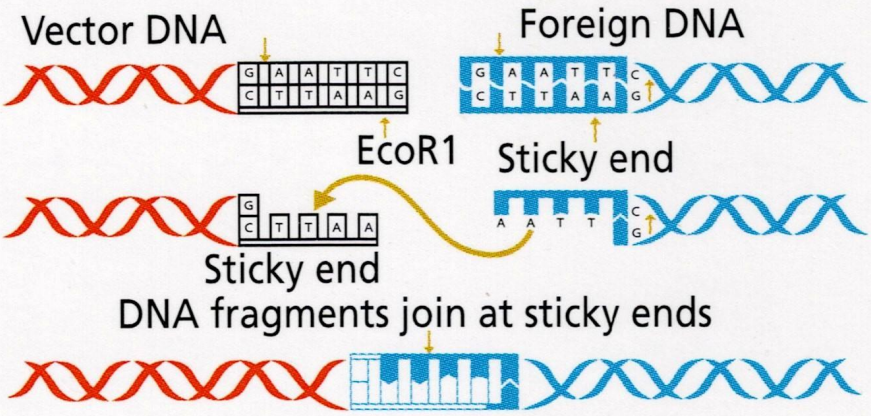
The enzyme cuts both DNA strands at the same site

EcoR1 cuts the DNA between bases G and A only when the sequence GAATTC is present in the DNA



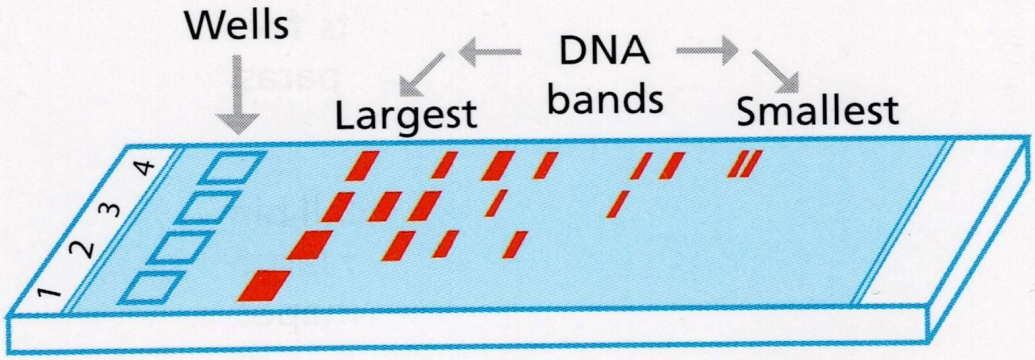
Recombinant DNA

- Restriction enzymes cut a little away from the center of palindrome site, but between the same two bases on the opposite strands. This leaves **sticky ends** on each strand which forms hydrogen bonds with their complementary counterparts with the help of DNA ligases.
- In the naming of restriction enzymes, the first letter comes from the genus. The second two letters come from the species of the prokaryotic cell from which they were isolated.
- Example- *Eco*RI isolated from ***E**scherichia **co**li* RY 13. In *Eco*RI, 'R' is the name of strain and roman

numbers following the names indicate the order in which the enzymes were isolated from that strain of bacteria.

2. Gel Electrophoresis:

- This technique helps in the separation of DNA fragments which are produced by the action of restriction endonucleases.
- The negatively charged DNA fragments are separated by forcing them towards the anode (+) by applying an electric field through an agarose medium.
- DNA fragments separate according to their size through sieving effect provided by the agarose gel. Therefore, smaller sized fragment moves farther.



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- The separated DNA fragments can be seen as bright orange-coloured bands when they are stained with **ethidium bromide** and exposed to UV light.
- Finally, the separated stained bands of DNA are cut out from the agarose gel and the process termed as **elution**.
- Thus, the purified DNA fragments with gene of interest are used in constructing recombinant DNA by joining them with cloning vectors with the help of DNA ligase.

3. Cloning vectors:

- These are small piece of DNA which has the ability to replicate within the bacterial cells independent of the chromosomal DNA. For e.g., Plasmids, bacteriophages, etc.
- Plasmids generally have 1-2 to 15-100 copy numbers per cell. Whereas, bacteriophages have high copy number of their genome within the bacterial cell. If foreign DNA is linked to these vectors, it is multiplied to the number equal to their copy number.

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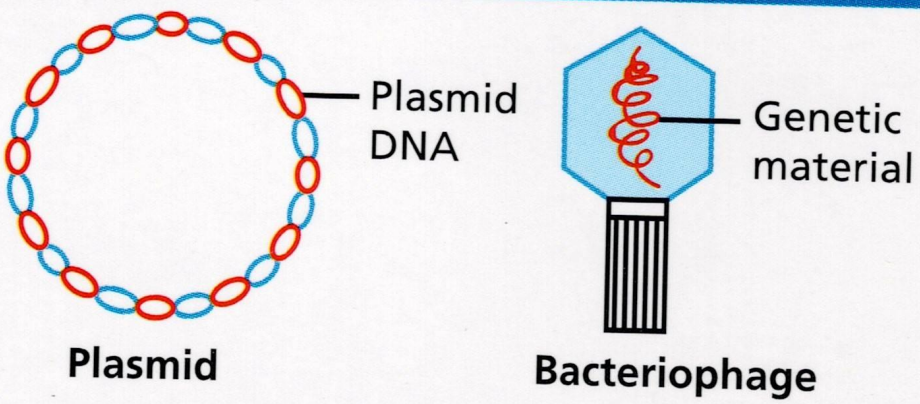
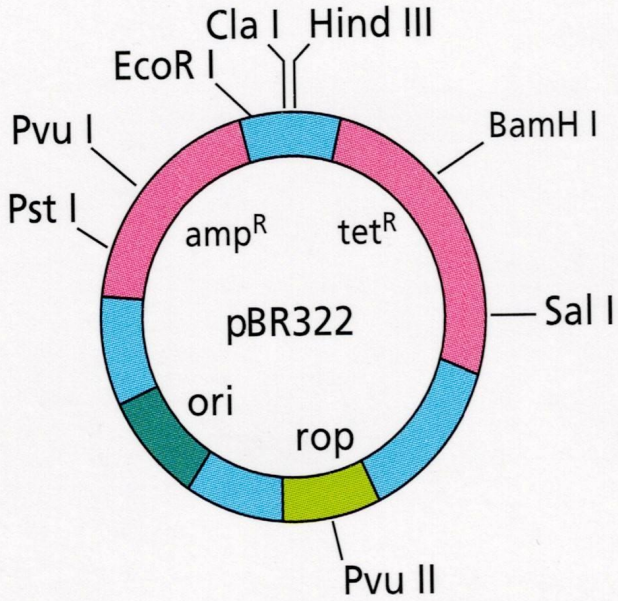


Plasmid **Bacteriophage**

- To facilitate cloning into a vector following features are required:
  - **Origin of replication (ori)** is the sequence from where replication starts and any piece of DNA when linked to *ori* can be made to replicate within the host cells. This sequence control and support the high copy number of the linked DNA.
  - **Selectable marker** is a gene sequence which permits the growth of transformants and eliminates non-transformants. Antibiotic resistance genes such as amp^R (ampicillin resistant), tet^R (tetracycline resistant) serve as useful selectable markers for *E.coli.*
  - **Cloning sites** are the recognition sites for restriction enzymes. Presence of more than one recognition sites generates several fragments,

which will complicate the gene cloning. The foreign DNA is usually ligated at a restriction site present in one of the two **antibiotic resistance** genes.



- Restriction sites: Hind III EcoR I, BamH I, Sal I, *Pvu* II, PSt I, Cla I.
- ori
- Antibiotic resistance gene: ampR and tetR.
- Rop: codes for the proteins involved in the replication of plasmid.

E.coli cloning vector pBR322

- When the desired gene is introduced at the site of the antibiotic resistance gene resulting in the loss of antibiotic resistance in the recombinant plasmid. So, recombinants can be selected from the non-recombinants.
- In the process of insertional inactivation, a recombinant DNA is inserted within the coding sequence of an enzyme, β-galactosidase, which

results into inactivation of the gene. Therefore, the bacterial colonies do not produce any color. Hence identified as recombinant. If the plasmid in the bacteria is devoid of the insert, it forms blue-coloured colonies due to the presence of a chromogenic substrate. Hence identified as non-recombinants.

- **Vectors for cloning genes in plants** are *Agrobacterium tumefaciens*, which delivers a piece of DNA known as 'T-DNA' to transform normal plant cells into a **tumor**. This is done by its tumor inducing (Ti) plasmid which has now been modified into a cloning vector by removing the genes responsible for pathogenicity.
- **Vector for cloning genes in animals** is retrovirus which transforms normal cells into cancerous cells. **Retroviruses** have also been disarmed and used to deliver desirable genes.

4. Competent host (For Transformation with Recombinant DNA):

- DNA is a hydrophilic molecule; thus it cannot pass through cell membranes. So bacterial cells are made 'competent' to take up alien DNA or plasmid by the following methods:

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- In **Chemical/$CaCl_2$ method,** bacterial cells treated with a specific concentration of calcium due to which DNA enters the bacterium through pores in cell wall. The bacterial cells with recombinant DNA are incubate on ice first, then briefly placed at 42°C **(heat shock)** and again on the ice.
- With **Micro-injection**, recombinant DNA is directly injected into the nucleus of an animal cell.
- In **Biolistics (gene gun)**, cells are bombarded with high velocity micro-particles of gold or tungsten coated with DNA. This method is suitable for plants.
- **Disarmed pathogen vectors** infect the cell and transfer the recombinant DNA into the host.

TEST

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1. The *Taq* polymerase enzyme is obtained from _____.
   (a) *Thermus aquaticus*
   (b) *Thiobacillus ferrooxidans*
   (c) *Bacillus subtilis*
   (d) *Pseudomonas subtilis*
2. Which of the following is an endonuclease?
   (a) DNase I
   (b) *Hind* II
   (c) Protease
   (d) RNase restriction
3. Which of the following is not a component of downstream processing?
   (a) Expression
   (b) Preservation
   (c) Purification
   (d) Separation
4. A foreign DNA and plasmid cut by the same restriction endonuclease can be joined to form a recombinant plasmid using _____.
   (a) *Taq* polymerase
   (b) Polymerase III
   (c) Ligase
   (d) *Eco* RI

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Solutions:

1. **Option (a) is correct.**

   The *Taq* Polymerase enzyme is obtained from a thermophilic bacterium, *Thermus aquaticus*. This enzyme remains active even at high temperature during the denaturation of double stranded DNA.

2. **Option (b) is correct.**

   Hind II was the first restriction endonuclease. It was isolated and characterized by Smith Wilcox and Kelley in 1968 from *Haemophilus influenzae*. This enzyme always cleaves at specific sequence within the DNA strand.

3. **Option (a) is correct.**

   In downstream processing, product is subjected through a series of processes such as separation and purification, and after that preservation process is performed for enhancing the product's storage period.

   Expression is not a component of the downstream processing.

4. **Option (c) is correct.**

   In order to create a recombinant DNA molecule, a foreign DNA and plasmid cut with the same restriction enzyme. Because of this the resultant DNA fragments have the same type of 'sticky-ends' and, these are joined together (end-to-end) by DNA ligases.

TEST

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5. DNA fragments separated on an agarose gel can be visualized after staining with _____.
   (a) ethidium bromide
   (b) bromophenol blue
   (c) acetocarmine
   (d) aniline blue

6. There is a restriction endonuclease called *Eco* RI. What does '*co*' part in it stand for?
   (a) *coli*
   (b) *colon*
   (c) *cofactor*
   (d) *none of the above*

7. Which of the following is not correctly matched for the organism and its cell wall degrading enzyme?
   (a) plant cells – cellulase
   (b) bacteria – lysozyme
   (c) fungi – chitinase
   (d) algae – methylase

8. For transformation, micro-particles coated with DNA to be bombarded with gene gun are made up of:
   (a) Platinum or Zinc
   (b) Silicon or Platinum
   (c) Gold or tungsten
   (d) Silver or Platinum

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Solutions:

5. **Option (a) is correct.**

The separated DNA fragments on agarose gel can be visualised only after staining the DNA with a compound known as Ethidium Bromide (EtBr) followed by exposure to UV radiation. Due to this, bright orange-coloured bands of DNA can be seen.

6. **Option (a) is correct.**

Restriction endonuclease called *Eco*RI is isolated from bacterium ***Escherichia coli*** RY 13. In *Eco*RI, the first letter, i.e., ***E*** comes from the genus ***Escherichia***. The second two letters, i.e., ***co*** come from the species ***coli***.

7. **Option (d) is correct.**

In algae, cell wall is made up of cellulose. Thus, it is degraded with the help of enzyme cellulase. Rest other options are correctly matched.

8. **Option (c) is correct.**

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