RECOMBINANT DNA TECHNOLOGY AND ITS APPLICATIONS

Author

Rajendra B. Kakde
Department of Botany
Swami Vivekanand Senior College
Mantha Dist. Jalna (M.S.) India
kakderb@gmail.com

I. WHAT IS RECOMBINANT DNA TECHNOLOGY?

Recombinant DNA technology means joining together of two DNA molecules from two different species which are later on inserted into a host organism to produce new genetic combinations which have importance in science, medicine, agriculture and industry.

II. STEPS IN RECOMBINANT DNA TECHNOLOGY

Following are the basic steps involved in recombinant DNA technology:

1. Selection and Isolation of foreign DNA (gene of interest)
2. Incorporating foreign DNA (the target gene) into a vector to generate a recombinant DNA entity.
3. Introducing the recombinant DNA entity into a compatible host.
4. Choosing the transformed host cell through a selection process.
5. Expression and multiplication of foreign DNA into host

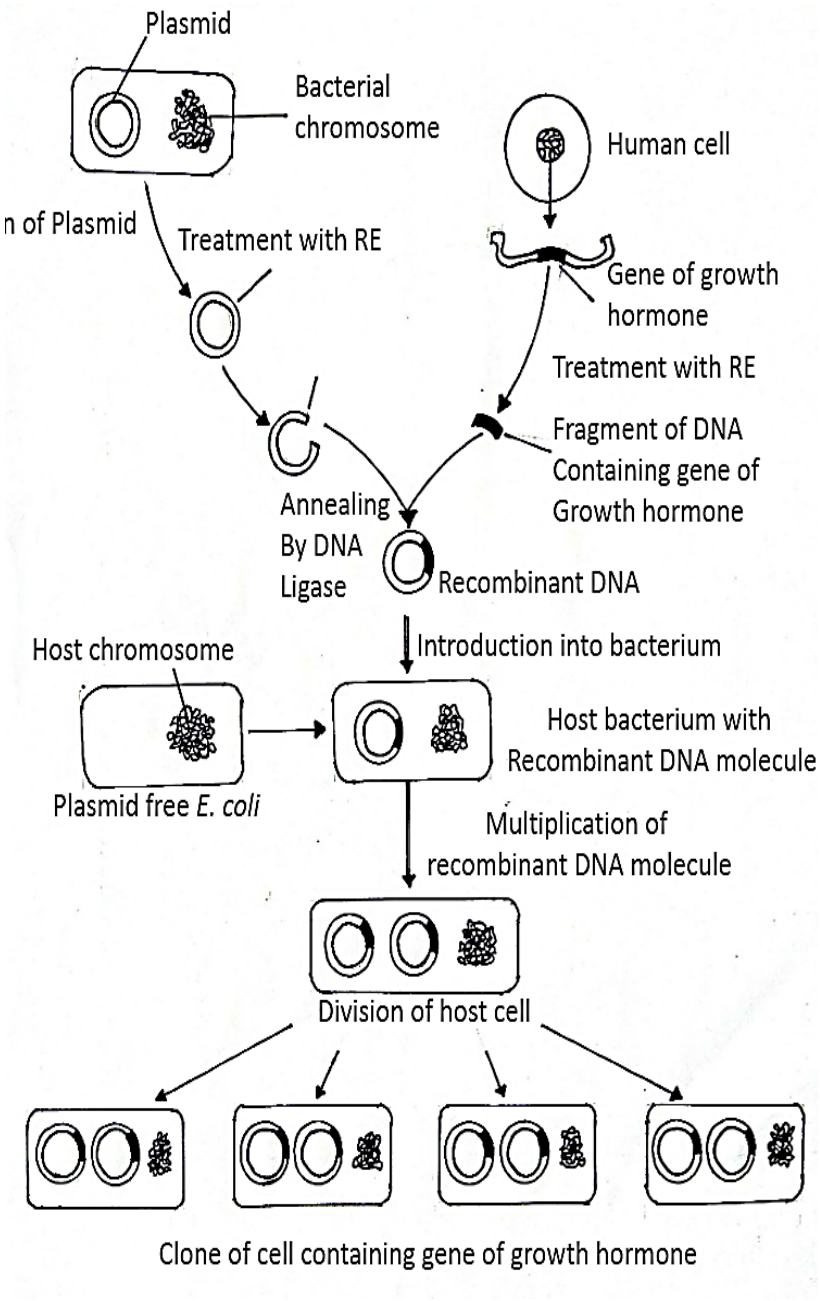

Figure 1: Steps in recombinant DNA technology

III. SELECTION AND ISOLATION OF FOREIGN DNA (GENE OF INTEREST)

The initial stage of DNA isolation involves the gentle extraction of DNA by employing the least disruptive techniques to avoid mechanical shearing and fragmentation. Since cell structures can vary, the approaches used to disrupt the cells differ accordingly.

The bacterial cell, such as E. coli, can be lysed through the action of the enzyme lysozyme and the chemical ethylenediamine tetraacetate (EDTA). EDTA serves to chelate the essential Mg++ ions required for the degradation of the DNase enzyme, which is responsible

for breaking down DNA. Subsequently, sodium dodecyl sulfate (SDS) detergent is introduced.

Animal cells in culture can be readily opened through the use of SDS treatment. In contrast, plant cells possess robust cell walls that necessitate more rigorous treatment for disruption. The cells are frozen and subsequently ground using a mortar and pestle, a highly effective method for breaking down the plant cell wall.

Furthermore, aside from DNA, all other cellular components are thoroughly eliminated. To achieve this, the cellular extract is subjected to centrifugation at a low speed to separate and remove cell wall debris, resulting in the formation of a pellet at the tube's bottom. The supernatant is then gathered and subjected to treatment with phenols or a phenol/chloroform mixture to precipitate proteins situated between the organic and aqueous layers. The aqueous layer, containing DNA and RNA, is isolated and treated with RNase (ribonuclease), leading to the degradation of RNA while leaving DNA intact. DNA can subsequently be precipitated by adding ethanol and isolated following centrifugation. Subsequently, the DNA pellet is redissolved in a buffer containing EDTA to inactivate any DNase present. This solution can be safely stored at -40°C.

Then to obtain desired gene the restriction digestions and gel electrophoresis are carried out. Restriction digestions are performed by incubating purified DNA molecule with the restriction enzyme. Further, by using agarose gel electrophoresis activity of restriction enzyme can be checked.

IV. AGAROSE GEL ELECTROPHORESIS

This technique is the most commonly employed method for isolating nucleic acid fragments of varying lengths. It is capable of separating DNA fragments up to 20 kb in size, but larger DNA molecules cannot be separated or do not penetrate the gel matrix. By reducing the agarose concentration to 0.1%, it becomes possible to separate DNA fragments as large as 500 kb. However, such low-percentage agarose gels are notably delicate and exceptionally challenging to manipulate. In this procedure, DNA is compelled to migrate through a densely cross-linked agarose matrix in response to an electric current. The rate of DNA migration through the gel is influenced by the size of the DNA fragments and the ionic strength of the running buffer. In solution, DNA's phosphate groups carry a negative charge, causing the DNA to migrate toward the positive pole.

V. PROTOCOL

1. Preparation of the Gel:

- Weigh the appropriate amount of agarose into a flask. Add appropriate amount of water to form gel. The agarose concentration within a gel is determined by the size of the DNA fragments intended for separation. Typically, gel concentrations fall within the range of 0.5-2%, with a commonly utilized concentration being 0.8% in many laboratories. This concentration is well-suited for separating DNA molecules within the 0.5-10 kb size range.

- Add the running buffer to the flask containing agarose and mix it by gently swirling. The most frequently used gel running buffers include TAE (comprising 40 mM Tris-acetate and 1 mM EDTA) and TBE (consisting of 45 mM Tris-borate and 1 mM EDTA).

- Dissolve the agarose-buffer mixture by heating it in a microwave. At intervals of 30 seconds, take out the flask and gently swirl its contents to ensure thorough mixing. Continue this process until the agarose has completely dissolved.

- Add ethidium bromide (EtBr) to a concentration of 0.5µg/ml to the gel. Gel will stained after electrophoresis in running buffer containing 0.5µg/ml EtBr for 15-30 minutes.

- Place the gel tray into casting apparatus. Then seal the open edges of the gel tray with the help of molten agarose. Then appropriately place the comb into gel mold to create the wells.

- Transfer the liquefied agarose into the gel mold, and then allow the agarose to solidify at room temperature. Standard-purpose gels typically measure around 25 cm in length and 12 cm in width.

- Remove the comb and place the gel into gel box.

- Add loading dye to the DNA samples intended for separation. The loading dye comprises a 6X concentration, which includes 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol. The function of the loading dye is twofold: it aids in tracking the distance the DNA sample has migrated within the gel and facilitates the sample's penetration into the gel matrix.

- Run the gel with power supply to desired voltage 1.5 V/cm overnight.

- Add enough buffer to cover the surface of the gel. Use the same running buffer as the one used to prepare the gel.

- Using a micropipette, meticulously and cautiously load the DNA sample into the gel. Ensure that alongside the experimental sample, a standard DNA marker is also loaded.
- The cathode (black knob) should keep closer to the wells than the anode (red knob).

- Activate the power supply and confirm the operational status of both the gel box and the power supply. Run the gel until the dye has migrated to an acceptable distance. When an electrical current is applied, the negatively charged DNA moves towards the positive electrode and undergoes separation according to its size.

- Turn off the power supply and remove the lid of the gel box when electrophoresis is completed.

- Remove the gel from the gel box. From the surface of the gel remove the excess buffer. Place the gel tray on paper towels to absorb any extra running buffer.

- Remove the gel from the gel tray and visualize under the UV light of 300 nm wavelength. Under the UV light EtBr build up at the site of DNA bands and these bands fluoresce orange-red. As little as 10 ng of DNA can be visualized as a 1 cm wide DNA. Wear goggle while observing the DNA bands because the UV light may damage the eye.

- The standard DNA should be separated to use determination of the sizes of the sample bands.

- Cut out the gel along with DNA bands by a scalpel blade. DNA is recovered by electroelution method. The gel fragment of desired DNA band is placed into a dialysis bag with buffer. The bag is then placed into a gel box containing buffer and start electric current. The extracted DNA is precipitated from the solution and dissolve the gel into agarase enzyme to digest the agarose and we get pure DNA.

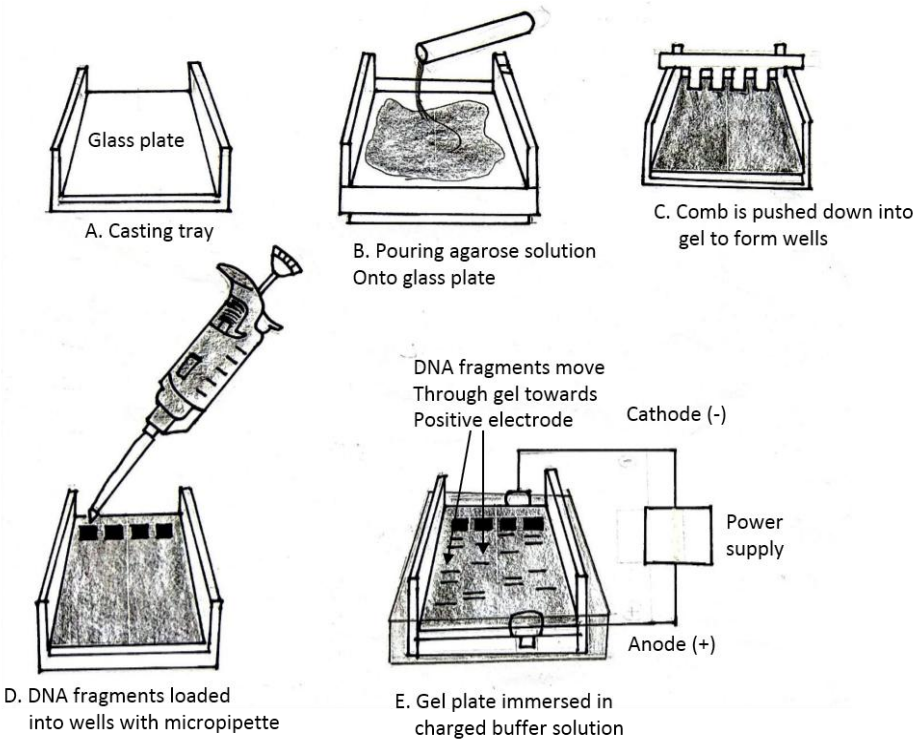

Figure 2: Agarose gel electrophoresis

2. **Polymerase Chain Reaction (PCR):** PCR is a laboratory method created by Kary Mullis in 1983 for amplifying substantial amounts of a specific gene (DNA) of interest.

3. **Principle** The double-stranded DNA of interest undergoes denaturation to separate into two individual strands. Each of these strands is then permitted to hybridize with primers, a process known as renaturation. The resulting primer-template complex serves as the

foundation for DNA synthesis. These three steps—denaturation, renaturation, and synthesis—are iterated multiple times to generate numerous copies of the desired DNA.

4. **Materials and Reagents:** Target DNA or DNA of interest

- **Forward and reverse Primers:** 0.2μM,
  Deoxynucleoside triphosphates (dNTPs): Each dATP, dTTP, dGTP, and dCTP is present at concentrations ranging from 50 to 200μM. These serve as the fundamental building blocks that DNA polymerase utilizes to synthesize a new DNA strand.
  Taq DNA polymerase

- **Buffer solution:** This solution is employed to create an optimal chemical environment that enhances the activity and stability of DNA polymerase.

- **Bivalent magnesium or manganese ions:** These ions are essential for maximizing the activity of Taq DNA polymerase and exert an influence on the efficiency of primer-to-template annealing.

5. **Procedure:**

- Prepare the reaction mixture into PCR tube by following way:

Ingredients for PCR	**Volume in µl**
Molecular biology grade water	30.5 µl
10X assay buffer	5 µl
Template DNA (DNA of interest)	2 µl
Forward primer	1 µl
Reverse primer	1 µl
25 mM $MgCl_2$	5 µl
2.5 mM dNTP mix	5 µl
Taq DNA polymerase	0.5 µl
Total volume	50 Ml

- Tap the tube for 2-3 seconds to mix the contents thoroughly.
- PCR consist of three defined steps such as **denaturation**, **renaturation** or **annealing** and.
- **Synthesis** or **extension**. Each of these steps is repeated 30-40 times, termed as **cycles**. All the cycles are run in the thermal cycler.
- During the initial cycle, the double-stranded template DNA is denatured by subjecting the reaction to a temperature exceeding 90°C for approximately 1 minute.
- The temperature of the mixture is subsequently gradually reduced to a range between 40°C to 60°C for duration of 1 minute. During this phase, the primers form complementary pairs with the flanking regions of the target DNA, a process known as renaturation or annealing.
- DNA synthesis commences at the 3' hydroxyl end of each primer, and the primers are elongated as they bind with bases complementary to the DNA strand. During the

synthesis step, the temperature is maintained at 75°C for 2 minutes, which represents the optimal condition for the stability of the Taq DNA polymerase enzyme.

- In this way 30-40 cycles of **denaturation**, **renaturation** and **synthesis** are run in the thermal cycler. New strand extend beyond the target DNA it will contain the complementary regions to the primers at the 3' end. Thus, if another round of DNA synthesis is allowed to take place, the original strand as well as new strand used as template.
- All the new strands will act as template so that there will be exponential increase in the amount of DNA produced.

6. **Insertion of foreign DNA (gene of interest) into vector to form recombinant DNA molecule:** The DNA of interest and suitable vector are cut with same restriction enzyme in order to obtain sticky ends. Both are ligated by mixing vector DNA, DNA of interest and enzyme DNA ligase to form recombinant DNA or chimeric DNA or hybrid DNA.

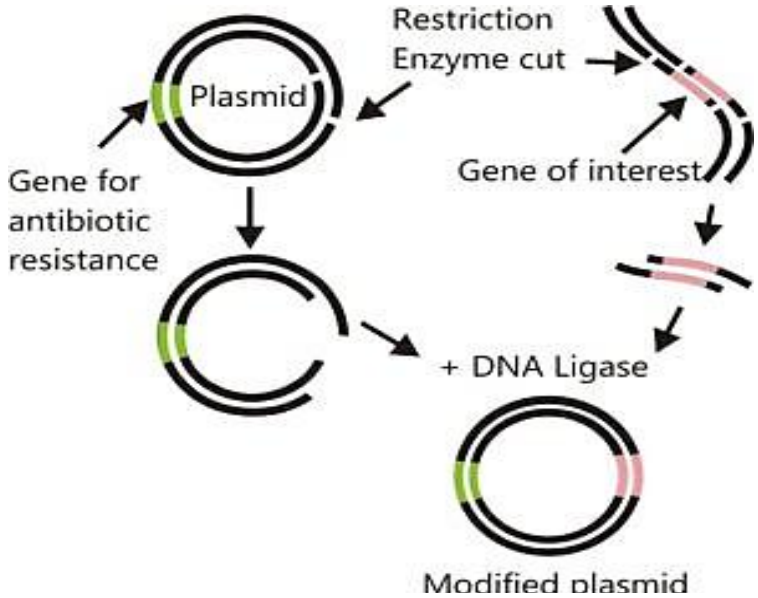

Figure 3: Restriction enzyme digestion followed by ligation

Enzyme DNA ligase is obtain from bacteriophage T4 and termed T4 DNA ligase. This enzyme forms covalent bond between the 5' phosphate at the end of one strand and then 3' hydroxyl of the adjacent strand.

7. **Introduction of recombinant DNA molecule into a suitable host i.e. transformation:** Introduction of recombinant DNA into host is achieved by two methods such as chemical methods and physical methods.

- **Chemical methods:**
  - Polyethylene glycol mediated
  - Calcium chloride mediated
  - DEAE dextran mediated

- **Physical methods:**
  - Electroporation
  - Microinjection
  - Liposome mediated
  - Particle bombardment
  - Sonoporation

8. **Selection of Transformed Host Cell:** Following the introduction of recombinant DNA into the host, three distinct types of cells emerge: those lacking the plasmid, those containing only the plasmid, and those harboring the recombinant DNA, referred to as chimeric DNA. It's important to note that not all bacterial cells take up the recombinant DNA; only a small fraction do. The plasmids utilized in cloning carry an antibiotic resistance gene. Consequently, all bacterial cells are placed onto an antibiotic plate to isolate the transformed cells that possess the recombinant DNA plasmid. Bacteria lacking the recombinant DNA plasmid do not survive. Each bacterium carrying the transformed plasmid generates a cluster of identical bacterial colonies. To identify the cells with the recombinant plasmid, several colonies are examined using PCR or restriction digestion techniques.

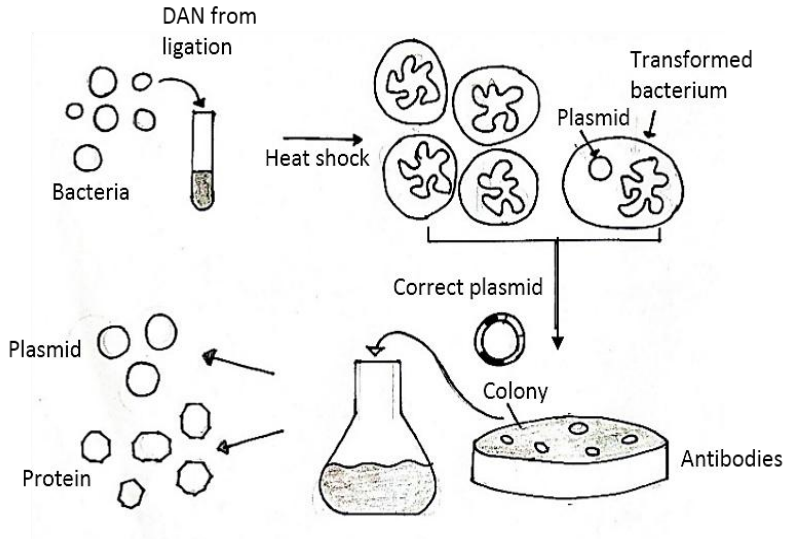

Figure 4: Bacterial transformation and selection

9. **Expression and multiplication of foreign DNA into host:** The colony containing the transformed plasmid is cultured in bulk and employed for protein expression. Within the host, the recombinant DNA undergoes replication, and the gene of interest generates mRNA, which is translated into a protein under ideal conditions. This resulting protein is now referred to as recombinant protein. A small-scale cell culture does not yield a significant quantity of recombinant protein. Consequently, large-scale production becomes essential to produce products with significant human applications. To accomplish this, vessels known as bioreactors are utilized. Bioreactors are sizable containers featuring a continuous culture system, in which fresh medium is introduced on one side, while used medium is removed from the other side. Bioreactors provide optimal conditions, including temperature, oxygen levels, pH, and essential vitamins, to facilitate

the conversion of raw materials into specific proteins, enzymes, and other desired products.

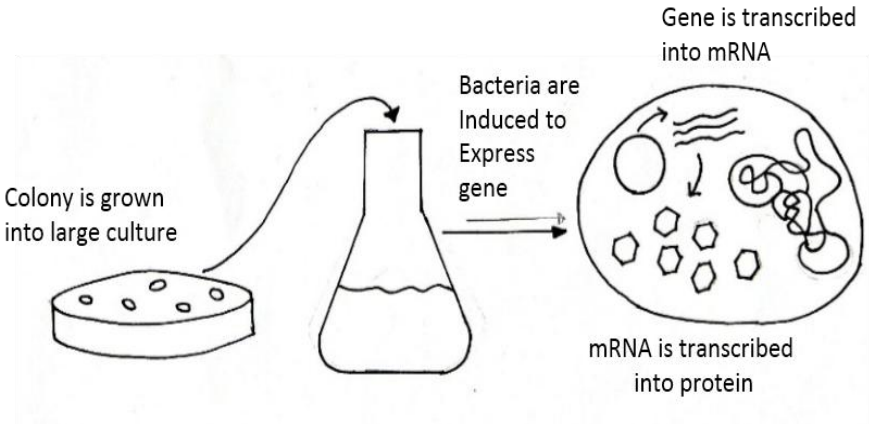

Figure 5: Expression of DNA of interest into host

10. **Purification of recombinant proteins:** Bacteria contain many proteins and macromolecules. So that newly formed proteins are need to be purified before it can be used. There are several methods are available for the purification of recombinant protein. One of the technique called **affinity chromatography** used for this purpose. A mixture of molecules obtained from the lysed bacteria is passed through a column that contains beads coated with antibodies. Consequently, an immune system protein selectively binds to the target molecule. Specifically, only the recombinant protein adheres to these beads, excluding other macromolecules. Consequently, the recombinant protein becomes immobilized within the column, while other molecules are removed during washing. In the last step, the recombinant protein is eluted from the column and collected for its intended use.

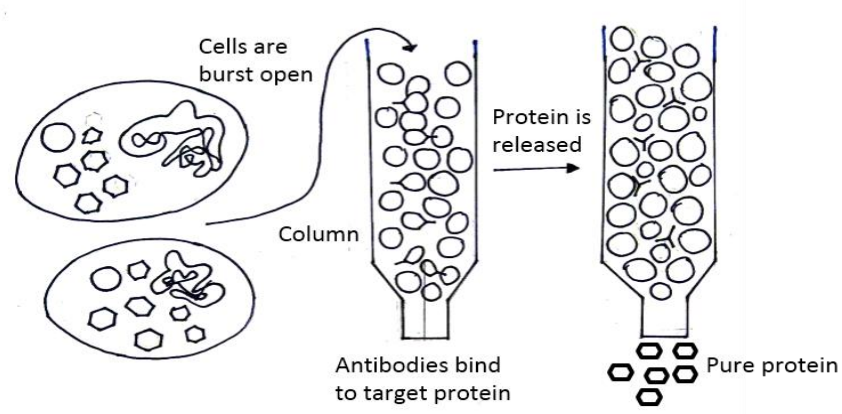

Figure 6: Purification of recombinant protein by affinity chromatography technique

VI. APPLICATIONS OF RECOMBINANT DNA TECHNOLOGY

There are three important applications of recombinant DNA technology:

- Applications in crop improvement
- Applications in medicine
- Applications in industry

1. **Applications in Crop Improvement:** rDNA technology has several potential applications in crop improvement.

    - **Distant hybridization:** Advancements in rDNA technology have made it feasible to transfer genes among species that are distantly related. Challenges related to gene transfer between different species or genera have been successfully addressed. The gene of interest can now be moved from lower organisms to higher organisms using recombinant DNA technology.

    - **Development of transgenic crops:** A crop that incorporates a foreign gene from another organism, enabling it to express a desired function, is termed a transgenic crop or genetically modified crop (GM crop). Using rDNA technology, various benefits can be achieved, including disease, insect, and pest resistance, herbicide tolerance, drought resistance, tolerance to metal toxicity, increased yield, induction of male sterility for plant breeding purposes, and improvements in crop quality. For instance, Bt-cotton and Bt brinjal have been developed to withstand pest attacks, reducing the need for pesticides. Additionally, recombinant DNA technology has been employed to create Golden rice, which is rich in vitamin A, as well as lysine-enriched pulses.

    - **Development of root nodule in cereal crops:**
      Leguminous plants host nitrogen-fixing bacteria called Rhizobium in their root nodules. Within these root nodules, Rhizobium bacteria convert atmospheric nitrogen into nitrates. Using rDNA technology, the genes in these bacteria responsible for nitrogen fixation can be transferred to cereal crops like wheat, rice, maize, barley, and others. This genetic modification equips these cereal crops with the ability to also fix atmospheric nitrogen.

    - **Development of C4 plants:**
      By means of protoplast fusion or recombinant DNA technology, it is possible to transform a C3 plant into a C4 plant, thereby enhancing the photosynthetic rate and consequently increasing crop yields. C4 plants exhibit a greater potential for biomass production compared to C3 plants.

2. **Applications in Health Care:** With the application of rDNA technology, scientists have the capability to manufacture antibiotics, hormones, vaccines, and interferon within the realm of medicine.

    - **Production of antibiotics:** Penicillium fungus and Streptomyces bacteria are employed in the large-scale production of widely recognized antibiotics: penicillin

and streptomycin, respectively. Utilizing rDNA technology, genetically optimized strains of these microorganisms are developed to enhance the yield of these antibiotics.

- **Production of hormone insulin:** Insulin has traditionally been derived from the pancreases of cows and pigs, and it possesses a slightly different structure than human insulin. As a result, it can lead to allergic reactions in patients. To overcome this issue, the human gene responsible for insulin production has been introduced into bacterial DNA. These recombinant bacteria are then employed for large-scale insulin production, and the insulin produced in this manner does not trigger allergic reactions in patients.

- **Production of vaccines:** Vaccines are created by introducing genes encoding antigens into pathogenic bacteria. These modified bacteria then generate antibodies, offering protection against infections caused by the same bacteria or virus.

- **Production of interferon:** Natural interferons are naturally generated in limited quantities from human blood cells, rendering them both scarce and expensive. Interferons play a crucial role in combating viral infections and serve as the first line of defense against viruses. However, thanks to rDNA technology, it is now feasible to produce interferons at a significantly lower cost.
- **Production of enzyme:** Some useful enzyme such as urikinase can be produce by rDNA technology. Urikinase enzyme used to dissolve blood clots.

- **Gene therapy:** Replacement of faulty gene with normal healthy gene is called as gene therapy. It is used to correct rare diseases like sickle cell anemia, hemophilia, phenylketonuria, alkaptonuria which are caused by the mutation in the single gene. rDNA technology is also used to produce human blood clotting factor VIII C. To treat the hemophilia, the clotting factor VIII C gene is cloned to express in mammalian cell lines and produce the protein VIII C which is responsible for blood clotting. In in vivo approach of gene therapy genes are direct introduced into target organ of patient. Therefore, this gene therapy is also called as **patient therapy**. On other hand, in in vitro approach of gene therapy, cells are isolated from the patient for the transfer of desired gene. Such transgenic cell are injected back into patient.

3. Applications in Industry:

- With help of recombinant DNA technology, a number of synthetic medical peptides are produced. All such products show least side effects. For example, octreotide, help in controlling diarrhea.

- Genes of biodegradable plastic polyhydroxylbutyrate (PHB) is isolated from bacterium Alcaligenes eutrophus and transferred to model plant Arabidopsis thaliana. Chloroplast of this plant produce PHB without producing plant's growth and development. This transgenic plant is used for the biodegradable plastic production.

- In oil industries such as groundnut, mustard, rapeseed, sesame, soybean and sunflower fatty acid quality and yield can be improved by recombinant DNA technology.

- Through recombinant DNA technology, chemical compounds with significant commercial value can also be manufactured. Enhancing current fermentation processes and generating proteins from waste materials can be accomplished by cultivating more efficient strains of microorganisms. Certain microbes are employed to remediate and detoxify environmental pollutants.