SEMESTER VI

17BTU601B BIOTECHNOLOGY AND HUMAN WELFARE 3H - 3C

Total hours/week: L:3 T:0 P:0 Marks: Internal: 40 External: 60 Total: 100

Scope: This paper deals with major techniques and methods involved in human welfare.

Objective: This paper will enable the students to learn the basics and lay strong foundation in understanding the biotechnological techniques in human welfare.

UNIT- I

Industry: Protein engineering; enzyme and polysaccharide synthesis, activity and secretion, alcohol and antibiotic formation.

UNIT-II

Agriculture: N2 fixation: transfer of pest resistance genes to plants; interaction between plants and microbes; qualitative improvement of livestock.

UNIT-III

Environments: Chlorinated and non-chlorinated organ pollutant degradation; degradation of hydrocarbons and agricultural wastes, stress management, development of biodegradable polymers such as PHB.

UNIT-IV

Forensic science: DNA finger printing and its applications in human welfare. Identification of origin-Paternity, crime.

UNIT-V

Health: Development of non-toxic therapeutic agents, recombinant live vaccines, gene therapy, diagnostics, monoclonal in *E.coli*, human genome project.

References

1. Sateesh, M.K. (2010). *Bioethics and Biosafety*. I. K. International Pvt Ltd.
2. Sree Krishna,V. (2007) *Bioethics and Biosafety in Biotechnology*. New age international publishers.

2017-2020 SEMESTER VI

17BTU601B BIOTECHNOLOGY AND HUMAN WELFARE LECTURE PLAN

S.No	Lecture Duration Period	Topics to be covered	Support Material/ Page No.
		UNIT I Industry	
1	1	Protein engineering	J1
2	1	Enzyme and polysaccharide synthesis	J2; J3
3	1	Activity and secretion	W1
4	1	Alcohol formation.	W1
5	1	Antibiotic formation.	W1
6	1	Unit test	
		Total No of Hours Planned for Unit I = 06	
		UNIT II Agriculture	
1	1	N2 fixation	J4
2	1	Transfer of pest resistance genes to plants	J5
3	1	Interaction between plants and microbes	J6
4	1	Qualitative improvement of livestock	T1
5	1	Unit test	
		Total No of Hours Planned for Unit II = 05	
		UNIT III Environments	
1	1	Chlorinated organ pollutant degradation	
2	1	Non-chlorinated organ pollutant degradation	
3	1	Degradation of hydrocarbons and agricultural wastes	
4		Degradation of agricultural wastes	
5	1	Stress management	
6	1	Development of biodegradable polymers such as PHB	
7	1	Unit test	
		Total No of Hours Planned for Unit III = 07	
		UNIT IV Forensic science	
1	1	DNA finger printing	
2	1	Applications in human welfare	
3	1	Identification of origin-Paternity	
4	1	Crime	
5	1	Unit test	
		Total No of Hours Planned for Unit IV = 05	
		UNIT V Health	
1	1	Development of non-toxic therapeutic agents	
2	1	Recombinant live vaccines	
3	1	Gene therapy	
4	1	Diagnostics	
5	1	Monoclonal Antibodies production in *E.coli*	
6	1	Human Genome Project	
7	1	Unit test	
		Total No of Hours Planned for Unit V = 07	

Prepared by Dr.T.Soundara Rajan, Assistant Professor, Dept. of Biotech., KAHE

Support Materials

Textbooks

T1: Abubaker et al. The Role of Biotechnology in Improvement of Livestock. Springer. 2015.

Website

https://nptel.ac.in › storage2 › nptel_data3 › html › mhrd › ict › text › lec1

Journals

J1: Turanli-Yildiz et al. Protein Engineering and Applications. Intechopen. 2012.

J2: Khan. Enzyme Technology – An Emerging Trend in Biotechnology. Enzyme Enginerring. 2018.

J3: Donato et al. Polysaccharides: Applications in Biology and Biotechnology/Polysachharides from Bioagro-waste new biomolecules-life. Polysaccharides. 2014.

J4: Oldroyd and Dizon. Biotechnological Solutions to the Nitrogen Problem. Current Opinion in Biotechnology. 2014.

J5: Khan. Gene Transfer Technologies in Plants: Roles in Improving Crops. Recent Research in Science and Technology. 2009.

J6: Shelake et al. Exploration of Plant-Microbe Interactions for Sustainable Agriculture in CRISPR Era. Microorganisms. 2019.

Protein engineering

Protein engineering is the design of new enzymes or proteins with new or desirable functions. It is based on the use of recombinant DNA technology to change amino acid sequences. Due to the development in rDNA technology and high-throughput screening techniques, protein engineering methods and applications are becoming increasingly important and widespread.

Protein engineering methods

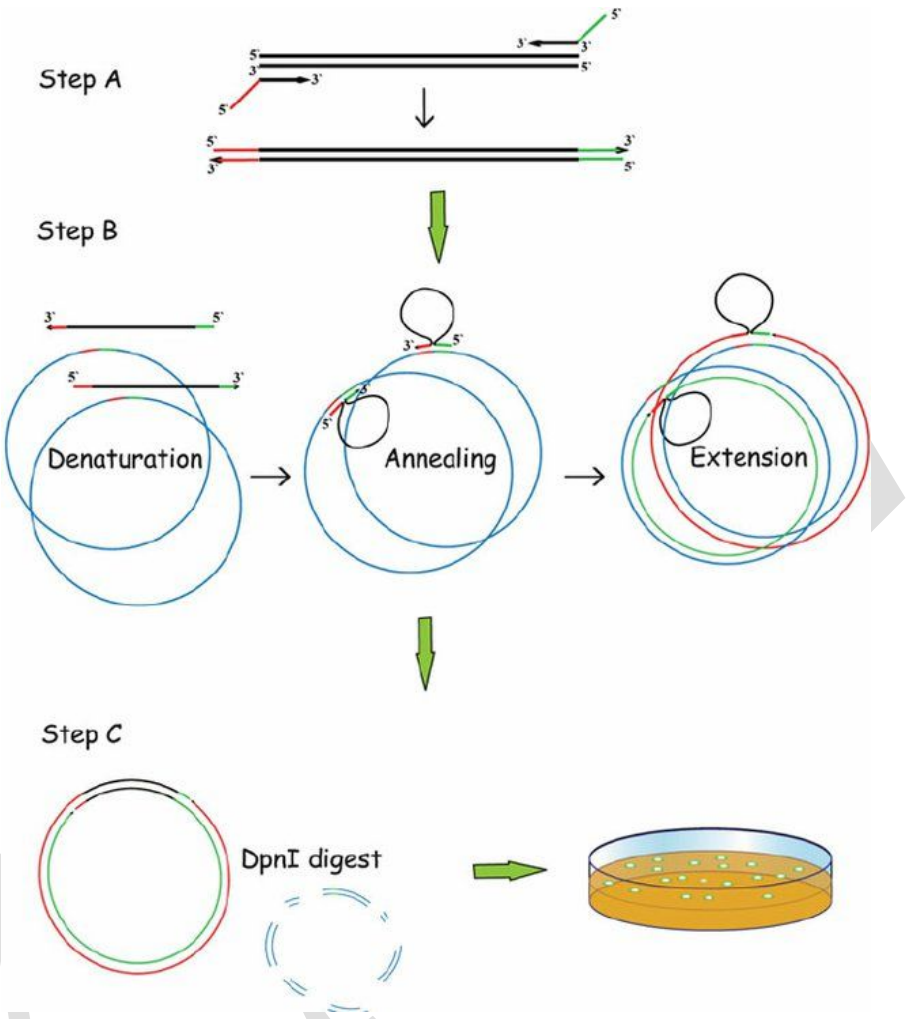
Many different protein engineering methods are available today, owing to the rapid development in biological sciences, more specifically, rDNA technology.

The most classical method in protein engineering is the so-called "rational design" approach which involves "site-directed mutagenesis" of proteins. **Rational design is an effective approach when the structure and mechanism of the protein of interest are well-known**.

Site-directed mutagenesis: This is a classical rational design method in protein engineering which allows introduction of specific amino acids into a target gene. There are two common methods for site-directed mutagenesis: **Overlap extension** & **Whole plasmid single round PCR**.

Overlap extension (also known as Circular Polymerase Extension Cloning):

- ***This method involves two primer pairs, where one primer of each primer pair contains the mutant codon with a mismatched sequence***.
- These four primers are used in the first polymerase chain reaction (PCR), where two PCRs take place (primary PCR), and two double-stranded DNA products are obtained.
- Upon denaturation and annealing of them, two heteroduplexes are formed, and each strand of the heteroduplex involves the desired mutagenic codon.
- DNA polymerase is then used to fill in the overlapping 3' and 5' ends of each heteroduplex and the second PCR (ligation PCR) takes place using the nonmutated primer set to amplify the mutagenic DNA.

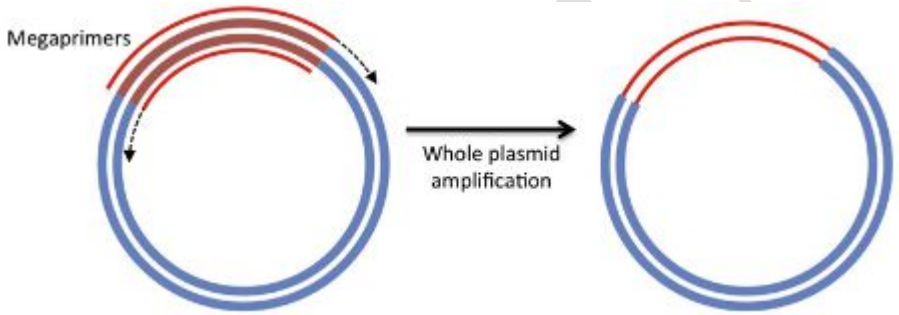


Overview of overlap extension PCR cloning. (Step A) The insert is PCR amplified with the chimericprimers. The ends of final PCR product overlap regions of the vector. (Step B) Vector and insert are mixed,denatured, and annealed. The hybridized insert is then extended by Phusion®polymerase using the vector asa template until polymerase reaches the 50-end of the insert. After several PCR cycles, the new plasmid withtwo nicks (one on each strand) accumulates as a product. (Step C) The parental plasmid is destroyed by DpnI digest, and new plasmid is used to transform E.coli.

Whole plasmid single round PCR (Megaprimer strategy):

- This method forms the basis of the commercial "QuikChange Site-Directed Mutagenesis Kit" from Stratagene. ***It requires two oligonucleotide primers with the desired mutation(s) which are complementary to the opposite strands of a double-stranded DNA plasmid template***.

- Using DNA polymerase PCR takes place, and both strands of the template are replicated without displacing the primers and a mutated plasmid is obtained with breaks that do not overlap.
- *Dpn*I methylase is then used for selective digestion to obtain a circular, nicked vector with the mutant gene.
- Upon transformation of the nicked vector into competent cells, the nick in the DNA is repaired, and a circular, mutated plasmid is obtained.



Overview of Whole plasmid Single round PCR: Gene of interest (GOI) is amplified with two primers containing 5′ sequences corresponding to the integration sites in the recipient vector. Following the initial amplification stage, the generated intermediate PCR products served as megaprimers for linear amplification of whole plasmid.

Random mutagenesis: This is an alternative approach when there is limited amount of information on the structure and mechanisms of the protein of interest.

Examples: **Saturation mutagenesis** & **Region specific random mutagensis**

Saturation mutagenesis: It involves the replacement of a single amino acid within a protein with each of the natural amino acids, and provides all possible variations at that site.

Region (localized) specific random mutagenesis: It is a combination of rational and random approaches of protein engineering.

It includes the simultaneous replacement of a few amino acid residues in a specific region, to obtain proteins with new specificities. This technique also makes use of overlap extension, and the whole-plasmid, single round PCR mutagenesis, as in the case of site-directed mutagenesis.

Peptidomimetics: It is another important method that finds applications in protein engineering.

- It involves mimicking or blocking the activity of enzymes or natural peptides upon design and synthesis of peptide analogs that are metabolically stable.
- It is an important approach for bioorganic and medical chemistry. It includes a variety of synthesis methods such as the use of a common intermediate, solid phase synthesis and combinatorial approaches.

A summary of different methods used in protein engineering

- Rational design
- Site-directed mutagenesis
- Evolutionary methods/directed evolution
- Random mutagenesis
- DNA shuffling
- Molecular dynamics
- Homology modeling
- 'MolCraft'in vitro protein evolution systems
- Computational methods (computational protein design)
- Receptor-based QSAR methods
- NMR
- X-ray crystallography
- Peptidomimetics
- De novo enzyme engineering
- mRNA display

Protein engineering applications

Protein engineering is used for food industry and environmental, medical and nanobiotechnology applications:

a) Food and detergent industry applications:

- Important application area of protein engineering regarding food industry is the wheat gluten proteins. Their heterologous expression and protein engineering has been studied using a variety of expression systems, such as E.coli, yeasts or cultured insect cells.

- Wildtype and mutant wheat gluten proteins were produced to compare them to each other for protein structure-function studies because of their availability, rapid and easy use, as well as high expression levels.

- Food industry makes use of a variety of food-processing enzymes, such as amylases and lipases, the properties of which are improved using rDNA technology and protein engineering.

- The deletion of native genes encoding extracellular proteases, for example, increased enzyme production yields of microbial hosts.

- Some large groups of enzymes like Proteases, Amylases and Lipases are important for both food and detergent industries, as they have a broad range of industrial applications.

- **Proteases** are used in several applications of food industry regarding low allergenic infant formulas, milk clotting and flavors. They are also important for detergent industry for removing protein stains.

- **Amylases** are also important for both food and detergent industries. In food industry, they are use for liquefaction and saccharification of starch, as well as in adjustment of flour and bread softness and volume in baking. The detergent industry makes use of amylases in removal of starch stains.

- **Lipases** are used in many applications of food industry such as for the stability and conditioning of dough (as an in situ emulsifier), and in cheese flavour applications. Lipases are also crucial for the detergent industry, as they are used in removal of lipid stains.

b) **Enviromental applications:**

- Environmental applications of enzyme and protein engineering are also another important field.

- Genetic methods and strategies for designing microorganisms to eliminate environmental pollutants and included gene expression regulation to provide high catalytic activity under environmental stress conditions, such as the presence of a toxic compound, rational changes introduced in regulatory proteins that control catabolic activities, creation of new metabolic routes and combinations.

- Many organic pollutants such as phenols, azo dyes,organophosphorus pesticides and polycyclic aromatic hydrocarbons can be detoxified using enzymatic oxidation.

- It is used in Petroleum biorefining.

c) **Medical applications:**

- The use of protein engineering for cancer treatment studies is a major area of interest. Pretargeted radioimmunotherapy has been discussed as a potential cancer treatment. By pretargeting, radiation toxicity is minimized by separating the rapidly cleared radionuclide and the long-circulating antibody.

- Advances in protein engineering and recombinant DNA technology are expected to increase the use of pretargeted radioimmunotherapy.

- The use of novel antibodies as anticancer agents is also an important field of application, where the ability of antibodies to select antigens specifically and with high affinity is exploited, and protein engineering methods are used to modify antibodies to target cancer cells for clinical applications.

- Recently, the term "modular protein engineering" has been introduced for emerging cancer therapies.

- Treatment strategies based on targeted nanoconjugates to be specificallydirected against target cells are becoming increasingly important. Additionally, multifunctional and smart drug vehicles can be produced at the nanoscale, by protein engineering. These strategies could be combined to identify and select targets for protein-based drug delivery.

- Pharmacokinetic properties of antibodies have been improved by protein engineering and antibody variants of different size and antigen binding sites have been produced for the ultimate use as imaging probes specific to target tissues.

- Molecular imaging tools based on antibodies will find more applications in the future regarding diagnosis and treatment of cancer and other complex diseases.

d) **Biopolymer production:**

- Protein engineering applications for biopolymer production are also promising. Particularly, peptides are becoming increasingly important as biomaterials because of their specific physical, chemical and biological properties.

- Protein engineering and macromolecular self assembly are utilized to produce peptide-based biomaterials, such as elastin-like polypeptides, silk-like polymers.

- The ability of protein engineering to create and improve protein domains can be utilized for producing new biomaterials for medical and engineering applications.

e) **Nanobiotechnology:**

- Nanobiotechnology applications of protein engineering are becoming increasingly important. The synthesis and assembly of nanotechnological systems into functional structures and devices has been difficult and limiting their potential applications for a long time.

- Biological macromolecules, such as proteins, carbohydrates and lipids are used in the synthesis of biological tissues in aqueous environments and mild physiological conditions, where this biosynthetic process is under genetic regulation.

- Particularly proteins are crucial elements of biological systems, based on their roles in transport, regulation of tissue formation, physical performance and biological functions. Thus, they are suitable components for controlled synthesis and assembly of nanotechnological systems.

- Combinatorial biology methods commonly applied in protein engineering studies, such as phage display and bacterial cell surface display technologies, are also used to select polypeptide sequences which selectively bind to inorganic compound surfaces , for ultimate applications of nanobiotechnology.

- Another interesting nanotechnology application is the use of amyloid fibrils as structural templates for nanowire construction. This application is based on the fact that some proteins form well-ordered fibrillar aggregates that are called amyloid fibrils.

- The self association of well-ordered growth fibrils through noncovalent bonds under controlled conditions was suggested to have a high potential to be used for nanobiotechnology. The use of amyloid fibrils as structural templates for nanowire construction.

f) **Novel applications:**

- "Insertional protein engineering" applications are also becoming important, particularly for biosensor studies such as analytical molecular sensing.

- "Zinc finger protein engineering" is another approach that has been used in gene regulation applications. The zinc finger design and principle is used to design DNA binding proteins to control gene expression.

- "Virus engineering" is another emerging field, where the virus particles are modified by protein engineering. Viruses have many promising applications in medicine, biotechnology and nanotechnology. They could be used as new vaccines, gene therapy and targeted drug

delivery vectors, molecular imaging agents and as building blocks for electronic nanodevices or nanomaterials construction.

Enzyme Engineering/Technology

Enzymes are biological molecules with a definite structural organization that influence their catalytic function. Currently enzymes are being employed in industrial biotechnology for numerous purposes for the production of novel and sustainable products at a speedy rate. Enzyme technology or Enzyme engineering encompasses **modification of enzyme structure or its catalytic function to yield novel metabolites or to take part in new reaction pathways**. Enzyme technology is primarily engaged in the production, isolation, purification and use of enzymes either in the soluble or immobilized form, for the benefit of humankind. With the advancements in recombinant DNA technology, enzyme engineering produces more effective and diverse group of enzymes with useful applications in microbiology, biochemistry, diagnostics, therapeutics, biocatalysis, structural biology etc. Tee overall objective of this emerging technology is to produce unique sustainable products with specific function to fulfill the need of growing population. This technology enables the commercial synthesis of numerous enzymes with diverse applications in **food processing and preservation, detergents, textile industry, leather industry, paper industry, medicine** etc.

Enzymes classification

Class	Properties
Oxidoreductases	Co substrate required
Transferases	Two substrates reaction
Hydrolases	Two substrates reaction
Ligases	Requires ATP as co substrate
Isomerases	One substrate reaction
Lyases	Require only one **substrate** for the **reaction** in one direction, but **two substrates** for the reverse **reaction**.

Functional capabilities of enzymes are determined by their structure. Active sites of enzymes are the sites for their substrate specific catalytic function, while the rest of the protein acts as scaffolding.

Objectives of enzyme engineering

1) Fabrication of high value specific products by superior enzyme

2) To yield greater concentration of enzymes.

3) Assembly of synthetic drugs, peptides and proteins

4) Producing enzymes with unique properties such as ability to function under extreme environmental conditions

5) Alteration in enzyme properties to produce the desired function such as kinetic features of enzyme-turnover, thermal stability, enzyme activity in nonaqueous solvent, reaction/substrate specificity subunit structure etc.

Enzyme production technology

The basic steps taken for enzyme production includes:

a) Organism selection: Selection of organism depends on the ability of microorganism to yield increased concentration of desired enzyme in a short time period with decreased amounts of secondary metabolites. Microbial inoculums introduced in sterilized liquid medium for fermentation, providing optimal growth conditions such as pH, temperature, O_2 supply, nutrient.

b) Isolation and purification of enzyme: Desired enzyme produced may be excreted into the culture medium or may be present within the microbial cells is recovered and purified by cell disruption techniques and downstream processing respectively ensuring minimal loss of enzyme activity.

c) Immobilization of enzyme: Immobilization of enzyme is achieved on cellulose, polyacrylamide, starch and beads etc., by the following methods:

• ***Adsorption***: It involves the attachment of enzyme molecule on an inorganic or organic inert solid support such as silica gel, beads or glass, starch, cellulose etc., by forming weak van dar Waals forces or hydrogen bonding. Later enzymes are removed by slight alterations in pH, ionic strength or temperature.

• ***Cross linking***: Enzyme can be immobilized by forming crosslinkage to another chemical such as cellulose or glyceraldehydes but it might cause denaturation of enzyme.

• ***Entrapment***: Enzymes can be immobilized by physical entrapment inside a mesh, capsule or a gel matrix of an inert material such as gelatin, polyacrylamide gel, starch, collagen, silicone, cellulose and rubber.

• ***Covalent binding***: Immobilization of the enzymes by means of covalent bonding with the support providing the strongest enzyme-support interaction.

Applications of enzyme technology

A) **Textile industry**

• Amylases enzyme is used as softening agents for starched clothes.

• Cellulases is useful for fabric finishing and often used for better dye uptake in cotton.

B) **Leather industry**

• Proteolytic enzymes is used in dehairing of the skin and for softening /plumping of dehaired skin.

C) **Detergent industry**

• Proteases are efficient in removing protein stains such as grass, blood, egg and human sweat.

• Lipases can easily break down lipid and protein based stains.

• Amylase is efficient in removing starch-based residues.

• Cellulase used in detergent causing restoring fibre smoothness, its original color and softening.

D) **Pharmaceutical industry**

• Pegadamase bovine is employed for SCID treatment.

• Pancreatic enzymes are effective against fat malabsorption in HIV patients.

• Spinal injuries could be treated with chondroitinases.

• Hyaluronidase aids in the rebuilding of damaged nerve tissues.

• Lysozyme is used as an antibacterial agent and also possesses activity against HIV.

• Human melanoma and hepatocellular carcinomas could be inhibited by PEGylated arginine deaminase.

E) Food industry

• Amylase is added to the dough to ensure its quality and increased shelf life.

• Enzymes are also employed in cheese making by milk coagulation.

• Proteolytic enzymes help to increase the shelf life of meat and sea food.

• Lipases used to control the lipid content of food products.

• Glucose oxidase and catalase are used as food preservative agents.

• Renin is used in the manufacture of cheese.

• Lactase enzymes is applied in the making of ice cream and yoghurt.

• Invertase used in the making of chocolate covered berries and candies.

• Glucose isomerase is used for the production of fructose and high fructose syrups.

Polysaccharide synthesis using Biotechnological approaches

Polysaccharides, in many forms, play a central role in all living organisms for supply and storage of energy and/or structural integrity and protection of cells. The science for use of polysaccharide-based substances is well evolved in manufacturing of health and cosmetic products, food and feed production, and for cellulose derived materials (wood products, paper and cellulose derivatives, or textiles). Advances in the use of polysaccharides are closely linked to the ability of the scientific community to unravel the complexity of polysaccharides in nature. Translation of this knowledge to practical applications is required to model and shape the various biological, physical and chemical inter-relations taking place and to invent new and improved characterisation tools.

Polysaccharides from Bioagro-Wastes: A Sustainable Source of Chemicals and Energy

Polysaccharides' market is continuously increasing because of the wide range of potential applications of this class of natural polymers that indeed, as such or by means of chemical and/or biological transformations, can constitute the main feedstock in many industrial activities such as food, materials, chemicals, and energy production.

Polysaccharides are produced by animals, microorganisms, and plants, but the latter are the main source of these biomolecules: indeed about 90 % of total natural polysaccharides produced on earth can be found in the vegetables.

Polysaccharides are the main constituents of vegetable biomass that, in turn, is currently exploited for the production of chemicals, materials, and energy: many examples of biorefinery facilities (a new production system resembling the petroleum refinery for the production of chemicals and fuels, but that is based on renewable feedstock like vegetables) can be found such as corn or sugarcane refineries that produce food and biofuel.

Notably polysaccharides are massively processed for different production chains; they are indeed the sources of the sugars that in turn are the starting materials for the production of those chemical compounds that have been included in the list of the so-called "building block" molecules, according to the National Renewable Energy Laboratory (NREL) of the United

States. These "building block" substances (i.e., succinic, fumaric, and malic acids; 2,5-furandicarboxylic acid, 3-hydroxypropionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol) are fundamental feedstock for the production of secondary and intermediate chemicals that are then used in several industrial sectors such as cosmetic, pharmaceutical, material, food, transportation, industry, etc.

For these reasons in the last years, an increasing demand of plant polysaccharide-derived goods (energy and foods) has caused an increase in the intensity of acreage, agricultural production, and agro-industrial activities that in turn generate million tons of wastes. Such wastes, i.e., bioagrowastes, comprise all the residues produced from horticulture and/or agricultural activities during cultivation, postharvest, and processing of plants. Indeed all the different phases of manufacturing of vegetable materials (e.g., selection of fruits and vegetables for the whole market or food industries, industrial processing of crops for the production of chemicals, energy, food, and so on) produce every year huge amounts of highly heterogeneous residues. Depending on the feedstock and on the production chain considered, such waste biomass is constituted by unemployed parts of plants, like roots, straw, leaves, cobs, etc., or by vegetable transformation residues like exhausted pulps, peels, and seeds, etc.

The proper disposal of such residual biomass represents a critical environmental concern and an economical problem that all agro-industries have to face with. Nonetheless, since bioagro-wastes still contain a variety of value-added chemicals, they could be considered as starting material for other production chains rather than residual matter. Indeed significant amounts of proteins, polysaccharides and fibers, polyphenols, carotenoids, fatty acids, etc., that are lost in the discarded materials could be used for the production of a variety of goods like biomaterials, food additives, nutraceuticals, antioxidant and antimicrobial agents, bioenergy, etc.

The reuse and then the valorization of bioagro-wastes could be implemented in the frame of an integrated biorefinery approach to biomass exploitation, in which the exploitation of full plant feedstock could be a solution to the issues of sustainability and of waste disposal.

Therefore, on these bases, vegetable biomass like bioagro-wastes is under investigation as sources of biologically and biotechnologically useful polysaccharides.

Biotechnological Approaches to Waste Polysaccharide Exploitation

Polysaccharides from renewable and sustainable sources, like food and agricultural wastes, can play a central role in the emerging biobased economy, i.e., the new economy system shifting from fossil resources of energy and chemicals to renewable resources such as biomass. Indeed the wide variety of structures and biological functions of polysaccharides that can be recovered from agro-wastes make them biotechnologically useful biopolymers that either are already exploited for several purposes or are under investigation for new applications.

Main industrial applications of polysaccharides:

a) for biomaterials and biodegradable plastics production

b) for food packaging

c) for pharmaceutical industry

d) for fuel ethanol production and

e)enzyme production

Since a great share of industrial production of chemicals and energy is based on the exploitation of plant polysaccharides, like starch, the search for new biotechnologies and strategies for exploiting and employing waste materials in several industrial fields is therefore the object of growing interest.

Such an approach to polysaccharide waste biomass exploitation comes under the frame of the so-called biorefinery, i.e., a new production system in which a variety of goods (fuels, chemicals, and biomaterials) can be recovered from renewable feedstock by using a single or a combination of chemical, physical, and biological treatments. Biorefinery of biomass is a multistep process that can be divided in: choice of starting material and of suitable pretreatment method, in order to render it more prone to the following processes, and conversion of the pretreated biomass by means of one or combined biological, chemical, and physical techniques

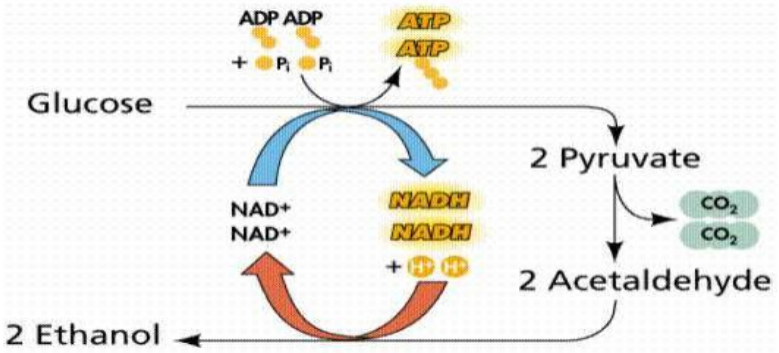
to obtain energy or value-added chemicals and/or building blocks for further production processes.

Polysaccharide rich waste biomass is the ideal feedstock for biorefinery since they are renewable, not in competition with the food chain and not impacting on land use and biodiversity.

In addition, such materials are available in significant amounts every year and therefore are under investigation for several industrial applications, for example, production of green chemicals, biomaterials, second-generation biofuel, etc.

Alcohol formation

Production of ethyl alcohol from sugary materials is one of the oldest known microbiological processes. Alcohol is an important solvent and raw material used in a variety of chemical industries. Although today industrial alcohol is also produced synthetically from ethylene, production of alcohol by fermentation of cheap sugary materials such as molasses by yeast is still an important industry.



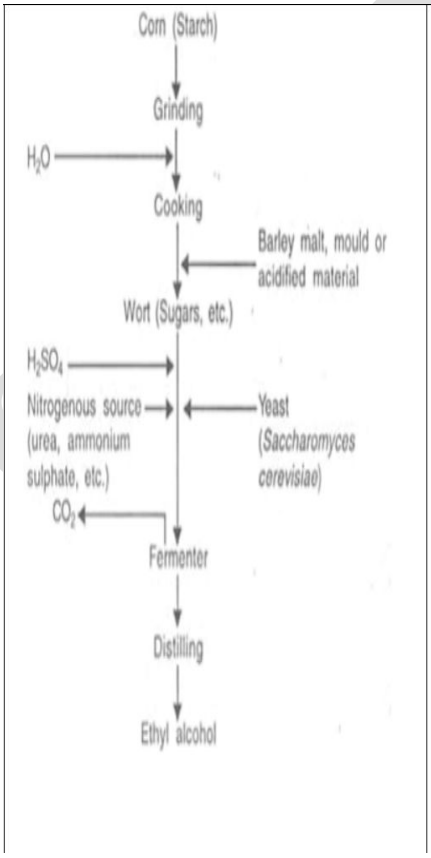
For ethyl alcohol production, selected strains of Saccharomyces cerevisae are employed since all the strains are not equally efficient. The alcohol tolerance and sugar tolerance are important criteria used in the selection of yeast strains. Strains tolerant to high sugar and alcohol concentration are desired.

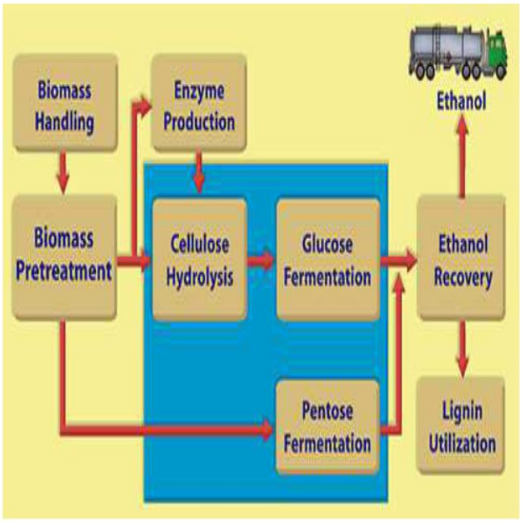
The raw material generally used is either crude cane molasses or best molasses which contain about 50 per cent fermentable sugars. The production process involves the dilution of molasses to a suitable sugar concentration(15- 16 per cent sugars), addition of small quantity of nitrogen source (urea, ammonium sulphate or ammonium phosphate), adjustment of pH to about 5.0 and the addition of anactively growing yeast culture.

The fermentation is carried out in big deep tanks of steel or stainless steel. The fermentation is allowed to continue for about 24 – 36 h at 25^0 C to 30^0 C after which the

cells are allowed to settle. The fermented mash is then distilled and passed through rectifying columns to recover ethyl alcohol. A large amount of carbon-di oxide is also produced during the fermentation which is purified and compressed. The yield of ethyl alcohol is about 50 per cent of the fermentable sugar concentration.

Further purification of ethyl alcohol is done by fractional distillation. In some distilleries, the yeast is recovered and used as animal feed while in most, it is discarded into the effluents, a procedure that is very undesirable. In recent years because of the possibility of using ethyl alcohol as a fuel supplement and a chemical feed stock, there is increased interest in increasing production but at a cheaper and economical rate.





For this, a variety of improvements in the traditional batch fermentation have been described in literature.

Among these, the one that has attracted attention is the cell recycle technique which does not involve much additional expenditure. Basically, the technique involves the reuse of cell mass that is produced during the fermentation.

It has been found that by doing so, about 5-10 per cent of the substrate which would have been otherwise used for cell growth is saved in addition to a great saving in the cost of inoculum and time.

By using recycling technology, fermentation time has been drastically reduced from 24-36 hours in a batch fermentation to as low as 5-6 hours.

Antibiotics Production

Production of Penicillin

- Penicillin (sometimes abbreviated PCN or pen) is a group of antibiotics derived from *Penicillium* fungi, including penicillin G (intravenous use), penicillin V (oral use), procaine penicillin, and benzathine penicillin (intramuscular use).

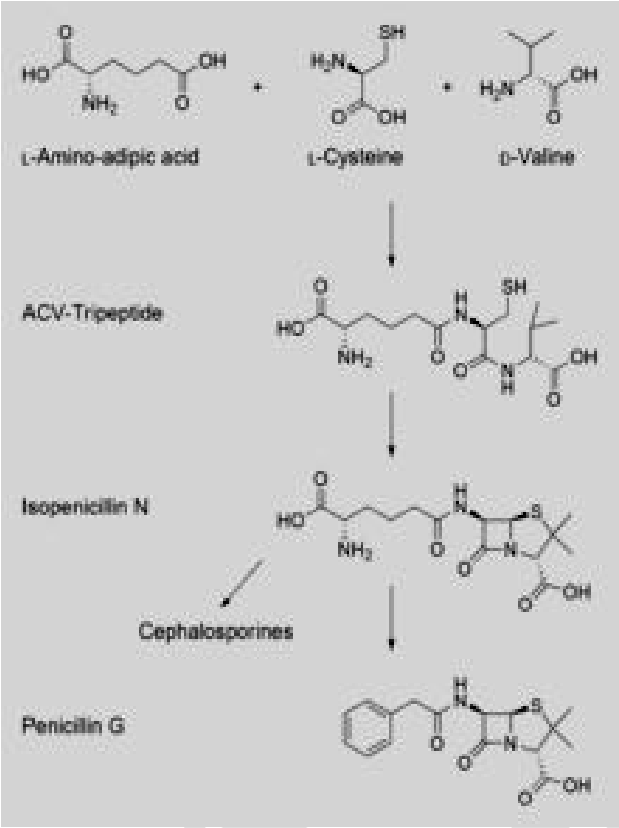
- Antibiotics such as penicillin are usually produced in large cylindrical vats, constructed of stainless steel, containing a liquid medium in which *Penicillium chrysogenum* is grown.

Penicillin biosynthesis in cell

- Overall, there are three main and important steps to the biosynthesis of penicillin G(benzylpenicillin).

- The first step is the condensation of three amino acids—L-α-aminoadipic acid, L-cysteine, L-valine into a tripeptide. Before condensing into the tripeptide, the aminoacid L-valine must undergo epimerization to become D-valine. The condensed tripeptide is named δ-(L-α-aminoadipyl)-L-cysteine-D-valine (ACV).

- The condensation reaction and epimerization are both catalyzed by the enzyme δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase (ACVS), anonribosomal peptide synthetase or NRPS.
- The second step in the biosynthesis of penicillin G is the oxidative conversion of linear ACV into the bicyclic intermediate isopenicillin N by isopenicillin N synthase(IPNS), which is encoded by the gene *pcbC*. Isopenicillin N is a very weak intermediate, because it does not show strong antibiotic activity.



- The final step is a transamidation by isopenicillin N N-acyltransferase, in which the α-aminoadipyl side-chain of isopenicillin N is removed and exchanged for aphenylacetyl side-chain.

- This reaction is encoded by the gene ***penDE***, which is unique in the process of obtaining penicillins.

Production

- Penicillin is a secondary metabolite of certain species of *Penicillium* and is produced when growth of the fungus is inhibited by stress.

- It is not produced during active growth. Production is also limited by feedback in the synthesis pathway of penicillin.

  α-ketoglutarate + AcCoA → homocitrate → L-α-aminoadipic acid → L-lysine + β-lactam

- The by-product, L-lysine, inhibits the production of homocitrate, so the presence of exogenous lysine should be avoided in penicillin production.

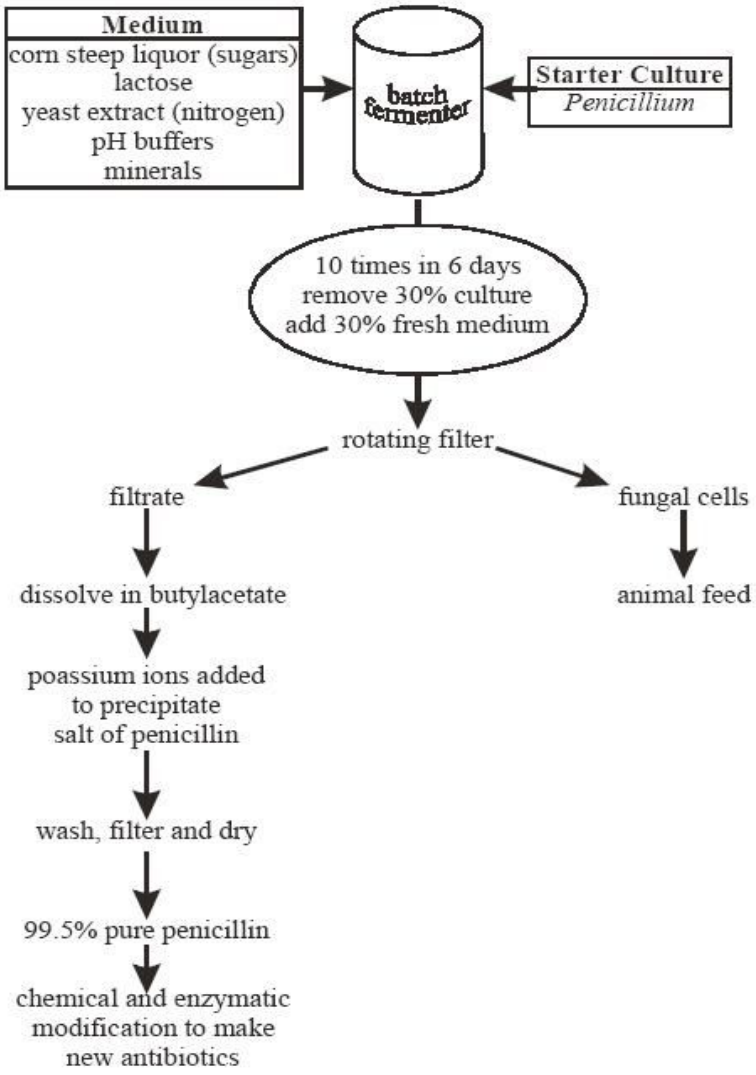
- The *Penicillium* cells are grown using a technique called fed-batch culture, in which the cells are constantly subject to stress, which is required for induction of penicillin production.

- The available carbon sources are also important: Glucose inhibits penicillin production, whereas lactose does not. The pH and the levels of nitrogen, lysine, phosphate, and oxygen of the batches must also be carefully controlled.

- The biotechnological method of directed evolution has been applied to produce by mutation a large number of *Penicillium* strains.

- These techniques include error-prone PCR, DNA shuffling, ITCHY, and strand-overlap PCR.

- Semisynthetic penicillins are prepared starting from the penicillin nucleus 6-APA. Before use, fermenters must be sterilised, usually with superheated steam.



Usually these fermenters are operated in a **batch process.** After a certain amount of time for fungal growth, followed by gradual production of antibiotic, the contents are removed and processed to extract the antibiotics, then the fermenter is cleaned, sterilised and the process is repeated.

Penicillin extraction

- After 6-8 days of batch culture, the liquid medium is pumped out, filtered and concentrated. The basic antibiotic - benzyl penicillin - is precipitated as crystals when potassium compounds are added.

- This antibiotic may then be modified by the action of other micro-organisms or by chemical means, before being mixed with inert substances and pressed into tablets or converted into syrup or injectable form.

- Although the molecular structure of penicillin is known, and it may be synthesised by chemical methods, it is not economic to do so.

- The production process still relies on fungal fermentation based on biological principles, although **modern strain**s are much **more productive** than the early strains.

- This has been achieved through screening programmes involving isolates from different sources, and treatment to encourage mutations.

Production of streptomycin

- Streptomycin is a water-soluble aminoglycoside derived from Streptomyces griseus. It is marketed as the sulfate salt of streptomycin.

- Streptomycin was first isolated on October 19, 1943 by Albert Schatz, a graduate student, in the laboratory of Selman Abraham Waksman at Rutgers University.

Uses

- Streptomycin can be used for the treatment of pneumonia, spinal meningitis, and typhoid fever.

- Streptomycin Sulphate Injection This product is used in the treatment of acute infections caused by various kinds of sensitive bacteria strains, like respiratory tract infections (pneumonia, laryngopharyngitis and bronchitis), urinary tract infections. Streptomycin is also used as a pesticide, to combat the growth of bacteria, fungi, and algae. A major use is in the control of fire blight on apple and pear trees.

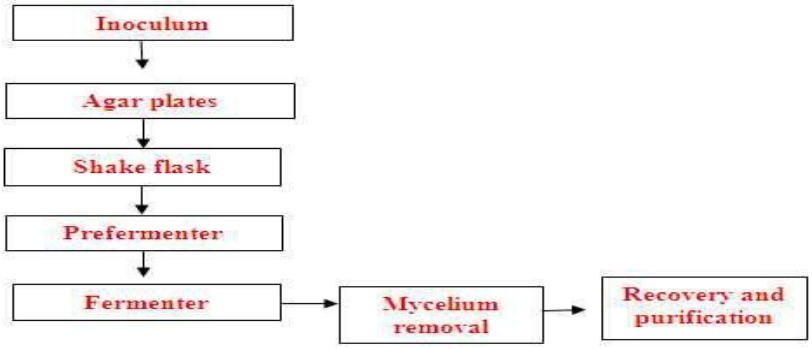
Culturing

Isolated strain of *S. griseus* was sub-cultured on Starch casein agar slant.

Production of streptomycin

- Starch casein broth medium was used as suitable medium for the production of streptomycin.

- Prepared starch casein broth medium by adding starch, peptone beef extract, casein (1% solution) in distilled water, casein solution pasteurized at 72ºC for 30 min., When medium is prepared then inoculate the *Streptomyces griseus* into the broth. Then fermentation broth incubated in the shaker incubator for 7-8 days at 37 ºC.



Purification of the streptomycin

- A general laboratory method was used for the purification of streptomycin. In this method Centrifuged the fermentation media for 10 min. at 6000rpm and 4ºC, Collect supernatant and filtered by using wattmann`s no.1 filter paper, Taken filtrate, added 2 present (2g. in 100 ml) activated charcoal kept the solution in the shaker incubator for 20 min, Filter the solution by using wattmann's no.1 filter paper, After 2 min. added 10 ml of phosphate buffer in the residue, Taken filtrate and allow to it for crystallization at room temperature, After crystallization added 2-3 ml of phosphate buffer for dissolving the crystal.

Activity and Purity screening

Using agar well and disc diffusion methods the purified streptomycin were screened their potentials with inhibiting the bacterial culture.

UNIT II

Agriculture

Nitrogen fixation

Nitrogen is an essential plant nutrient and a key determinant of crop productivity. Nitrogen is a critical limiting element for plant growth and production. It is a major component of chlorophyll, the most important pigment needed for photosynthesis, as well as amino acids, the key building blocks of proteins. It is also found in other important biomolecules, such as ATP and nucleic acids. Even though it is one of the most abundant elements (predominately in the form of nitrogen gas (N_2) in the Earth's atmosphere), plants can only utilize reduced forms of this element.

Plants acquire reduced forms of "combined" nitrogen by:

1) the addition of ammonia and/or nitrate fertilizer (from the Haber-Bosch process) or manure to soil,

2) the release of these compounds during organic matter decomposition,

3) the conversion of atmospheric nitrogen into the compounds by natural processes, such as lightning, and

4) biological nitrogen fixation.

Biological nitrogen fixation (BNF), discovered by Beijerinck in 1901, is carried out by a specialized group of prokaryotes. These organisms utilize the enzyme **nitrogenase** to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Plants can readily assimilate NH_3 to produce the nitrogenous biomolecules. These prokaryotes include aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as *Azotobacter*, bacteria that form associative relationships with plants, such as *Azospirillum*, and most importantly, bacteria, such as ***Rhizobium*** **and** ***Bradyrhizobium***, that form symbioses with legumes and other plants.

The Process

The reduction of atmospheric nitrogen is a complex process that requires a large input of energy to proceed. The nitrogen molecule is composed of two nitrogen atoms joined by a triple covalent bond, thus

making the molecule highly inert and nonreactive. Nitrogenase catalyzes the breaking of this bond and the addition of three hydrogen atoms to each nitrogen atom.

Microorganisms that fix nitrogen require **16 moles of adenosine triphosphate (ATP)** to reduce each mole of nitrogen. These organisms obtain this energy by oxidizing organic molecules. Non-photosynthetic free-living microorganisms must obtain these molecules from other organisms, while photosynthetic microorganisms, such as cyanobacteria, use sugars produced by photosynthesis. Associative and symbiotic nitrogen-fixing microorganisms obtain these compounds from their host plants' **rhizospheres**.

Industries use the **Haber-Bosch** process to reduce nitrogen essentially in the same way. Conventional agriculture has depended upon this process to produce the commercial fertilizer needed to grow most of the world's hybrid crops. But this approach comes with many consequences, including using fossil fuels for the energy needed to produce this fertilizer, the resulting carbon dioxide emissions and pollution from burning these fuels, and adverse affects on human health.

Overuse of these chemical fertilizers has led to an upset in the nitrogen cycle and consequently to surface water as well as groundwater pollution. Increased loads of nitrogen fertilizer to freshwater, as well as marine ecosystems, has caused eutrophication, the process whereby these systems have a proliferation of microorganisms, especially algae. This "greening" of the water column has caused decreased levels of dissolved oxygen (DO) in bottom waters as planktonic algae die and fuel microbial respiration. These depleted DO levels result in massive mortality of aquatic organisms and create so-called **dead zones**, areas where little or no aquatic life can be found.

Nitrogen Fixation by Free-Living Heterotrophs

Many heterotrophic bacteria live in the soil and fix significant levels of nitrogen without the direct interaction with other organisms. Examples of this type of nitrogen-fixing bacteria include species of *Azotobacter*, *Bacillus*, *Clostridium*, and *Klebsiella*. These organisms must find their own source of energy, typically by oxidizing organic molecules released by other organisms or from decomposition. There are some free-living organisms that have chemolithotrophic capabilities and can thereby utilize inorganic compounds as a source of energy.

Because nitrogenase can be inhibited by oxygen, free-living organisms behave as anaerobes or microaerophiles while fixing nitrogen.

Associative Nitrogen Fixation

Species of *Azospirillum* are able to form close associations with several members of the *Poaceae* (grasses), including agronomically important cereal crops, such as rice, wheat, corn, oats, and barley. These bacteria fix appreciable amounts of nitrogen within the rhizosphere of the host plants.

The level of nitrogen fixation is determined by several factors, including

Soil temperature (*Azospirillum* species thrive in more temperate and/or tropical environments),

The ability of the host plant to provide a rhizosphere environment low in oxygen pressure,

The availability of host photosynthates for the bacteria,

The competitiveness of the bacteria, and

The efficiency of nitrogenase.

Symbiotic Nitrogen Fixation

Many microorganisms fix nitrogen symbiotically by partnering with a host plant. The plant provides sugars from photosynthesis that are utilized by the nitrogen-fixing microorganism for the energy it needs for nitrogen fixation. In exchange for these carbon sources, the microbe provides fixed nitrogen to the host plant for its growth.

One example of this type of nitrogen fixation is the water fern *Azolla*'s symbiosis with a cyanobacterium *Anabaena azollae*. *Anabaena* colonizes cavities formed at the base of *Azolla* fronds. There the cyanobacteria fix significant amounts of nitrogen in specialized cells called heterocysts. This symbiosis has been used for at least 1000 years as a biofertilizer in wetland paddies in Southeast Asia.

Another example is the symbiosis between actinorhizal trees and shrubs, such as Alder (*Alnus* sp.), with the actinomycete *Frankia*. These plants are native to North America and tend to thrive in nitrogen-poor environments. In many areas they are the most common non-legume nitrogen fixers.

The most important nitrogen-fixing symbiotic associations are the relationships between **legumes and *Rhizobium* and *Bradyrhizobium* bacteria.** Important legumes used in agricultural systems include alfalfa, beans, clover, cowpeas, lupines, peanut, soybean, and vetches.

Legume Nodule Formation

The *Rhizobium* or *Bradyrhizobium* bacteria colonize the host plant's root system and cause the roots to form nodules to house the bacteria.

The bacteria then begin to fix the nitrogen required by the plant. Access to the fixed nitrogen allows the plant to produce leaves fortified with nitrogen that can be recycled throughout the plant. This allows the plant to increase photosynthetic capacity, which in turn yields nitrogen-rich seed.

The consequences of legumes not being nodulated can be quite dramatic, especially when the plants are grown in nitrogen-poor soil.

Steps involved in nodulation process

The nodulation process illustrates an orchestrated interaction between the bacteria and host plant. The process begins when the rhizobia are attracted to flavonoids released by the host legume's roots. For legumes like alfalfa, clover, and soybeans the bacteria then begin to attach themselves to extensions of root epidermal cells called root hairs.

The attachment process is actually a two-step process where

(a) the bacteria first attach using a Ca^{2+} - binding protein called **rhicadhesin**. After the bacteria accumulate and anchor themselves to the root hair surface,

(b) a firmer attachment that involves lectins and/or cellulose firbrils and fimbriae produced by the host plant and bacteria, respectively.

(c) The host legume then senses chemicals produced by the rhizobia called Nod factors (lipochition oligosaccharides) that cause the colonized root hairs to curl and form what is called a shepherd's crook.

(d) Then rhizobia penetrate the root hairs and typically form a tubular structure called an infection thread.

(e) Once the bacteria reach the root itself, they stimulate cortical cell divisions that lead to the formation of a nodule.

(f) As the nodule begins to form, the bacteria become surrounded by a plant-derived membrane and are released inside plant cells forming the nodule.

(g) The bacteria subsequently lose their cell walls and undergo a profound change in cell morphology to form large, irregularly shaped branching cells called bacteroids. They then are entirely dependent on the host plant for their energy needs. In return, the bacteria fix nitrogen for the plant.

The interaction between the bacteria and host legume is so intricate that a particular *Rhizobium* or *Bradyrhizobium* will only nodulate a select number of plant genera. For example, ***Rhizobium melilotii*** **will only nodulate alfalfa, while** ***Rhizobium leguminosarum biovar trifolii*** **will only nodulate clover (*Trifolium*)**. This host specificity is referred to cross inoculation group cell signaling between the bacteria and the legume host.

Another example of the intricate relationship between the rhizobia and the host legume is the production of leghemoglobin. This molecule is similar in structure and function to hemoglobin found in human blood. It is only produced in fully functioning root nodules, and functions to bind and regulate the levels of oxygen in the nodule. Because the enzyme nitrogenase is sensitive to oxygen, free oxygen in nodule cell cytoplasm would inhibit the levels of nitrogen fixation in the nodule. **Leghemoglobin** seems to transport enough oxygen to allow the rhizobia to carry out cellular respiration, but not too much to inhibit the action of nitrogenase. **This heme protein is jointly produced by the legume and bacterium**; the legume produces the apoprotein while the bacterium produces the heme (porphyrin ring bound to an iron atom).

Biotechnological solutions to the nitrogen problem

The availability of nitrogen is one of the major limiting factors to crop growth. In the developed world, farmers use unsustainable levels of inorganic fertilisers to promote crop production. In contrast, in the developing world inorganic fertilisers are often not available and small-holder farmers suffer the resultant poor yields. Finding alternatives to inorganic fertilisers is critical for sustainable and secure food production. Bacteria and Archaea have evolved the capability to fix atmospheric nitrogen to ammonia, a form readily usable in biological processes. This capability presents an opportunity to improve the nutrition of crop plants, through the introduction into cereal crops of either the nitrogen fixing bacteria or the nitrogenase enzyme responsible for nitrogen fixation.

Three approaches have been envisioned to increase biological Nitrogen fixation (BNF) using biotechnology, especially for non-legume species that have no or poor symbiotic relationships, e.g. cereals. In this context, the most important cereals are wheat, rice and maize (corn). Together, they constitute 75% of the world's total calorific uptake.

1) Modification of naturally cereal-associated bacteria

In the first strategy, bacteria already naturally associated with cereals are modified to improve their colonization ability, numbers, N_2-fixing capabilities and release of NH_3 produced to plant cells. Such bacteria can be loosely associated in close proximity to the plant root, or invade and spread within the plant tissue. This approach can be considered to face lower technical hurdles. Also, as it does not require genetic modification of the plant it has advantages in countries where transgenic plants are banned. One such example is the transfer of a genomic island with N_2-fixing activity (X940) from *Pseudomonas stutzeri* A1501 to the aerobic root-associated beneficial bacterium *P. protegens* Pf-5. Maize and wheat inoculated with the N_2-fixing strain *P. protegens* Pf-5 X940 showed increased biomass accumulation, nitrogen content and seed yield.

2) Engineering the legume symbiosis into cereals

This strategy aims to develop new symbiosis in non-legume plants, i.e. to engineer cereals to sense/associate with N_2-fixing bacteria and to form nodules (to make cereals into legumes). The fact that cereals possess the symbiosis signalling pathway (Nod factor-based) provides an opportunity to engineer this signalling pathway for recognition of rhizobia by cereal crops. The approach must focus on the legume-specific

components that regulate upstream and downstream of the symbiosis signalling pathway and allow this signalling pathway in legumes to be activated by Nod factor and to coordinate nodulation.

Recognition of Nod factor involves two receptor-like kinases (NFR1 and NFR5) that specifically function during nodulation and show direct Nod factor binding. The Nod factor receptors and nodulation-associated transcription factors are important targets for engineering the legume symbiosis in cereals. While engineering Nod factor signalling in cereals is an important first step, the appropriate processes necessary for nodule organogenesis, bacterial infection and ultimately providing a suitable environment for nitrogenase function are all important.

3) Engineering expression of nif genes/ nitrogenase in cereal crops

This strategy involves transfer of prokaryotic *nif* genes into the plant genome itself. The plant would then synthesize its own N_2-fixing machinery without the need for bacterial interactions. Introduction of nitrogenase into plant cells is an alternative approach to engineer nitrogen fixation in cereals. Nitrogenase is a complex enzyme consisting of two proteins: the reductase component, known as the Fe protein and the catalytic component termed the MoFe protein. Both of these components are irreversibly damaged by oxygen and the catalytic process requires 16 moles of ATP for every mol of dinitrogen gas that is converted to 2 moles of ammonia. This energy expenditure is associated with the electron transfer process necessary for substrate reduction.

Nitrogenase contains three metalloclusters; one of these located within the MoFe protein, termed FeMo-co, is one of the most complex heterometal clusters known in biology. FeMo-co provides the catalytic site for nitrogen reduction and contains molybdenum, iron, sulphur, a central carbon atom and homocitrate as an organic moiety. The remaining two metalloclusters comprise a [4Fe-4S] cluster in the Fe protein and the P cluster, a [8Fe-7S] cluster located in the MoFe protein. These two clusters provide an electron transfer conduit from the Fe protein via the P cluster to the FeMo-co catalytic site. A large number of Nitrogen fixating (*nif*) genes are required for the assembly and function of nitrogenase. For example, 18 nif genes are present in *Klebsiella oxytoca*, excluding regulatory genes.

Nitrogenase can only function in cellular compartments rich in reducing power and energy. **Chloroplasts and mitochondria (two plant organelles of endosymbiont origin) are promising candidate compartments** for nitrogenase assembly and function. Engineering the chloroplast and mitochondria genome in

order to express nif genes/ nitrogenase enzyme would help to produce plants with innate Nitrogen fixation capacity.

Transfer of pest resistance genes to plants


Insects not only kill crops and reduce crop yield they also spread other diseases such as viruses, bacteria and fungi from plant to plant

Plants are attacked by a variety of insect-pests (herbivores). Some insects suck the nutritious juice from phloem of the host plant (sucking insects-hoppers, whitefly, aphids, thrips etc.), some eat or chew the tissues (chewing insects-caterpillars) and some others bore the plant parts (fruit fly, beetles etc.). Damage by such insect-pests may cause severe crop loss and/or poor food quality. For instance, an outbreak of brown plant hopper (BPH) in rice during 1970s caused economic havoc in South East Asia. Insect-pests can be managed by application of chemicals (pesticides) that would kill them; but this approach is not desirable because traces of pesticides (residues) may stay in the food items or in the surroundings, which would cause food and environmental safety concerns. Therefore, it is important to develop crops that could inherently withstand pest attack and would require no or only minimum application of chemicals to protect them.

Scientists have long been exploring the possibility and found that some plants in a species do resist the insect attack, which is generally referred to as “Host Plant Resistance”. Plants have different mechanisms to defend themselves from insect-pests: constitutive or induced defenses. Host plant resistance is mostly of constitutive defense mechanism and is more exploited in development of crop plants resistant to insect-pests.

Host plant characteristics enable a plant to avoid, tolerate or recover from attacks of insects under conditions that would cause greater injury to other plants of the same species, have evolved through insect-plant interactions. These characteristics are grouped under three categories:

1) Antixenosis (non-preference),

2) Antibiosis and

3) Tolerance.

1) **Antixenosis** refers to plant traits (morphological, physical or structural and biochemical) that keep the insects away from it so that no colonization takes place. This happens by causing disturbances in the insect behaviour (settling, mating, oviposition, feeding and food ingestion). Pubescence, tissue hardness, repellents and antifeedents or feeding deterrents contribute to antixenosis.

2) **Antibiosis** operates after the insects have colonized and started utilizing the plant. It affects insect's growth, development, reproduction and survival. Under antibiosis, insects cannot gain weight, turn restless and finally may die. Toxins, growth inhibitors and nutritional imbalances are some of the antibiotic factors. Glandular trichomes which produce secondary metabolites contribute to antibiosis in plants.

3) **Tolerance** refers to plant's ability to grow, reproduce, repair injury and yield satisfactorily under insect-pest attack. Only moderate level of resistance is exercised in tolerance (horizontal) while the antixenosis/antibiosis offer high level of resistance (vertical) and exert heavy pressure on the insect population. Due to this pressure, the insect-pest population adapts (called as biotype) and defeats the host resistance. Emergence of biotypes has been a serious consequence of exploiting vertical resistance against insect-pests in crops. Therefore, tolerance is considered a safer mechanism to exploit but is very challenging to study it.

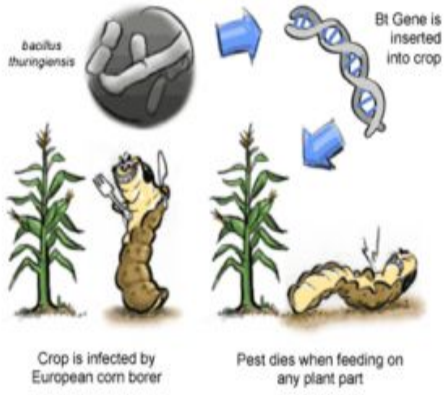
How do we find if a plant has resistance against an insect-pest?

We need to 'infest' the plant with insects artificially and monitor its response using standard procedures. As exemplified by the complex nature of resistance categories, resistance can be visualized in many different forms (phenotypes). However, using a simple measure such as 'seedling survival after insect infestation,' geneticists report 'major genes' that confer vertical resistance against insect-pests in plants. These genes act in 'dominant or recessive' manner and have been exploited in cultivar development.

For instance, to combat the outbreak of BPH in rice, three genes (*Bph1*, *bph2* and *Bph3*) were incorporated into the cultivars one after the other, which could curb the severity of the pest. Today, developments in plant molecular biology and genomics have enabled us to expand our knowledge on molecular basis of plant's responses to insects. A number of genes, their functions, and defense pathways are being unravelled, which would help developing pest resistant cultivars that can be grown free of pesticides and offer us a healthy food.

Insect-resistant genetically modified (GM) crops have been one of the major successes of applying plant genetic engineering technology to agriculture; cotton (*Gossypium hirsutum*) resistant to lepidopteran larvae

(caterpillars) and maize (*Zea mays*) resistant to both lepidopteran and coleopteran larvae (rootworms) have become widely used in global agriculture and have led to reductions in pesticide usage and lower production costs.



Plants have been developed with short sequences of genes from *Bt* (*Bacillus thuringiensis*-this bacterium produce proteins that kill certain insects with alkaline digestive tracts) to express the crystal protein *Bt* makes. With this method, plants themselves can produce the proteins and protect themselves from insects without any external *Bt* and/or synthetic pesticide sprays. Using this technology, *Bt* corn, potato and cotton were developed.

Methods of gene transfer in plants

Presently, a number of methods exist for the genetic manipulation of plant cells. These procedures range from exploitation of the natural gene transfer system of Agrobacterium to the chemical treatment of isolated protoplasts by polyethylene glycol. It also includes physical procedures of DNA introduction, including electroporation of protoplasts and tissues, microinjection and silicon carbide fibre-mediated transformation. Moreover microprojectile bombardment has also received much attention as a physical method of DNA transfer and in many laboratories, is now a routine and reliable technique for the production of transgenic plants.

The **importance of gene transfer technologies** to plants are listed below:

1. Provide resistance against viruses.
2. Acquire insecticidal resistance.
3. To strengthen the plant to grow against bacterial diseases.
4. Develop the plants to grow in draught.
5. Engineering plants for nutritional quality.
6. Make the plants to grow in various seasons.
7. Herbicide resistant plant can be made.

8. Resistance against fungal pathogens.

9. Engineering of plants for abiotic stress tolerance.

10. Delayed ripening can be done.

Different gene transfer (DNA delivery) methods in plants and their salient features.

TABLE 49.1 Gene transfer (DNA delivery) methods in plants

Method	*Salient features*
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

Gene transfer by Agrobacterium tumefaciens

Agrobacterium tumefaciens has been extensively used to introduce gene into plant cells. This bacterium is responsible for crawn gall disease in a variety of dicotyledonous plants. A plasmid carried within this bacterium cause crown gall disease. This plasmid is called tumor inducing plasmid (Ti).

Ti plasmid is upto 200 bp large at an apparently random position through non homologous recombination. The size of T-DNA is approximately 23 kbp and is responsible for the cancerous properties of the transformed cells. It also synthesizes opines. In the T plasmid, T-DNA is flanked by two 25 bp and carries genes that are required for infection. This plasmid has T-DNA that becomes integrated into plant genome

imperfect direct repeats. These sequences play roles in the integration of T-DNA into the plant genome. *Agrobacterium* has proved to be an incredible useful tool for the integration of genes into plants.

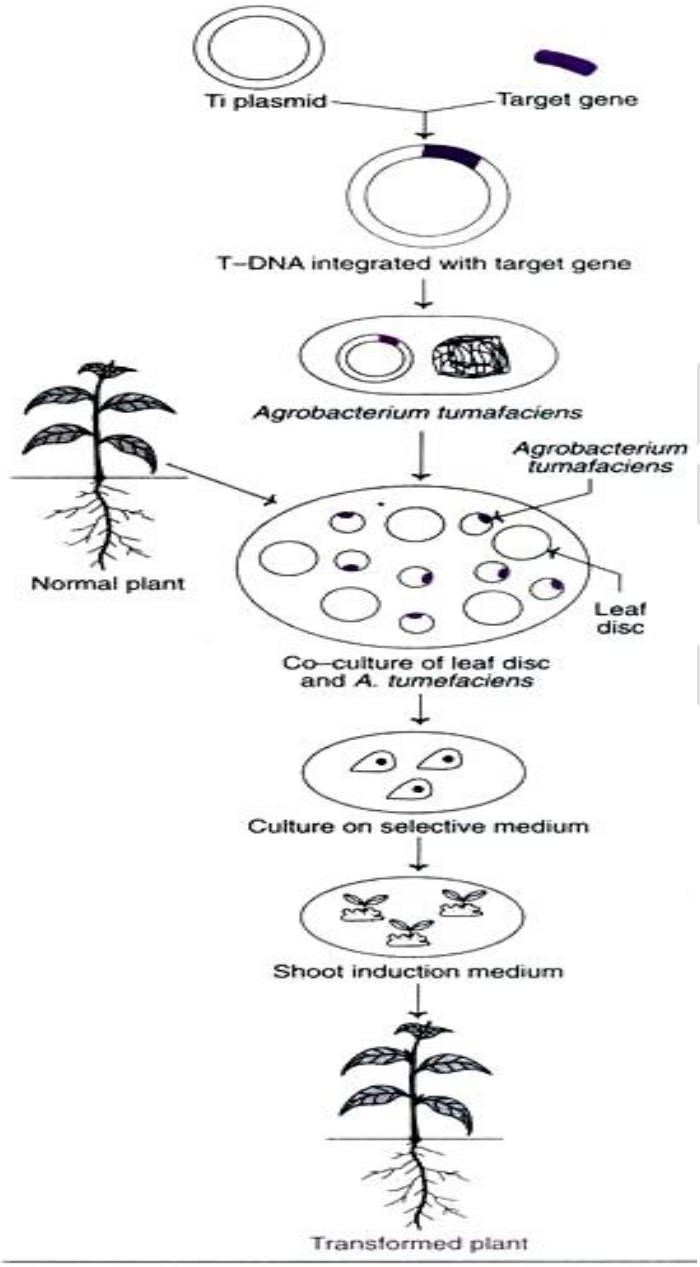


Fig. 49.7 : Transformation technique using Agrobacterium-mediated gene transfer.

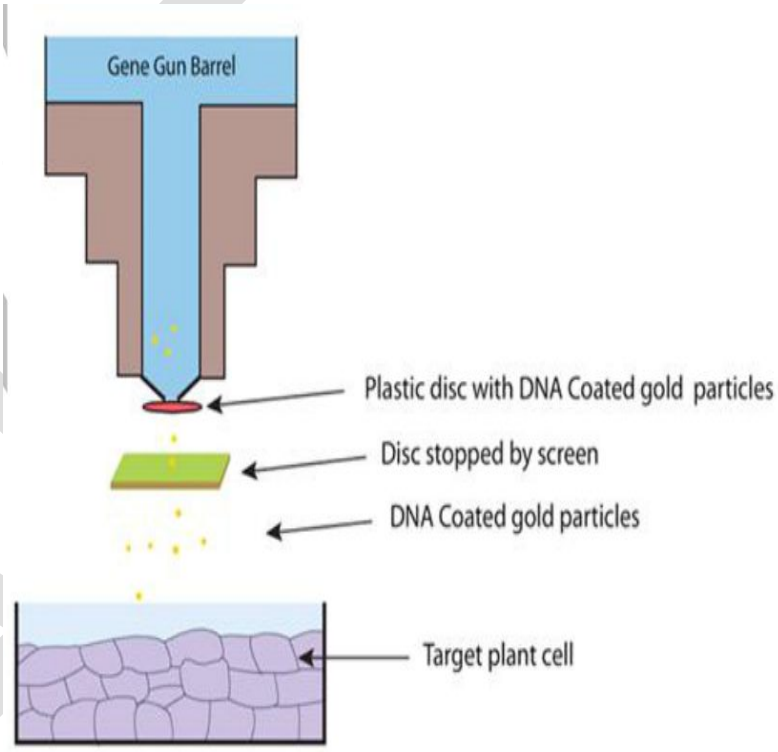
The most widely used technique for plant transformation is based on *Agrobacterium*, in which novel genes, linked to the Ti or Ri plasmid T-DNAs, are inserted into the host plant cells during T DNA transfer. This approach has been used to transform numerous plants, but is almost exclusively restricted to dicotyledons.

Despite recent reports using a strain of *A. tumefaciens* carrying a vector with the *vir B* and *vir G* genes from the supervirulent Ti plasmid pTiBo542 to transform rice, attempts to infect monocotyledonous plants, which constitute some of the world's most important food crops, with *Agrobacteria* have been rarely successful.

Gene transfer by microprojectile bombardment

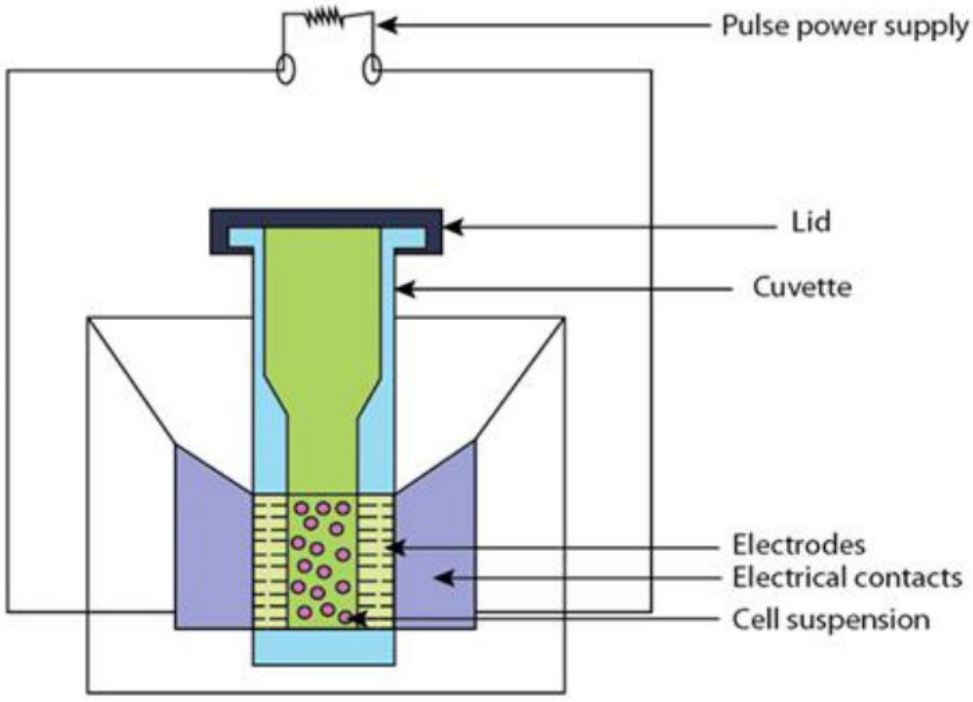
The concept of transfering DNA-coated particles directly into cells was first conceived by Sanford and co-workers in 1984.

Microprojectile bombardment has got much attention and attraction as a physical procedure of DNA transfer in many research laboratories during the past years. This method is routine and reliable way of producing transgenic plants. The method relies on a device which utilizes a propelling force, such as compressed gas or gunpowder, to accelerate inert (usually metal) particles (the microprojectiles), coated with DNA, into target cells. This technique is also referred to as particle bombardment, particle gun method, particle acceleration and Biolistics (Biological ballistics).



Electroporation of protoplast

High concentration of plasmid DNA containing the gene of interest is added to a suspension of protoplast and the mixture is given a shock with an electric field of 200-600 V/cm. The protoplasts are then grown in tissue culture for a period of one or two weeks. The selection pressure is then applied to select the transformed one. Both maize and rice protoplast have been successfully transformed with efficiencies of between 0.1 and 1%. Introduction and expression of transgenes in plant protoplasts were also reported. Moreover transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells were also explained.



Electroporator

Gene transfer by polyethylene glycol

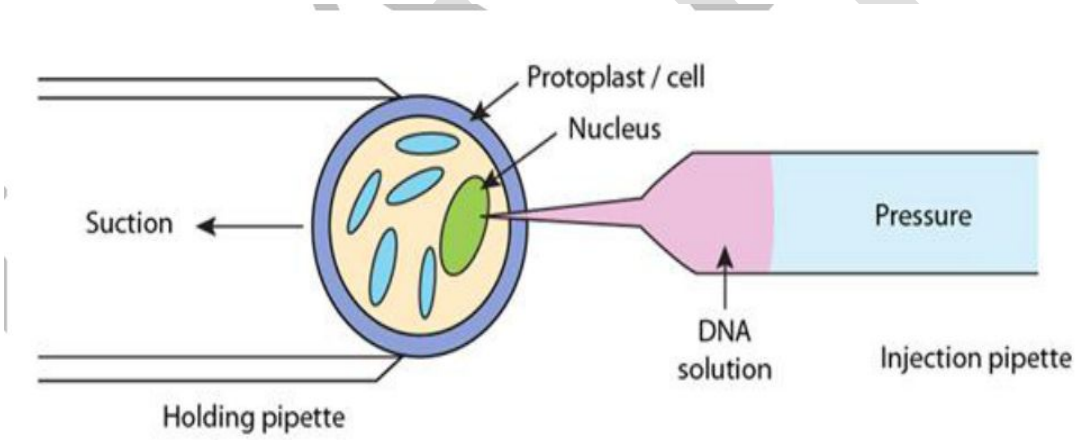
This technology is applicable for protoplast only. The chemical used is polyethylene glycol. It stimulates endocytosis and thereby causing the uptake of DNA. In this method protoplast are kept in polyethylene glycol (PEG) solution. The concentration of PEG used is 15% having 8000 dalton molecular weight. After exposure of protoplasts to exogenous DNA in presence of PEG and other chemicals, PEG is removed and intact protoplast are then cultured to form cells with walls and colonies in turn. Selection pressure is then applied to get the transformants.

The transfer of gene across the protoplast membrane can be initiated by a number of chemicals of which polyethylene glycol is the most important. It has become the most widely used due to the availability of simple

transformation protocol. Method was developed using calcium alginate micro beads to immobilize DNA molecules in combination with polyethylene glycol treatment also.

Gene transfer through microinjection

Transformation through microinjection is based on introducing DNA into the cytoplasm or nucleus by using a glass micro capillary-injection pipette. This operation requires a micromanipulator. During the introduction of DNA into the nucleus, cells are immobilized with a holding pipette and gentle suction. Microinjection is mainly used for the transformation of large animal cells. Its importance for plant transformation is rather limited due to the characteristics of plant cell walls, which contain a thick layer of lignins and cellulose. The plant cell wall is a barrier for glass micro tools. The method allowed the incorporation not only of DNA plasmids but also of whole chromosomes into plant cells.



Although it has a fairly high transformation frequency (20–50%), microinjection is a time consuming process that requires specific equipment and considerable training. This technique was used to study the cellular functions of plant cells and plastid physiology, e.g. in tobacco and *Vicia faba*.

Silicon carbide mediated transformation

Silicon carbide mediated method is also one of the transformation method used to transform plants. This method is least complicated. In this technique fibres are used which are single crystals of silica organic minerals like silicon carbide which possess an elongated shape, having a diameter of 0.6 mm and a length of 10–80 mm. Moreover it also exhibits a high resistance to expandability. In this method silicon carbide fibers' are added to a suspension containing plasmid DNA and plant tissue (immature embryos, callus, cell cluster). It is then mixed in

commercial shakers or in vortex. Fibres coated with DNA penetrate the plant cell wall in the presence of small holes produced at the time of collision between fibres and plant cells.

The factors on which the efficiency of transformation depends are the plant material, fiber size, parameters of vortexing, shape of the vessels used, and the characteristics of the plant cells, especially the thickness of the cell wall. This process is easy and quick. It is not so expensive and useful for various plant materials. The main drawback of this technique is low transformation efficiency, damage to cells negatively influencing their further regeneration capability, and the need of following extraordinarily rigorous precaution protocols during laboratory work, as breathing the fibers in, especially asbestos ones, can lead to serious sicknesses. Silicon carbide whisker-mediated embryogenic callus transformation of cotton (*Gossypium hirsutum* L.) and regeneration of salt tolerant plants were also reported.

Liposome *mediated gene transfer*

Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. It encapsulates the DNA fragments and then adheres to the cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. It is a very efficient technique used to transfer genes in bacterial, animal and plant cells.

Pollen *tube pathway method*

The transformation method via pollen-tube pathway has great significance in agriculture molecular breeding . After pollination the styles were cut. The DNA was then applied. The DNA reaches the ovule by flowing down the pollen-tube. This procedure, the so-called pollen-tube pathway (PTP), was applied first time for the transformation of rice. Here the transgenic plants were obtained at remarkably high frequency. Afterward PTP was used for other species e.g. wheat, soybean, *Petunia hybrida* and watermelon.

Sonication *assisted Agrobacterium mediated transformation*

Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is an efficient transformation technology, reported by Trick and Finer. It is *Agrobacterium* based technology. This method consists of subjecting the target plant tissue to brief periods of ultrasound while immersed in an *Agrobacterium* suspension. SAAT overcomes certain barriers such as the host specificity and the inability of *Agrobacterium* to reach proper cells in the target tissues.

This method also enhances DNA integration in many plant groups including dicots, monocots, and gymnosperms. It is likely that the enhanced transformation rates using SAAT result from micro-wounding both on the surface and deep within the target tissue. Therefore, unlike other transformation methods, this system also has the potential to transform meristematic tissue buried under several cell layers. Cotton transformation based on cavitations caused by sonication which results in thousands of micro wounds on and below the surface of plant tissue and allows *Agrobacterium* to travel deeper and completely throughout the tissue. This wounding fashion increases the probability of infecting plant cells lying deeper in tissue.

Interaction between plants and microbes

In nature, plants and animals continuously interact with numerous microbial species during all the stages of the life cycle. From early times of evolution, humans are exposed to a rich microbial world that extends the human capacity to adapt to a healthy life. Similarly, plants cohabit with microbes, including archaea, protists, bacteria, and fungi, together called microbiota. The beginning of microbial life dated back to the beginning of life (more than 3.5 billion years), suggesting that microbe–microbe interactions have evolved and diversified over time, long before the adaptation of plants to the land life, i.e., before 450 million years. Higher plants and photosynthetic algae assimilated cyanobacterial endosymbionts in the process of evolution, now we know them as chloroplasts or plastids. Thus, the evolutionary history of plant and microbes share common origins, and their survival is interdependent. Consequently, the "plant microbiota" has gained more attention that exists within or nearby surfaces of the plant parts.

Profiling of the plant-associated microbiome (genome assemblies of all microbes) is an emerging concept to understand the plant–microbe (PM) interactions. Microbiota extends the plant capacity to acclimatize fluctuating environmental conditions through several mechanisms. Beneficial PM interactions include plant growth promotion (PGP), protection against biotic and abiotic stresses through the priming of plant immune system or induction of plant defence pathways, adaptation to a variable environment, mycorrhizal symbiosis, nutrient uptake, and conversion of the unavailable nutrient forms into plant-accessible form. The PM interactions are bidirectional, and microbes also obtain nutrients from the host plants. The trade-off between plant and microbe may develop into distinguishing partnerships depending on its impact on plant health, i.e., mutualistic (beneficial to both the partners, symbiotic), neutral (beneficial to only one partner, commensalistic), or harmful (deleterious to the host plant, pathogenic). These PM interactions are crucial in sustainable agriculture and the environment for food production and health management, respectively.

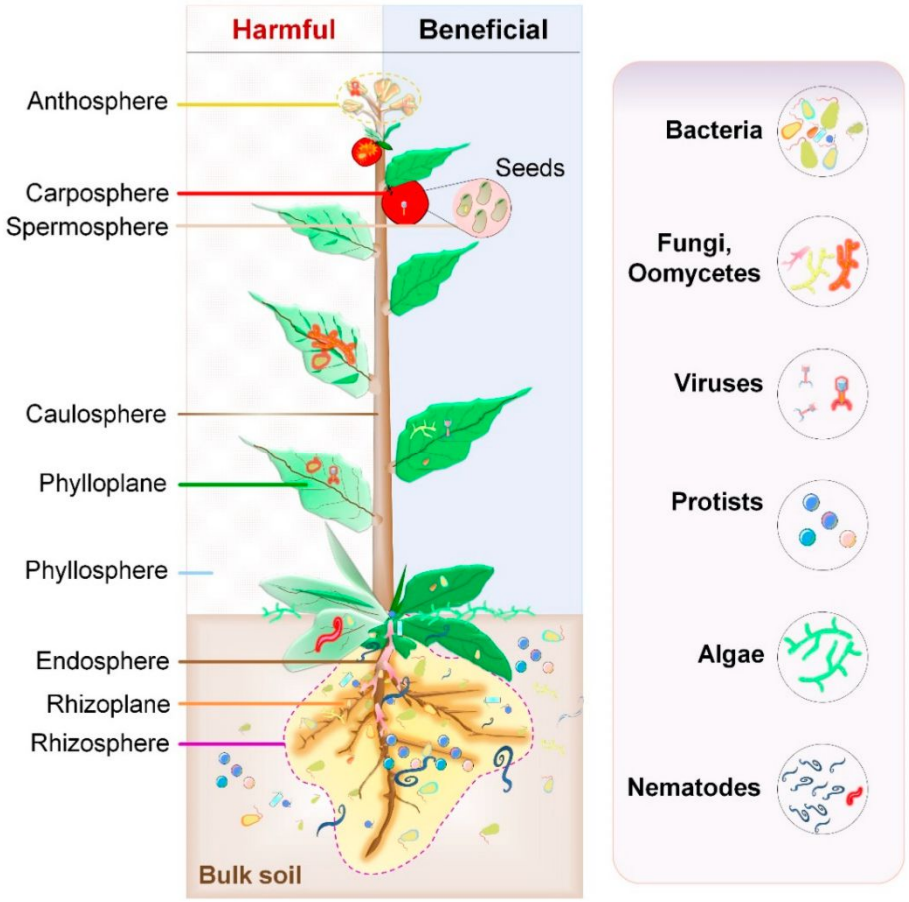


Figure. Microbiome in plant ecosystem. Schematic plant and plant-associated microbiota colonizing different niches on and inside the plant tissue. All the above-ground plant parts together, called the phyllosphere, are a continuously evolving habitat due to ultraviolet (UV) radiation and altering climatic conditions. It is primarily composed of leaves. Below-ground plant parts, mainly roots, are generally influenced by soil properties. Harmful interactions affect the plant growth through pathogenic activities of some microbiota members (left side). On the other hand, beneficial microbial interactions promote plant growth (right side).

An investigation of the host plant together with associated microbiome (also called holobiont) suggests the coevolution of plant–microbe, plant–plant, and microbe–microbe interactions. Modern technologies such as next-generation sequencing (NGS), omics approaches (metagenomics, transcriptomics, proteomics, metabolomics), and computational tools enable the understanding of community-level molecular aspects of the PM interactions governing the plant traits.

Table 1. Metagenomic-based studies of plant microbiota are summarized.

Host	**Sampling**	**Key Findings**
Agave	Rhizosphere, whole plant	Microbial composition was mainly regulated by the plant compartment, while the fungal community composition was primarily determined by the plant host biogeography.
Arabidopsis	Root, rhizosphere	The composition of rhizospheric microbiota was found reliant on the environment rather than host species.
Arabidopsis	Leaf, root	Genome drafts of 400 isolates revealed a substantial overlap of genome-encoded functional capabilities between leaf- and root-derived bacteria with few significant differences.
Arabidopsis	Root, rhizosphere	Explored genetic network controlling the phosphate stress response influences the structure of the root microbiome community, even under non-stress phosphate conditions.
Arabidopsis	Roots, rhizosphere	Bacterial microbiota is indispensable for plant survival and protection against root-filamentous fungi.
Barley	Root, rhizosphere	Rhizospheric and root microbiota affect plant growth. The interactions between microbe–microbe and plant–microbe drive distinct microbiota.
Citrus	Root, rhizosphere	The core rhizosphere microbiome comprises several potential beneficial plant microbial species and detected over-represented microbial functional traits.
Grapevine	Grape must	Environmental factors, variety, and regional origins determine the unique grapevine-associated microbiota. These factors are the key to the unique taste and wine quality.
Grapevine	Rhizosphere, whole plant	Microbial composition of soil and root is primarily influenced by plant-selective pressure, soil C:N ratio, and pH. Leaf and fruit microbiota alterations correlated with soil carbon, cultivation practices, and geography.
Maize	Roots, rhizosphere	Associated microbiota showed heritable variation in community composition of rhizosphere and significant field-specific heritable variation.
Maize	Roots, rhizosphere	Assembled a simplified and representative synthetic bacterial model community containing seven dominant strains to study the community assembly dynamics that interfered with the growth of a plant-pathogenic fungus.
Maize	Root,	Microbiome composition varies with plant genotype, plant age, and climate

Host	Sampling	Key Findings
	rhizosphere	events.
Petunia, Arabidopsis	Root, rhizosphere	Root microbiota composition and responses vary substantially in response to the varying phosphorus (P) application.
Potato	Roots, rhizosphere	Early stages of the plant showed the cultivar-dependent composition of bacterial communities, but in the flowering and senescence stages, this was not the case. Furthermore, the population of some species flourished under different ecological conditions more than the other species.
Rice	Root, rhizosphere	Endosphere, rhizoplane, and rhizosphere consist of a diverse microbiome. Cultivation practices influence the diversity of microbiome compositions at each compartment.
Rice	Root, rhizosphere	Type of soil environment (i.e., rhizosphere versus bulk soil) is a driving factor of the structure of the microbial community than the plant age.
Soybean, Wheat	Rhizosphere, root	Soil properties such as pH and nitrate content may influence the composition of root microbiome in agricultural fields.
Sugar beet	Soil after harvesting	Identified crucial bacterial taxa and genes suppressing a fungal root pathogen and showed that plant protection depends on the rhizospheric microbial community.
Sugarcane	Rhizosphere, whole plant	Microbial communities enter primarily from native rhizospheric soil and colonize plant organs in distinct patterns.
Tomato	Rhizosphere, whole plant	Distinct microbial communities found associated with different plant organs.
Tomato	Rhizosphere, whole plant	The study explored the protection role of rhizosphere microbiota against soil-borne pathogen causing wilt disease.
Wheat, Cucumber	Roots from pots	Genus or species level differences observed between the rhizospheric microbiome from diverse plant species related to environmental factors.
Wild mustard	Leaf and root	Leaf microbiome genetically controlled by the host and several bacterial species of leaf microbiomes shared with root microbiomes, suggesting acquisition from the soil.

Genetic information about the PM interactions is becoming available for several crops and associated microbes. Understanding the molecular aspects of PM interactions at gene level will be a crucial step toward the better use of microbiome in agriculture. In this regard, revolutionary techniques such as CRISPR (clustered regularly interspaced short palindromic repeats)-based genome editing (GE) capable of inducing precise genetic modifications, are an ideal platform to know the basics of the PM interactions in a fast-forward way and enable precise genetic modifications for higher crop productivity and disease resistance.

Composition of the Plant–Microbe (PM) Interactions

Below-ground microbial habitat consists of the rhizosphere (soil close to the root surface), rhizoplane (root surface), and endosphere (root interior). On the other and, above-ground habitat (phyllosphere) of microbes comprises of leaves (phylloplane), stem (caulosphere), flowers (anthosphere), seeds (spermosphere), and fruits (carposphere). Plants actively recruit microbes from the environment through the soil or air. Some microbes such as endophytes (dwell inside the plant tissues without causing any harm to host plants) follow either horizontal (acquiring from the environment with each new generation) or vertical (transfer from parental seeds) routes of transmission.

Driving Factors of the Plant–Microbe (PM) Interactions

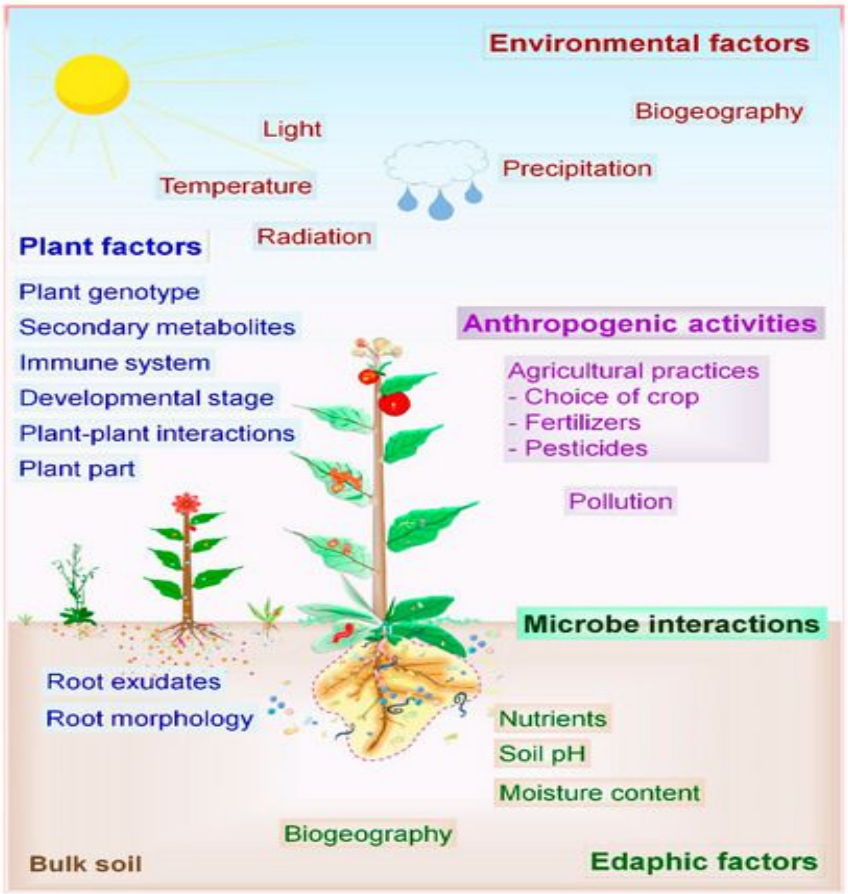
The microbial composition of a plant microbiota is not the result of random selection, and instead, it determined by assembly rules. Microbial diversity decreases sequentially from bulk soil to rhizosphere. Plant-associated factors promote the preferable growth of a set of microorganisms and inhibit the other. Recent advancement in the mapping of plant microbiota with NGS provides deep insights about biotic (plant-related factors, microbial factors, anthropogenic activities) and abiotic factors (soil properties, environmental factors) affecting the composition and structure of the microbial community.

Biotic Factors

Plant factors include host genotype, the immune system, plant compartment, metabolite secretions, plant age, plant–plant interactions, root morphology, and root exudates. Among them, plant genotype is the major driving factor governing the composition and structure of the microbial community in the rhizosphere. Different plant species vary in their rhizosphere and phyllosphere communities. It is not surprising given that plant genotype decides the properties of root and leaf surface, the type of exudates secreted by roots, chemical signalling pathways, and nutrient quantity plus quality available for microbes. Plant metabolites such as coumarins affect the host microbiota, the assembly of root microbiome, and act as semiochemicals in PM interactions. Microbial species available at specific geographical locations per se also influence the composition of the microbiota and their interactions with plants. The joint action of the plant–plant, microbe–microbe, and PM interactions determines the distinct microbiome assemblies. Symbiotic microbial species are abundant in the niche of rhizosphere and phyllosphere due to positive and

selective plant pressure. On the other hand, plant-pathogenic microbes cause a dramatic shift in the population of antagonistic microbes as well as plant immune responses, leading to the control of pathogen.

Anthropogenic factors such as agricultural practices, including higher dosages of fertilizers, pesticide sprays, cultivation practices pollution, and several other human activities disturb the quality of soil, air, and water, thereby influencing microbial structures and PM associations. Regarding fertilizer use, the composition of root microbiota of petunia and Arabidopsis varied significantly in response to the phosphorus (P) application and plant species responded differently to low-phosphate conditions. Furthermore, similar effects were observed for leaf microbiota in maize and soybean in response to nitrogen (N) dosages. In the future, it will be exciting to study the impact of intensive agricultural practices on changes in PM associations and structure of plant microbiota.



Abiotic Factors

Soil properties have a profound impact on the composition of bacterial and fungal communities in the rhizosphere. The soil is the natural resource of nutrients and hence acts as a microbial seed bank for the rhizosphere community. Soil properties such as soil pH, soil type, macronutrient distribution, soil organic matter, salinity, soil structure, and moisture content drive the microbial community formation. Plant species recruit distinct microbial communities in both rhizosphere, rhizoplane (epiphytes, colonize plant surfaces), and endosphere (endophyte, colonize internal plant parts) even if grown in a similar soil environment. On the other hand, certain plant species or genotype recruit the matching group of microorganisms irrespective of environmental and soil conditions, known as the core plant microbiota. Environmental factors also significantly influence the assemblies of phyllosphere microbes that include climate, light, water, ultraviolet (UV) radiation, and geographic location. Generally, plant phenotype is the outcome of interactions between plant genotype, associated microbiota, and environmental factors. Overall, plant microbiota is vertically (through seed, propagation material) or horizontally (through soil, air) acquired and resides on or in the inside of plant tissues where all of the above-discussed factors shape the structure of the microbial community.

Beneficial PM Interactions in Agriculture

Plant–microbe interactions regulate the process of soil carbon sequestration by the modulating of the terrestrial carbon cycle. The plant microbiota includes beneficial, neutral, or pathogenic microbial species that decompose the plant residues and dead animals. The beneficial plant microbiota is vital for plant growth, flowering time, and crop yields directly or indirectly. Furthermore, microbial responses drive the impact of climate changes on agriculture, so there is growing interest to use plant-associated microbiota to mitigate the influence of climate change on sustainable agricultural practices and food production. The consequence of a specific PM interaction is reliant on its effect on plant health, and it may be beneficial under the distinct set of conditions and damaging under the others. Well-known examples of beneficial PM associations include symbiotic interactions of legumes with N-fixing rhizobia and arbuscular mycorrhizal (AM) fungal taxa that helps host plants to access Nitrogen and Phosphorous, respectively, under nutrient-deficit environments. Symbiotic behavior helps PGP microbes to dominate the population of other microbial species. Many PGP bacteria affect plant growth via the production of phytohormones (auxin, cytokinin, gibberellin) and plant-beneficial enzymes (1-aminocyclopropane-1-carboxylate deaminase). Some PM

interactions are beneficial under heavy-metal stress through enhanced uptake, and detoxification by either or both the partners, i.e., plant or microbe.

Harmful PM Interactions

Some microbes are harmful to plants causing disease symptoms, for example, Pseudomonas syringae, Erwinia amylovora, Ralstonia solanacearum, Xanthomonas sp., and Xylella fastidiosa. Plant-pathogenic microbes infect the plant tissues through natural openings or wounds for nutrient acquisition and trigger the immune responses. Various factors regulate the outcome of plant–pathogen interaction like population size, the host vulnerability, the climate, and biotic factors like plant microbiota. Several members of plant microbiome known to enhance plant resistance against phytopathogenic microbes are called biocontrol agents. Some non-pathogenic microbes can act as pathogens under some circumstances such as change of host plants or alteration in microbial population size. Therefore, modern tools could be an ideal platform to understand such mysterious behavior of PM associations. In general, precise information of PM interactions at the molecular level is needed.

Modern Tools to Explore PM Interactions

Understanding the basic mechanisms of the plant-specific microbiome is a suitable approach for its use in agriculture since the plant-associated microbiota greatly influences the host's phenotype, as described above. More precisely, the investigation of microbial and plant genes involved in the PM interactions is vital for the future application of plant microbiome. Molecular biology, omics tools (genomics, transcriptomics, proteomics, metabolomics), and NGS technologies have significantly improved our understanding of plant microbiome that includes the PGP microbes as well as phytopathogens. Although some metagenomic and proteogenomic reports provided assembly level data of augmented functional categories, alternate methods are required to gain functional insights at the gene or protein level.

One of the research themes about PM application in agriculture deals with the use of microbial consortia (a group of species) whereas another research theme involves a precise genetic modification (GM) of either plant or microbe. Transgenic technology (GM method) is a promising approach to accomplish a faster outcome, but the integration of foreign genetic material limits its widespread use due to regulatory issues.

GE (Genome editing) tools are of great interest that allows scientists to edit genomic sequences in a more precise manner without the integration of a foreign gene. Genome editing technology employs the engineered endonucleases to create a double-strand break (DSB) that undergo DNA repair by endogenous mechanisms and generate different types of mutations.

DSB repair mechanisms occur through two major pathways, non-homologous end-joining (NHEJ) and homology-directed repair (HDR). HDR is precise but less common than NHEJ, and applicable in specific donor-dependent gene replacement.

Targeted genetic modifications can be accomplished through several ways. Three meganucleases (or site-specific nucleases (SSNs) or site-directed nucleases (SDN)), are the most commonly used recently that consist of

Transcription activator-like effector nucleases (TALENs),

Zinc finger nucleases (ZFNs), and

CRISPR/Cas (Cas, CRISPR-associated) system.

Genome editing by ZNFs and TALENs is based on the ability of DNA-binding domains that can specifically recognize almost any target DNA sequence. Therefore, the GE ability of ZNF/TALEN is mainly governed by the DNA-binding affinity and specificity of the assembled zinc-finger and TALE proteins. The CRISPR/Cas system is adventitious compared to ZFNs and TALENs in terms of simple designing, versatility, cost-effective, higher efficiency, multiplexing, and specificity.

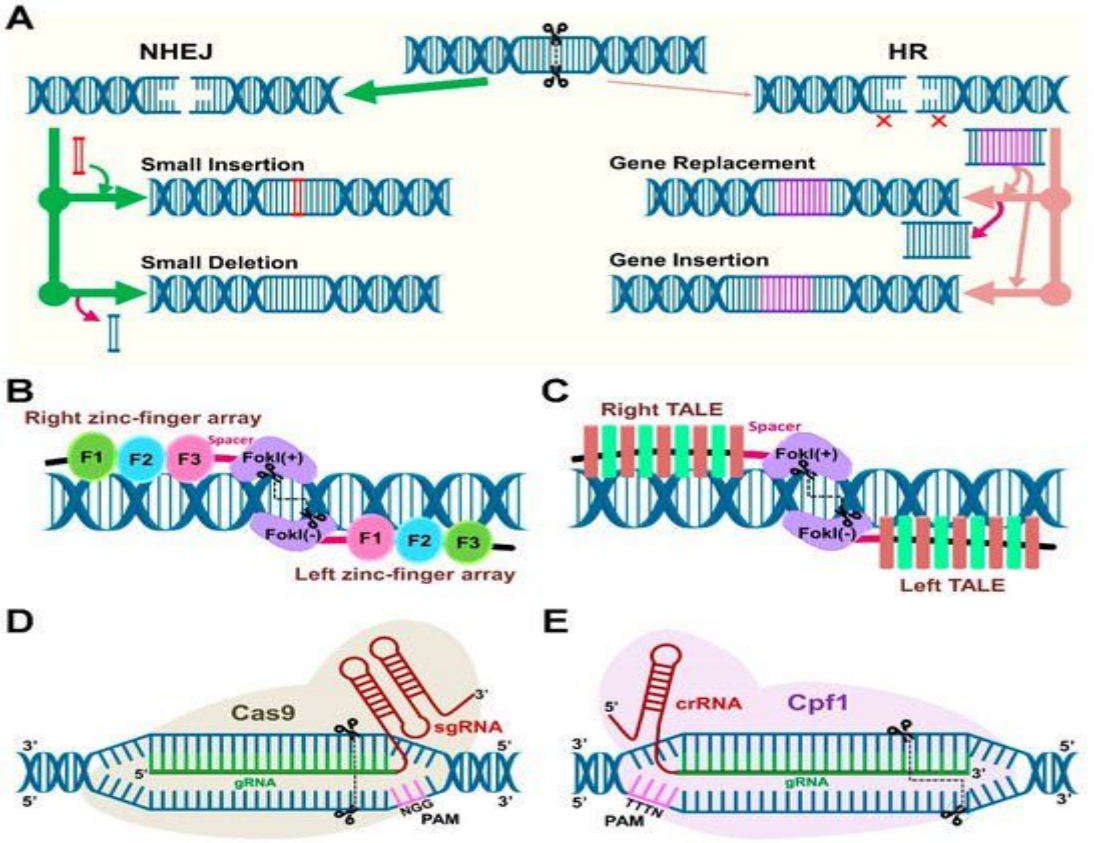


Figure. Biology and components of site-specific nucleases (SSNs) modified for genome editing applications. (**A)** Repair of double-strand breaks (DSBs) in damaged DNA strands occurs through two main pathways that consist of non-homologous end-joining (NHEJ) and homology-directed repair (HDR). NHEJ is most common in cells. It is an error-prone pathway that introduces indel mutations (small insertions or deletions). HDR is more precise compared to NHEJ, but it requires a donor template that results in either insertion or replacement. (**B**) Zinc finger nuclease (ZNF) is designed using an array of DNA-binding domains from zinc-finger proteins. Each ZFN comprises DNA-binding domain at N-terminus and FokI nuclease at C-terminus. The linker and spacers are shown in black and pink, respectively. (**C**) Design of transcription activator-like effectors (TALE) protein-based nuclease (TALEN) bound to DNA. (**D**) Illustration of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 with a sgRNA (red) encoding gRNA (green) bound to a target DNA (blue) adjacent to PAM, i.e., protospacer adjacent motif (magenta). Cleavage sites on both strands shown with scissors and dotted line depict the blunt ends produced by Cas9. (**E**) CRISPR/Cpf1 (Cas12a) system shown with a crRNA (red), a gRNA (green), target DNA (blue), PAM (magenta). Cleavage sites of both strands (scissors) produce staggered ends (dotted line) with Cpf1.

Table. List of genome-edited plant–pathogen interactions is summarized.

	Pathogen	**Disease**	**Host**	**Target Gene (plant or pathogen), Interaction**	**Genome Editing Tool**
				Bacteria	
1.	Xanthomonas oryzae pv. oryzae	Bacterial blight	Rice	OsSWEET14 (plant); Pathogen interacts with the promoter of gene and hijacks plant sugars	TALEN
2.	Xanthomonas oryzae pv. oryzae	Bacterial blight	Rice	OsSWEET14 and OsSWEET11 (plant); Pathogen interacts with the promoter of gene and hijacks plant sugars	CRISPR/Cas9
3.	Xanthomonas oryzae pv. oryzae	Bacterial blight	Rice	OsSWEET13 (plant); Pathogen hijacks sucrose from plant cells	TALEN
4.	Pseudomonas syringae pv. tomato, Xanthomonas spp., Phytophthora capsici	Bacterial speck, Blight, and spot	Tomato	SlDMR6-1 (plant); Knock-out of DMR6 increases salicylic acid levels that induces production of secondary metabolites and PR genes	CRISPR/Cas9
5.	Xanthomonas citri subsp. citri	Citrus canker	Citrus	CsLOB1 (plant); Susceptibility gene induced by pathogen	CRISPR/Cas9
6.	Xanthomonas citri subsp. citri	Citrus canker	Citrus	CsLOB1 (plant); Susceptibility gene induced by pathogen	CRISPR/Cas9
7.	Erwinia amylovora	Fire blight	Apple	DIPM-1, 2 and 4 (plant); Directly interact with the disease-specific gene of bacterial pathogen	CRISPR/Cas9
8.	Pseudomonas syringae pv. tomato (Pto) DC3000	Bacterial speck	Tomato	SlJAZ2 (plant); Directly interact with coronatine produced by bacteria that helps in leaf colonization	CRISPR/Cas9
				Fungi and Oomycetes	
9.	Magnaporthe grisea, Burkholderia glumae	Fungal blast, bacterial blight	Rice	OsMPK5 (plant); A negative regulator of rice defense response	CRISPR/Cas9
10.	Blumeria graminis f. sp. tritici	Powdery mildew	Wheat	MLO-A1, B1, and D1 (plant); Confer susceptibility to fungi	CRISPR/Cas9
				Viruses	
29.	BSCTV	Viral (DNA)	Arabidopsis	Replication origin (microbe)	ZNF
30.	TYLCCNV, TbCSV	Viral (DNA)	Tobacco	AC1 replication-associated (Rep) protein (microbe)	ZNF

	Pathogen	Disease	Host	Target Gene (plant or pathogen), Interaction	Genome Editing Tool
31.	TYCCNV, TbCSV, TLCYnV	Viral (DNA)	Tobacco	AC1 replication-associated (Rep) protein (microbe)	TALEN
32.	TuMV	Viral (RNA)	Arabidopsis	eIF4E/exon (plant); Directly interact with viral protein and helps viral replication	CRISPR/Cas9
33.	CVYV, ZYMV, PRSV-W	Viral (RNA)	Cucumber	eIF4E/exon (plant); Directly interact with viral protein and helps viral replication	CRISPR/Cas9
34.	RTSV	Tungro (RNA)	Rice	eIF4G (plant); Directly interact with viral protein and helps viral RNA replication	CRISPR/Cas9
35.	TYLCV, BCTV, MeMV	Viral (DNA)	Tobacco	Intergenic region of origin of replication (IR), capsid protein (CP), RCRII motif of Rep protein (microbe)	CRISPR/Cas9
36.	BeYDV	Viral (DNA)	Tobacco	Long intergenic region (LIR), Rep protein encoding gene (microbe)	CRISPR/Cas9
37.	BSCTV	Viral (DNA)	Arabidopsis, Tobacco	IR, CP and Rep (microbe)	CRISPR/Cas9
38.	CBSV	Brown streak (RNA)	Cassava	nCBP-1 & nCBP-2/exon (plant); Directly interact with viral protein and helps viral replication	CRISPR/Cas9

BCTV, Beet curly top virus; BeYDV, Bean yellow dwarf virus; BSCTV, Beet severe curly top virus; CBSV, Cassava brown streak virus; CLCuKoV, Cotton Leaf Curl Kokhran Virus; CRISPR/Cas9, clustered regularly interspaced palindromic repeat-CRISPR-associated protein 9; CVYV, Cucumber vein yellowing virus; CYVV, Clover yellow vein virus; eBSV, Endogenous Banana streak virus; eIF4E, eukaryotic translation initiation factor 4E; MeMV, Merremia mosaic virus; ORF, open reading frame, PmCDA1-Petromyzon marinus cytidine deaminase 1 base editor; PR genes, Pathogenesis-related genes; PRSV-W, Papaya ring spot mosaic virus-W; RTSV, Rice tungro spherical virus; SWEET, sugar will eventually be exported transporter; TALEN, transcription-activator-like effector nuclease; TbCSV, Tobacco curly shoot virus; TLCYnV, Tomato leaf curl Yunnan virus; TMV, Tobacco mosaic virus; TuMV, Turnip mosaic virus; TYLCCNV, Tomato yellow leaf curl China virus; TYLCSV, Tomato yellow leaf curl Sardinia virus; TYLCV, Tomato yellow leaf curl virus; UTR, untranslated terminal repeat; WDV, Wheat dwarf virus; ZNF, Zinc finger protein; ZYMV, Zucchini yellow mosaic virus

Qualitative improvement of livestock

Biotechnology is one of the frontier areas of scientific development in the world today. Advances in the fields of biotechnology catered to a wide area of science viz, Agriculture, Animal Sciences, Environmental Science, Food Science, Medicine etc. in order to improve the overall living standards of the human beings. This sphere of science is increasingly becoming sustainable means of improving livestock production by influencing animal health, nutrition, reproduction, genetics and breeding. The main impediment to the successful application of biotechnology relates to the cost of adoption and acceptability. The application of in-genuine biological principles in manipulating living organisms or their derivatives to either improve or multiply a product is biotechnology in simple means. The term can broadly be defined as the technology by which one can produce useful products from raw materials with help of living organisms or other biological processes. Biotechnological achievements of recent years have emerged as powerful tool to improve various livestock products including milk and meat products.

The biotechnological tools can be applied in production of high yielding animal, improvement in the quality attributes of animal origin products, production of hormones, functional and designer livestock products, enzymes, bio-preservation of livestock products, efficient byproduct utilization, quality control and meat authentication as outlined below:

Production of good quality and high yielding animal

Livestock production is expected to grow tremendously in line with the projected demand for animal products. Therefore, the methods of livestock production must be changed to allow for efficiency and improvement in productivity. Biotechnological research is important in order to respond to the pressure of producing more food from animals to cater food requirement of the ever-growing human population. Transgenic animals such as mice, rats, rabbits, pigs, sheep, and cows have been developed with the help of biotechnology. Transgenesis is the technique that permits the manipulation of genes of one organism which can subsequently be introduced into genome of another organism of same or other species in such a way that the genes are not only expressed but also gets transmitted to its progeny. Transgenic animals thus produced will have enhanced growth rate and improved food quality. For example, transgenic cows are developed to produce milk containing specific human proteins that helps in the treatment of human

emphysema. Cloned transgenic cattle produced increased amount of beta and kappa casein in milk fat and increased level of human lacto-ferrin. So also, such cows have been known to produce more milk or milk with less lactose or cholesterol, pigs and cattle that have more meat on them, and sheep that yield more wool. Pigs with human IGF 1 had 30 per cent more loin mass, 10 per cent more carcass lean tissue and 20 per cent less total carcass fat. Transgenic pigs carrying plant gene had increased amount of unsaturated fatty acids in their muscle to produce a meat called "Healthy Pork".

Improvement in quality of livestock products

Major genes for meat quality offer excellent opportunities for increasing level of meat quality and decreasing variability. Most scientists say that tenderness is 30 per cent and Pale Soft Exudate (PSE) condition in swine is 50 per cent genetically governed characters. Gene that affects tenderness of meat before slaughter are CLPG in sheep, myostatin in beef, RN in pork. Rate of rigor mortis is governed by RYRI in pork ;during storage – Calpain : Calpastatin ratio and Cathepsin. Identification, isolation and modi-fication of useful genes are some of the important aspects of biotechnology research and development . The quality of carcass can be improved by manipulating the lipoprotein receptor and leptin genes thereby the cholesterol and fat content of meat can be governed.

Production of hormones

There is a growing database supporting the use of pituitary derived somatotropin (ST) as an agent to improve growth and carcass composition. Pigs injected with pST had 35 per cent less fat and 8 per cent more protein. However, purification of ST from Pituitary gland is uneconomical as production of single dose requires collection and processing of 25-100 Pituitary glands. More recently, the development of recombinant DNA technology has provided a mechanism for large scale production of somatotropin. The gene for ST protein is inserted in laboratory strain of *E. coli* which can be grown on a large scale and from which ST can be purified and concentrated. Bovine and ovine ST improves growth rate by 20 per cent and lean to fat ratio by 40 per cent in ruminants. While experimentation in growing pigs, significant improvement of 40 per cent in average daily gain and 30 per cent in feed conversion efficiency had been achieved by administration of pST. Furthermore 60 per cent reduction in carcass fat and 70 per cent increase in carcass protein had been attained. No significant difference between effectiveness of pST and rpST had been observed.

Production of Enzymes

Dairy industry requires large amount of rennet to produce cheese in bulk. Traditionally rennet is procured from calves. However due to global imbalance between production of cheese and calf slaughter and worldwide shortage of calf rennet, exploration of alternative source of rennet is required to be investigated. In certain countries like India, religious feeling has aggravated the need to rennet substitute. Treatment of milk with galactosidase results in hydrolysis of lactose, there by making it digestible by lactose intolerant people. The enzyme galactosidase hydrolyses lactose into glucose and galactose. Since these enzymes are costly, biotechnology can help in its economic production as well application. Commercial -galactosidase is produced from yeasts such as *Kluyveromyces lactis* and *Kluyveromyces marxianus* (formerly known as *Kluyveromyces fragilis* and *Saccharomyces fragilis*), and moulds such as *Aspergillus niger* and *Aspergillus oryzae.* The production and optimization of -galactosidase enzyme using synthetic medium by *Kluyveromyces lactis* NRRL Y-8279 in shake flask cultures was found suitable. Pure chymosin produced from genetically engineered *E. coli* reduced coagulation time by 5 folds. Cheese prepared with use of bacterial chymosin and ripened at 6 – 7 C for 7 months showed similar characteristics and proteolysis to cheese made with calf chymosin. The DNA of calf chymosin has been successfully cloned into yeast (*Kluveromyces lactis*), bacteria (*E. coli*) and moulds *(Aspergillus niger).* These can be produced on large scale by fermentation methods and extracted by down stream processing. Technological performance of recombinant chymosin is excellent since the cheese produced using recombinant chymosin is essentially indistinguishable from traditional cheese.

Functional and designer livestock products

In order to improve the product, attempts can be made to develop strains of starter cultures capable of enhanced antichlolestermic attributes, enhanced anti- carcinogenic attributes, enhanced antagonistic influence on enteropathogenic microorganism. The genetic stability of starter strains, bacteriophage infection, production of off flavour and insufficient development of acid during fermentation are costly problems to dairy industry. Hence there is a great deal of interest in the development of new and improved strains, using modern techniques of molecular biology viz. plasmid transfer, transduction, protoplast fusion and cloning. Plasmid biology of lactic acid bacteria have opened new vistas for exploring possibilities for using recombinant DNA technology and genetic engineering to improve the nutritional or therapeutic value of these products. A complete phage resistant strain can be achieved by incorporating plasmid which are

inhibitory for phage absorption, penetration and DNA metabolism. Genetically engineered strains can play a vital role in manufacture of tailor made high quality fermented livestock products. Cloning of genes from lactic acid bacteria could be carried out in food grade strains of *E. coli* for which vectors & transformation systems are available. A detailed restriction endonuclease map of the citrate plasmid from *Streptococcus lactis sub sp diacetylactis* have been produced and various parts of molecules are cloned to define citrate permeate genes for production of diacetylactis.

Biopreservation

Livestock products being highly prone to microbial contamination and they undergo microbial deterioration. The recent trend of consumers towards food without artificial preservatives has led processors to search for natural preservatives. The antimicrobial system possessed by lactic acid bacteria offer scope for the development of an effective natural preservation process for application in food either as purified chemical agents or as viable cultures. Suppression of spoilage and food borne pathogens by lactic acid bacteria could be extremely beneficial to human health and dairy industry as these attributes can considerably improve the shelf life and safety of fermented foods. Lactic acid bacteria can act antagonistically against a wide range of food borne pathogens and spoilage organisms like *Salmonella*, *Staphylococcus, Clostridium, Listeria monocytogenes, Yersinia enterocolitica and Psuedomonas*. Incorporation of certain antimicrobial agents to livestock products viz bacteriocin, nisin, H_2O_2, diacetyl, microgard, reutrin and pimaricin has yielded highly promising results and the enhanced shelf life of products thus attained would be economically, nutritionally and therapeutically beneficial both to manufactures & consumers. Biotechnological techniques can now be applied to develop strains of lactic acid bacteria capable of enhanced production of these natural food grade preservative and also to combine within a single strain the ability of produce a number of such bacteriocin to extend their antibacterial spectrum.

Byproduct utilization

A major concern in the food processing industry is the development of methods to convert inedible and waste materials into new value-added products. Environmental and economic concerns necessitate a reduction of food processing waste, better use of raw materials and by products to new value added products. The cheese industry generates billions of pounds of whey that must be disposed off. Ultrafiltration has provided the cheese maker with a means of concentrating the protein component of

whey into a value-added item with significant dollar value. A recently developed bioconversion system employing selected strains of yeast can convert these solids to ascorbic acid with a market value of about $10 per kilogram. The yeast biomass could be dried and used as single-cell protein supplements in animal feed. Enzymatic treatment of food processing waste streams can produce materials readily metabolized by genetically engineered micro-organisms to produce antibiotics, hormones or peptides of interest in the pharmaceutical or chemical industries.

Quality control

Ensuring an acceptable level of food quality and safety is absolutely necessary to provide adequate protection for consumers and to facilitate trade. There must be a strong network of efficient quality assurance programme to monitor the quality and safety of these foods before reaching to the consumers. This can be largely possible with the application of recent developments in biotechnological tools in quality assurance programmes. The use of modern biotechnology has proved to be rapid, sensitive and accurate methods for detection and analysis of bacterial contaminants and pathogens or their toxins. Some of the most powerful tools of biotechnology which have already made great strides include rDNA technology, Genetic engineering, PCR, Microarray etc. Random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) molecular marker systems can also be used for the comparison of genetic differences between species, subspecies and strains, depending on the reaction conditions used. The use of combination of these technologies and other genetic tests allow the characterisation and identification of organisms at the genus, species, sub-species and even strain levels, thereby making it possible to pinpoint sources of food contamination, to trace microorganisms throughout the food chain or to identify the causal agents of food borne illnesses. Microarrays are biosensors which consist of large numbers of parallel hybrid receptors (DNA, proteins, oligonucleotides). Microarrays are also referred to as biochip, DNA chip, DNA microarray or gene arrays and offer unprecedented opportunities and approaches to diagnostic and detection methods. They can be used for the detection of pathogens, pesticides and toxins and offer considerable potential for monitoring the quality and safety of raw materials. Biotechnology has led to the development of tests that can be completed in one fifth of the time required by conventional methods. Development of new innovative methods for rapid detection of emerging high risk food pathogens such as *E. coli O157, Listeria monocytogenes, Salmonella* and *Yersinia enterocolitica* in food of animal origin is extremely important in context of food safety.

Meat authentication

In food production, raw materials from farm animals are typically processed to meat and cheese products. Ef? cient assays to detect the origin of meat precisely and rapidly are always in demand. Identi? cation of the meat origin from processed meat products is a current matter of concern for a variety of economic, religious, and health reasons. The extensive development of nucleic acid based technologies over the past decade re? ect their importance in food analysis. DNA analytical methods offer a promising alternative for reliable species differen-tiation even in heated food samples, since the thermostability of DNA is much higher than that of proteins. Most of the DNA-based methods used rely on the simplicity and the sensitivity of the polymerase chain reaction (PCR). Species differentiation is achieved either by amplification of characteristic DNA fragments with species-specific primers or in the consensus PCR approach, by use of universal primers followed by sequencing or restriction fragment length polymorphism (RFLP) analysis for the species-specific identification of the resulting amplicons.

Applications of Biotechnological Techniques: Their Adverse Effects

During the past couple of years the science of engineering and modifying organism to meet and comply with requirements have been quite significant. The commercial applications of these animals have been quite significant; however, the role of these interventions have been under considerable scrutiny. The insertion of genes has been used in mice and numerous other transgenic animals which have been used for animal experimentation. Genetically engineered mice have become the hallmark of vivisection and research especially in case of animal studies. Hence, genetically altered mice have become widespread in their usage in biomedical research. The question arises, however, if these mice were allowed to interact with other mice in the environment, then what would the resulting interactions result in.

Since it is now possible to generate animals which have important functions in biotechnology and biomedical research, similar techniques are being adopted to engineer animals having superior weight gain and physical characteristics which is what farmers and animal breeders are looking for. Methods that originally originated from mice as well as Drosophila melanogaster have been adapted to be used in other animals as well. Manipulation of the fertilized egg, and in vivo implantation are a part of the biotechnological techniques used. Sperm manipulation before it reaches the egg, also the usage of embryonic stem cells post manipulation ex vivo, somatic cell culture as well as the usage of embryonic stem cells are a part of the techniques used to manipulate the animal's characteristics. The nuclei of these animals

can be used to produce generic transgenic animals, which are then used in various frontiers of biotechnology. Some of these methods have the advantage of the geneticist to incorporate particular character-istics into the transgenic animal of interest which is engineered as a result of manipulation.

The selection of certain genes that have characteristic features of particular interest to the animal breeder will be incorporated or selected for the production of these transgenic animals. The implications, however, will result in the formation of animals which possess a certain set of "selected genes," these selected genes are a representation of the breeder/geneticist's interpretation of characteristic traits and may not be the appropriate controller. The greatest issue relating to this method is depletion in the gene pool. Due to increased artificial selection and prevention of appropriate natural selection, genes continue to be lost from the abundant gene pool. This can be seen in animal species, particularly in case of cattle. Here simple inbreeding or forced selection will result in the prevention of appropriate genetic diversity within a species. Variations in the genome are what give a species its various traits, particularly in case of animals where traits are dependent on genes for their phenotypic expression. These expressed traits are selected on the basis of required characteristics enhancing the overall feed conversion and efficiency in case of animals. However, it has been noticed that when genes are introduced then genetic instability is noted in the genome. This instability may have adverse effects on the overall health status of the animal in question.

The biggest question that looms over animal breeders and scientists is the use of transgenic animals for human use. The reason being that the incorporation of recombinant animals may have inadvertent adverse effects on the human population which is in direct contact with these animals. Many chemicals and growth promoters used to enhance the vigor and muscle to fat ratio in bulls may have effects on the physiology of the bull. When that bull is sacrificed for human consumption, the resulting accumulation of toxic biotransformed prod-ucts present in various tissues will result in negative effects on humans on con-sumption. A novel inclusion of certain genes in animals used for formation of transgenic animals may cause the production of protein structures that can induce allergenicity and toxicity in the consumer. These variations in proteins that are produced as a result of the toxicity of the included genes or chemicals used to enhance the vigor of animals, and the sheer variations in the expression of proteins, will result in complex immune responses to the change occurring.

Dead carcasses when consumed by other animals also may result in the transference of these toxic compounds to other animals resulting in the death of animals in the ecosystem. When certain essential animals in the ecosystem are affected, the delicate fabric of biodiversity is affected and the equilibrium is lost. This loss of equilibrium will result in a failure of the complex ecological system in place; a failure that can be translated as disastrous for the environment.

Biodegradation of Chlorinated Compounds

Chlorinated organic molecules constitute the largest single group of compounds on the list of priority pollutants compiled by the U.S Environmental Protection Agency (U.S. EPA). They are extraneously added into the environment in large quantities as a result of their widespread use as herbicides, insecticides, fungicides, solvents, hydraulic and heat-transfer fluids, plasticizers, cleaning agents, fumigants, aerosol propellants, gasoline additives, degreasers, and intermediates for chemical syntheses.

The ability of chlorinated compounds to impart toxicity, bioconcentrate, and persist and con-sequently their ubiquitous distribution in the biosphere has caused public concern over their possible effects on the quality of life. Some chlorinated compounds also occur naturally in the environment, although in lower concentrations. For example, many different genera of wood rotting fungi produce chlorinated anisyl metabolites in their natural environments. These chloroanisyl-derivative-producing fungi are widespread in nature.

As is true for many organic compounds, the turnover of chlorinated molecules in the environment is largely determined by their susceptibility to biotransformation by microorganisms. Many of the chloro-organics that are not degraded by bacteria and fungi have the potential to persist in the environment and express their toxicity over extended periods of time.

Thus, identification and application of novel organisms that use chlorinated pollutants for growth have become an important area of research today. Further, process optimization for biodegradation of these hazardous chemicals requires an understanding of microorganisms involved in the degradation, their nutrient requirements, the biochemistry of their mediated reactions, and why they promote these reactions.

Biodegradation of Chlorinated Compounds

TABLE 1. Major Chlorinated Hydrocarbons (HC) and Their Applications

Chlorinated HC	Major uses
Chloromethanes	
Monochloromethane	Production of silicones, tetramethyllead, methylcellulose, other methylation reactions
Dichloromethane	Degreasing agent, paint remover, pressure mediator in aerosols; extract technology
Chloroethanes	
Monochloroethane	Production of tetraethyllead, production of ethylcellulose; ethylating agent for fine chemical production, solvent for extracting processes[3]
1,1- Dichloroethane	Feedstock for the production of 1,1,1-trichloroethane
1,2-Dichloroethane	Production of vinyl chloride, production of chlorinated solvents such as 1,1,1-trichloroethane and tri- and tetrachloroethane, synthesis of diethylenediamines
Chloroethenes	
Monochloroethene	Production of polyvinyl chloride (PVC), production of (vinyl chloride) chlorinated solvents, primarily 1,1,1-trichloroethane
Trichloroethene	Solvent for vapor degreasing in the metal industry and for dry cleaning, extraction solvent, solvents in formulations for rubbers, elastomers and industrial paints
Chlorinated paraffins	Plasticizers in PVC; flameproofing agents in rubber textiles, plastics, H_2O repellent and not—preventive agents; elastic sealing compounds paints & varnishes; metalworking agents (cutting oils); leather finishing

The biological destruction of toxic and hazardous chemicals is also based on the principles that support all ecosystems. These principles involve the circulation, transformation, assimilation of energy and matter. Microorganisms convert complex organic compounds, via their central metabolic routes, to CO_2 or other simple organic compounds. The oxidation yields energy and reducing equivalents that are used for conversion of a part of the intermediates to cell mass (assimilation), enabling growth of the organisms that carry out the degradation process.

Degradation of compounds of natural origin is usually easy to achieve, and organisms that bring about their degradation can be easily isolated from their natural environments. However, in general, compounds having a structure that is different from naturally occurring compounds (xenobiotics, most of which are toxic and hazardous) are more difficult to degrade. Never-theless, in the recent past, an array of microorganisms has been identified that use xenobiotics such as chlorinated alkanes, chlorinated halohydrins, polychlorinated biphenyls, and chlorobenzenes for their survival.

Biodegradation of chlorinated compounds follows two pathways namely "**aerobic** degradation" or "**anaerobic** degradation." However, irrespective of the pathway followed, the extent of degradation depends on the struc-ture of the compound, the number of chlorine substituents, and the posi-tion of chlorine in the molecules. Depending on the structure, chlorinated compounds can be either oxidized or reduced. Reduction is possible be-cause of their electronegative character, which makes them highly electron deficient.

Aerobic Degradation

During aerobic degradation of chlorinated compounds by microorganisms, molecular oxygen serves as the electron acceptor. Several chloroaliphatic compounds have been shown to degrade aerobically. Sustained degradation of TCE in a suspended growth reactor is noticed by an *Actinomycetes* enrichment culture.

Aerobic mineralization has been well documented for chlorobenzenes with up to four chlorine substituents in microcosms and by pure cultures. Several of the chlorobenzenes (containing one, two, three, or four chlorine substituents) could be biotransformed only under aerobic conditions and were unstable in the absence of molecular oxygen.

4-chlorophenol (4-CP) can be partially or completely degraded aerobically by several bacteria, including *Pseudomonas*, *Alcaligenes*, *Rhodococcus*, Azotobacter[58] etc.

Pentachlorophenol (PCP) is degraded by *Phanerochaete* spp. A list of microorganisms degrading some aliphatic chlorinated compounds aerobically is given in Table.

Aerobic Degradation of Some Chlorinated Aliphatics

Number	Compound	Microorganism
1	1,2-Dichloroethane	*X. autotrophicus*
2	1,1,2-trichloroethane	*P. putida*
3	Trichloroethylene	*Methylomonas methanica*
4	CCl_4	*Escherichia coli* K-12

Anaerobic Degradation

In the anaerobic mode of degradation the **electron acceptor is a molecule other than O_2**. This could be NO^-_3, SO^{2-}_4, Fe^{3+}, H^+, S, fumarate, trimethylamine oxide, an organic compound, or CO_2. The term "dehalorespiration" has been coined for anaerobic bacteria that couple the reductive dehalo-genation of chlorinated aliphatic and aromatic compounds to ATP synthesis via an electron transport chain.

Reductive dechlorination or reductive de-hydrogenolysis is a common biotransformation pathway for chloroaliphatics containing one or two carbon atoms, under methanogenic conditions. A strictly anaerobic homoacetogenic bacterium and an uncharacterized anaerobic mixed culture were shown to use chloromethane as a 'C' and energy source.

Most of the chlorinated aromatic compounds and several pesticides are known to be best degraded under anaerobic conditions. Beside Benzenes and Toluenes, several chlorinated aromatic compounds have been shown to be degraded under methanogenic conditions. These include 2,4,5-trichlorophenoxyacetate, 3-chlorobenzoate, 2,4-dichlorophenol, 4-chlorophenol, 2,3,6-trichlorobenzoate, and dichlorobenzoates.

Dehalo-coccoides sp. completely detoxified VC by an anaerobic enrichment culture. Under anaerobic conditions, degradation of pesticide hexachlorocyclohexane (HCH) is occurred in sewage sludge. Anaerobic reductive dechlorination of chlorobenzene congeners is occurred by cell extracts of *Dehalococcoides* strain CBDB1. A list of microorganisms degrading some aliphatic chlorinated compounds anaerobically is given in Table.

Anaerobic Degradation of Some Chlorinated Aliphatics

Number	Compound	Microorganism
1	Carbon tetrachloride	Methanogenic enrichment *Clostridium sp.*
2	1,1,1-Trichloroethane (TCE)	Sulfate reducing enrichment, methanogenic enrichment
3	Vinyl chloride	Anaerobic mixed culture
4	Dichloromethane (DCM)	Anaerobic consortia
5	Trichloroethylene	Anaerobic consortia

Sequential Degradation

Although degradation of chlorinated aliphatic and aromatic compounds has been reported both under aerobic and anaerobic conditions, sequential use of these processes always has an advantage over using them individually for complete mineralization of heavily chlorinated compounds.

It is generally implied that aerobic microbes often fail to metabolize heavily chlorinated compounds. For example, several bacteria capable of oxidizing TCE, DCE, and VC by using nonspecific enzymes cannot oxidize TeCE by any of these enzyme systems. Aerobic bacteria that rapidly biodegrade monochlorinated benzenes are usually unable to degrade heavily chlorinated benzene compounds.

Therefore, it has been suggested that detoxification and complete mineralization of chlorinated wastes can be easily achieved by using a sequential treatment process, that is, anaerobic followed by aerobic treatment.

For instance, the fungicide HCB (hexachlorobenzene) and polychlorinated biphenyl (PCB) undergo reductive dechlorination in anaerobic environments. Then, the The products are biodegraded by aerobic bacteria. A sequential treatment step will ensure total mineralization of these chlorinated toxic compounds.

DNA FINGER PRINTING AND ITS APPLICATIONS

DNA fingerprinting

Introduction

DNA fingerprinting or **DNA profiling,** any of several similar techniques for analyzing and comparing DNA from separate sources, used especially in law enforcement to identify suspects from hair, blood, semen, or other biological materials found at the scene of a violent crime. It depends on the fact that no two people, save identical twins, have exactly the same DNA sequence, and that although only limited segments of a person's DNA are scrutinized in the procedure, those segments will be statistically unique.

DNA fingerprinting: Applications

In criminal investigations, the DNA fingerprint of a suspect's blood or other body material is compared to that of the evidence from the crime scene to see how closely they match. The technique can also be used to establish paternity. First developed in 1984 by Alec Jeffreys, a British professor of genetics at the Univ. of Leicester, DNA fingerprinting has been accepted in most courts in the United States, and has in several notable instances been used to exonerate or free persons convicted of crimes, but the Supreme Court has ruled (2009) that convicted criminals do not have a constitutional right to DNA testing.

DNA fingerprinting is generally regarded as a reliable forensic tool when properly done, but some scientists have called for wider sampling of human DNA to insure that the segments analyzed are indeed highly variable for all ethnic and racial groups. It is possible to create false genetic samples and use them to misdirect forensic investigators, but if those samples have been produced using gene amplification techniques they can be distinguished from normal DNA evidence.

The techniques used in DNA fingerprinting also have applications in paleontology, archaeology, various fields of biology, and medical diagnostics. It has, for example, been used to match the goatskin fragments of the Dead Sea Scrolls.

In biological classification , it can help to show evolutionary change and relationships on the molecular level, and it has the advantage of being able to be used even when only very small samples, such as tiny pieces of preserved tissue from extinct animals, are available.

DNA fingerprinting: Methods

A common procedure for DNA fingerprinting is restriction fragment length polymorphism (RFLP). In this method, DNA is extracted from a sample and cut into segments using special restriction enzymes. RFLP focuses on segments that contain sequences of repeated DNA bases, which vary widely from person to person.

The segments are separated using a laboratory technique called electrophoresis, which sorts the fragments by length. The segments are radioactively tagged to produce a visual pattern known as an autoradiograph, or DNA fingerprint, on X-ray film.

A newer method known as short tandem repeats (STR) analyzes DNA segments for the number of repeats at 13 specific DNA sites. The chance of misidentification in this procedure is one in several billion.

Yet another process, polymerase chain reaction , is used to produce multiple copies of segments from a very limited amount of DNA (as little as 50 molecules), enabling a DNA fingerprint to be made from a single hair. Once a sufficient sample has been produced, the pattern of the alleles (see genetics) from a limited number of genes is compared with the pattern from the reference sample.

A nonmatch is conclusive, but the technique provides less certainty when a match occurs.

DEVELOPMENT OF NON TOXIC THERAPEUTIC AGENTS

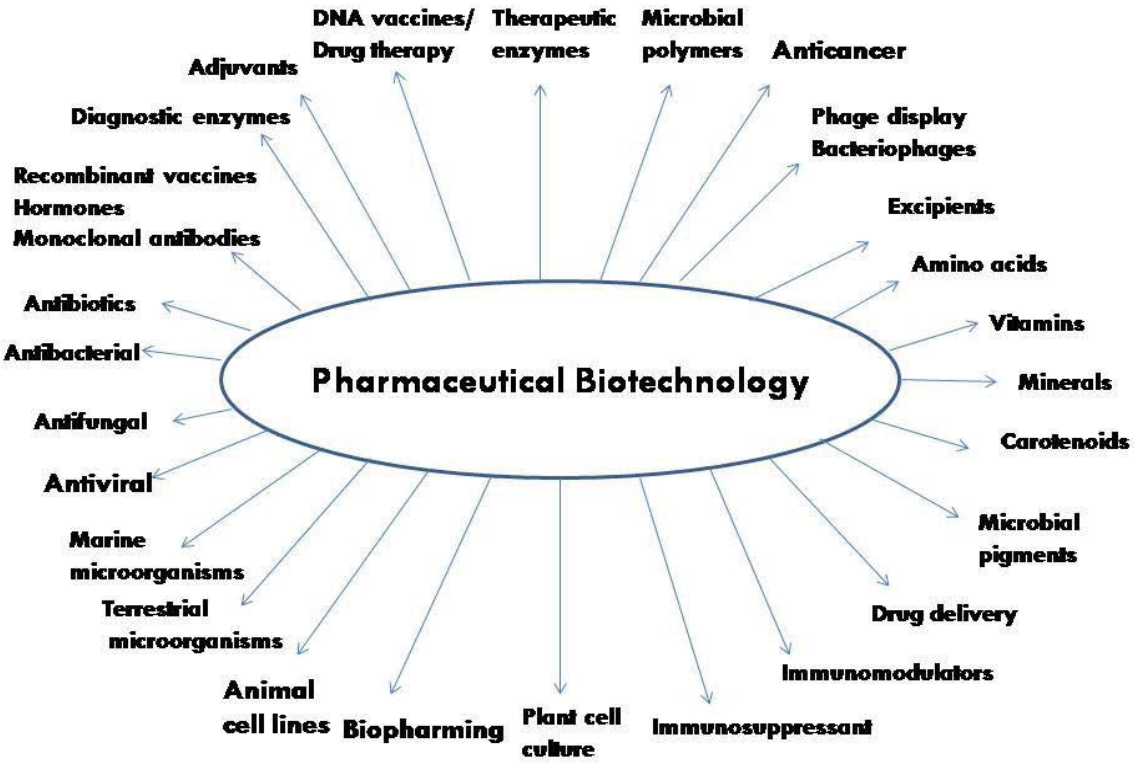
In recent years, the number of drugs of biotechnological origin available for many different diseases has increased exponentially, including different types of cancer, diabetes mellitus, infectious diseases (e.g. AIDS Virus / HIV) as well as cardiovascular, neurological, respiratory, and autoimmune diseases, among others. The pharmaceutical industry has used different technologies to obtain new and promising active ingredients, as exemplified by the fermentation technique, recombinant DNA technique and the hybridoma technique.

Introduction

The word biotechnology was first used by Karl Ereky (Hungarian agricultural engineer) in 1919, featuring the use of living organisms on a given raw material for the purpose of obtaining a particular product and introducing the concept of genetic change. Biotechnology is based on scientific knowledge from different disciplines such as Microbiology, Biochemistry, Genetics, Chemistry, Engineering and Computer Science for biological agents such as microorganisms, cells or molecules (enzymes, antibodies, DNA, etc.) to provide goods and ensure services. This multidisciplinary approach is the most important characteristic of this scientific field of study in constant evolution.

In recent years, the biotech industry has been remarkable since it is associated with high efficiency production processes, low manpower, low costs, an environmentally friendly industry, with low energy consumption and reduced emission of greenhouse gases. The pharmaceutical industry, in their attempts to discover new molecules, has found an ally in the biotechnology industry, with exponential growth. Thus, the biggest global pharmaceutical companies are buying companies linked to biotechnological research and production.

The most important biopharmaceuticals are **blood factors, hormones, cytokines, enzymes, vaccines and monoclonal antibodies**.



Biotechnology Processes

Current biotechnological processes essentially involve five different groups of organisms:

Bacteria (e.g. Escherichia coli, Pseudomonas spp. Serratia mascescens, Erwenia herbícola, Lactococcus lactis and Bacillus subtilis),

Fungi (e.g. Saccharomyces cerevisiae, Pichia and Hansenula, Trichoderma and Aspergilli),

Plants (e.g. tobacco plant, rape and transgenic potatoes)

Insects (e.g. Spodoptra frugiperda) and

Mammalians (e.g. Chinese hamster ovary cells (CHO), baby hamster kidney cells (BHK) and transgenic animals).

The application of different techniques allows changes to be made in microorganisms, in order to highlight a particular feature or increase their production and ultimately the production of new products. For this, conventional genetic techniques such as mutagenesis, fermentation, sexual and parasexual processes or modern techniques such as recombinant DNA techniques or the hybridoma technique, can be used.

Examples of drugs obtained by biotechnology processes

Biopharmaceutical forms are potent, reactive, unstable and very expensive. They have several advantages such as the provision of effective treatments in chronic and uncommon diseases. Recombinant drugs (Factor VIII for hemophilia), offer safer and reduced side effects, improve on existing therapies and can be produced on a large scale by biotechnological processes.

TABLE – Some examples of diseases and respective biopharmaceuticals used in treatment

Disease	**Active substance**
Hepatitis C	Interferon α
Multiple Sclerosis	Interferon β
Renal Cancer	Interleukin
Hemophilia	Factor VIII and Factor IX
Diabetes	Human Insulin
Anemia	Erythropoietin alfa
Crohn's Disease	MoAb Infliximab cA2
Immune system rejection	MoAb muromonab-CD3
Cystic fibrosis	Dornase α

In the future, biopharmaceuticals may be used against the AIDS virus, different types of cancer, asthma, Parkinson's and Alzheimer's disease.

There are different groups of biopharmaceuticals: **antibiotics**, **blood factors**, **hormones**, **growth factors**, **cytokines**, **enzymes**, **vaccines** and **monoclonal antibodies**.

Antibiotics

Antibiotics are the largest group in terms of economic importance among the products obtained by fermentation. Some examples of antibiotics whose synthesis involved microorganisms include penicillin produced from Penicillium notatum; cephalosporins from the genus Streptomyces; chloramphenicol from Streptomyces venezuelae; streptomycin from Streptomyces griseus; cycloserine from Streptomyces orchidaceus; clindamycin from Streptomyces lincolnensis; vancomycin isolated from cultures of Streptomyces orientalis (Nocardia orientalis); teicoplanin from Actinmoplanes teichomyceticus and mupirocin from Pseudomonas fluoresces.

Blood Factors

Even with identical causes, two types of hemophilia can be distinguished:

Hemophilia A (the deficient or abnormal element is Factor VIII or antihemophilic factor A) and

Hemophilia B (the deficient or abnormal element is Factor IX or antihemophilic Factor B).

These two blood clotting factors are produced by recombinant techniques. The recombinant **Factor VIII** produced in CHO cells, containing 1438 a.a. is used in the treatment of hemophilia A (a hereditary disease characterized by slow blood clotting and difficulty controlling blood loss). Another example is the **Factor IX** produced in CHO cells, containing 415 a.a. used in the treatment of hemophilia B. The gene that produces this factor was cloned in a sheep by a Scottish laboratory in 1997, and this sheep subsequently produced milk that contained this factor.

In 2009, the FDA approved **Atryn**® (antithrombin recombinant), the first medicine produced using genetically engineered animals. This protein with anticoagulant and anti-inflammatory properties is produced in the milk of goats that have been genetically modified.

Hormones

In 1982, the FDA approved the first dosage form obtained through biotechnological processes, recombinant human insulin for the treatment of patients with diabetes, using recombinant DNA techniques in the bacteria E. coli (**Humulin**®, **Novolin**®, **Velosulin**®). Today, recombinant human insulin is available in different concentrations under different forms of therapeutic action (insulin lispro, insulin aspart, insulin glargine - respectively, very fast, fast, long acting) and for different applications (intramuscular, sub-cutaneous, etc.). The recombinant human growth hormone improved the long-term treatment of children whose body was not producing enough growth hormone. **Somatropin** is a recombinant human growth hormone, marketed under different brand names such as Saizen®, Nutropin®, Humatrope® and Serostin®.

Growth Factors

Many Hematopoietic Growth Factors (HGFs) have been isolated, and the understanding of their clinical potential continues to grow. HGFs have had a significant impact on the prevention of infections associated with chemotherapy-induced neutropenia, chemotherapy induced thrombocytopenia, and chemotherapy-induced anemia. Patients with HIV/AIDS can also been helped by the administration of recombinant HGFs.

Erythropoietin, a hormone produced by the kidneys, stimulates the bone marrow to produce red blood cells. The recombinant human erythropoietin (Procrit®, Epogen®, Eprex®, NeoRecormon®) may appear in different forms: alpha (produced in CHO), beta (produced in CHO) and gamma (produced in BHK). This recombinant growth factor is used in the treatment of anemia associated with renal failure, HIV infections, surgery, etc. Erythropoietin alpha is targeted for the treatment of anemia due to chronic renal failure, HIV infection and cancer.

Another example is **Mircera**® (beta methoxypolyethyleneglycol-epoetin) used for the treatment of anemia associated with chronic renal failure (Fajardo et al., 2010). On the other hand, **Palifermin** (Kepivance®) is very similar to a natural growth factor that exists in the

human body, known as keratinocyte growth factor (KGF). **Kepivance**® stimulates the growth of cells, helping to reduce the incidence, severity and duration of oral mucositis in cancer patients subjected to intensive care.

Cytokines

Cytokines are molecules that activate the immune cells (e.g. lymphocytes and macrophages), regulate growth and differentiation of immune cells, also important messengers in cells, influencing the response in inflammation, response immune and tissue repair.

Interleukins are molecules that act as leukocytes messengers, for example the interleukin-2 stimulates T lymphocytes. **IL-2 recombinant interleukin, approved by FDA, produced by E. coli, which differs from the natural interleukin by the alanine absence on the N-terminal and by the fact that serine is replaced by cysteine at 125 amino acid 125**, as exemplified in **aldesleukin** (Proleucina®). This drug is used in the treatment of renal cell cancer, and its effect is proportional to the amount of recombinant drug administered.

There are other drugs that block interleukin, for example, **Arcalyst**® (rilonacept) used for the treatment of CAPS - Cryopyrin Associated Periodic Syndromes. This drug blocks a chemical messenger called interleukin-1-beta and interleukin1-alpha.

The recombinant interferons (potent cytokines that act against viruses and against uncontrolled proliferation of cells) exist in three forms: alpha, beta and gamma, and feature a wide variety of applications. The α recombinant interferon is used in patients with Kaposi's sarcoma, hepatitis B, hepatitis C and renal cell cancer. The β recombinant interferon (produced by E. coli containing 165 a.a.) is used in patients with secondary progressive sclerosis, because it inhibits the production of Th1 cytokines and activates the monocytes involved in the immune response.

Examples of **α recombinant interferons** are **Intron-A**®, **Roferon-A**® and **Actimmume**® whereas **β recombinant interferons** include **Avonex**®, **Rebif**® and **Betaseron**®. Finally, γ recombinant interferon (produced by E. coli containing 139 a.a.) is used in patients with infections associated with chronic granulomatous disease.

Enzymes

Recombinant **dornase alpha** (formulated in the form of an aerosol - **Pulmozyme**®) is an enzyme produced by CHO cells, used in the treatment of patients with cystic fibrosis, a genetic disorder marked by excessive mucous secretions and frequent lung infections.

Another example of a recombinant enzyme is a **plasminogen activator**, known as **alteplase** (Activase®), used to dissolve blood clots formed in the circulatory system, which can cause heart attacks, pulmonary embolisms and strokes. On the other hand, **Naglazyme**® (Galsulfase) is a form of recombinant enzyme used for the treatment of patients with mucopolysaccharidosis VI (MPS VI or Maroteaux-Lamy). This disease is caused by the lack of an enzyme called B arylsulfatase, required in the degradation of substances, known as glycosaminoglycans (GAGs). If the enzyme is not present, the GAG cannot be degraded and accumulates in cells, causing large head and movement difficulties.

Elaprase® (idursulfase) is another enzyme produced by biotechnological processes used in the treatment of patients with Hunter syndrome (patients are not able to degrade glycosaminoglycans, which gradually accumulates in cells, affecting most organs, causing difficulty breathing and walking).

Another case of using biotechnology to produce drugs is the production of essential enzymes in patients with Gaucher syndrome type 1 and 3 (a disease characterized by deficiency of the beta-glucosidase enzyme). This disease is usually characterized by a neurological disorder that includes mental degeneration and seizures. There are a few effective therapies for treatment including VPRIV® (velaglucerase alpha - a human cell line derived enzyme replacement therapy - for the long-term treatment of type 1 **Gaucher disease**), the Protalix Biotherapeutics (taliglucerase alpha - a plant cell-expressed recombinant glucocerebrosidase enzyme), Cerezyme® (imiglucerase - produced by recombinant DNA technology using mammalian cell culture, CHO) and Zavesca® (miglustat -reduces the harmful build up of fatty substances throughout the body by reducing the amount of glycosphingolipids produced by the body - used in patients who cannot be treated with enzyme replacement therapy).

A different enzyme produced using human cell lines is **alfagalsidase** (Replagal®). This enzyme is a copy of the human enzyme used in enzyme replacement therapy for **Fabry's disease**

(chronic and progressive genetic diseases caused by absence or deficiency of an enzyme called alpha-galactosidase A, responsible for the decomposition of lipids in the body, consequently the lipids accumulate in vital organs causing serious problems).

Vaccines

Currently, vaccines are not only developed against infectious diseases, but also against drug abuse (nicotine, cocaine) and against allergies, cancer and Alzheimer's disease. Despite the success of conventional vaccines, there are still many infectious diseases and other chronic diseases against which no effective vaccine exists. In addition, the growing resistance to the existing arsenal of antibiotics increases the need to develop vaccines against common bacterial infections.

Although conventionally produced vaccines are generally harmless, some of them may, rarely, contain infectious contaminants. Vaccines whose active ingredients are recombinant antigens do not carry this slight risk. Vaccines produced by recombinant DNA techniques have been used to combat seasonal influenza virus (**Fluarix**®, **Istivac**®, Fluzone®, FluMist®, Agriflu® etc.) and hepatitis A and B. The first vaccine against hepatitis B was made from plasma derived from patients with chronic hepatitis B, and a recombinant vaccine whose sole active ingredient is a recombinant antigen has now replaced it.

There are also other types of vaccines produced by genetic engineering, using the yeast Saccharomyces cerevisiae for the production of HBsAg or by entering the HBsAg gene in mammalian cells (**Recombivax** HB®, Engerix B®). The **Ambirix**® vaccine is another example of a bivalent vaccine used to protect against hepatitis A and hepatitis B (diseases affecting the liver) in children aged between 1 and 15 years old, who have no immunity to these diseases. This vaccine consists of inactivated hepatitis A virus (produced in human diploid cells, MRC-5) and surface antigen of hepatitis B (produced in Saccharomyces cerevisiae yeast cells by recombinant DNA technology). Another example of a vaccine used to protect against hepatitis A and B infection is **Twinrix**®, which contains inactivated hepatitis A virus and parts of the hepatitis B virus as active substances (surface antigens obtained by recombinant DNA technology). On the other hand, the Myobloc® vaccine is a botulinum toxin type B vaccine for the treatment of cervical dystonia, produced by fermentation using the bacterium Clostridium botulinum type B.

Botulinum toxin type A (**Botox**®) is indicated for the treatment of **cervical dystonia**. The Botox Cosmetic® is used in adults under 65 years to raise and fix tissue firmness.

Another example of a vaccine produced by genetic engineering is **Dukoral**®, used in protection against cholera (an extremely serious disease caused by V. cholerae, which is contracted from contaminated food or water and causes severe diarrhea). This vaccine contains small amounts of dead cholera bacteria and a part of the cholera toxin called "B subunit" (produced by recombinant DNA).

Monoclonal antibodies

Monoclonal antibodies provide targeted immunosuppression that, when used in conjunction with specific maintenance immunosuppressants, may allow more specific therapy and can be used not only for tumor therapy but also in other therapies or diagnoses. In recent years, this group of drugs has undergone more extensive research, and shown a very promising.

TABLE II – Examples of monoclonal antibodies on the market and their therapeutic indications

Medicine	Active Substances	Therapeutic Indications	Mechanism
Leukoscan®	Sulesomab	Imaging of bone infection or inflammation	Antigranulocyte scintigraphy
Verluma®	Nofetumomab	Detection Kit for Lung Cancer	Directed against a 40-kilodalton (Kd) glycoprotein antigen expressed on the surface of numerous tumors
ProstaScint®	Capromab Pendetide	Diagnosis of prostate cancer	Recognizes a prostate specific membrane glycoprotein that is chiefly expressed by prostatic epithelial cells, (PSMA)
Rituxan®	Rituximab	B cells Non-Hodgkin lymphoma	Chimeric monoclonal antibody against the protein CD20 found on the surface of B cells
Mabthera®	Rituximab	Follicular lymphoma, diffuse non-Hodgkin lymphoma	Chimeric monoclonal antibody against the protein CD20 found on the surface of B cells
Simulect®	Basiliximab	Reduces the incidence and severity of acute rejection in kidney transplantation	Blocks the receptor for IL-2, a protein that simulates proliferation of T-lymphocytes, which play a key role in organ transplant rejection
Remicade®	Infliximab	Rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, psoriasis	Blocks the effects of tumor necrosis factor alpha (TNF-alpha)
Synagis®	Palivizumab	Against the Human respiratory syncytial virus (RSV)	Targets the fusion protein of RSV, inhibiting its entry into the cell thereby preventing infection
Zenapax®	Daclizumab	Reduces the incidence and severity of acute rejection in kidney transplantation	Binds specifically to the alpha subunit (p55 alpha, CD25, or Tac subunit) of the human high-affinity interleukin-2 (IL-2) receptor that is expressed on the surface of activated lymphocytes
Herceptin®	Trastuzumab	Breast Cancer	Attaches itself to the HER2 receptors on the surface of breast cancer cells blocking them