SRINIVASAN COLLEGE OF ARTS & SCIENCE

(Affiliated to Bharathidasan University, Trichy)

PERAMBALUR – 621 212.

DEPARTMENT OF MICROBIOLOGY

Course : M.Sc Year: **II** Semester: **IV**

Course Material on:

MICROBIAL BIOTECHNLOGY

Sub. Code : P16MBE5A

Prepared by :

Dr. A.CHOLARAJAN, M.Sc., M.Phil., Ph.D

ASSISTANT PROFESSOR / MB

Month & Year : APRIL – 2020

ELECTIVE COURSE - MICROBIAL BIOTECHNOLOGY

OBJECTIVES:

To impart the potential applications of microbial and molecular biotechnology in medicine, agriculture and various other current industrial processes.

UNIT I: Microbial production of therapeutic agents and vaccines: History – Microbial vs molecular biotechnology and Commercialization – concerns and consequences - Pharmaceuticals - interferons and growth hormones, enzymes: DNase I and alginate lyase, Monoclonal antibodies - HIV therapeutic agents. Subunit vaccines: Herpes simplex virus, Foot and mouth disease virus, TB, Peptide vaccines – genetic immunization – vector vaccines.

UNIT II: **Microbial production of commercial products Microbial production of restriction endonucleases:** PstI, Dye: Indigo, Antibiotics: Synthesis of Novel antibiotics. Biopolymers: Xanthan gum and PHA. Microbial production of alcohol, lactic acid, streptomycin, L- glutamic acid, lipase and riboflavin.

UNIT III: **Production of PGPR, biofertilizers and biocontrol agents**: Plant growth promoting bacteria (PGPR) – genetic engineering of nitrogenase gene cluster, hydrogenase and Nodulation. Mass cultivation of microbial biofertilizers: Cyanobacteria (Sprirulina), Azolla and other nitrogen fixers (Rhizobia, Azospirillum, Azotobacter and VAM) Biocontrol of pathogens: Siderophores, antibiotics and enzymes. Release of genetically engineered organisms - Ice nucleation and anti-freeze proteins. Microbial herbicides. Microbial insecticides (Pseudomonas and Bacillus thrungiensis): - genetic engineering of Bt strains – Bt cotton – viral insecticides – entomopathogenic fungi.

UNIT IV: Plant and algal biotechnology and bioremediation: Ti plasmid derived vector systems - Development of insect, virus and herbicide resistant plants, stress and senescence tolerant plants, modification of flower nutritional content, sweetening by genetic engineering. Plant as bioreactors. Production of food, colourant and fuel from microalgae.

UNIT V: Animal biotechnology and IPR Transgenic animals: methods of creating transgenic mice, cattle and sheep. Human gene therapy – in vivo and ex vivo gene therapy. Molecular diagnostics for genetic diseases. Biosafety and Bioethics. Intellectual Property Rights: Patents - copy right and neighboring rights, patents for invention, - Drafting and filing a patent application, exploitation of patented invention. Indian patent laws.

UNIT I: Microbial production of therapeutic agents and vaccines:

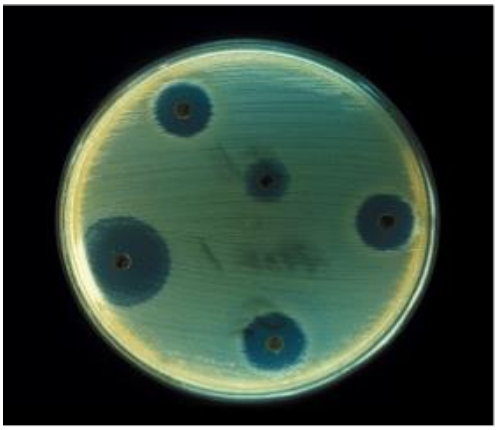
- The term 'biopharmaceutical' was first used in the 1980s and came to describe a class of therapeutic protein produced by modern biotechnological techniques, specifically via genetic engineering.
- Biomedical research continues to broaden our understanding of the molecular mechanisms underlining both health and disease.
- Research undertaken since the 1950s has pinpointed a host of proteins produced naturally in the body which have obvious therapeutic applications.
- Examples include the **interferons,** and **interleukins**, which regulate the immune response; growth factors such as **erythropoietin,** which stimulates red blood cell production; and **neurotrophic factors**, which regulate the development and maintenance of neural tissue.

Antibiotics:

Antibiotics are microbial poisons made paradoxically by microbes. Antibiotics arise as a result of resource competition. To minimize competition some microbes produce antibiotics to restrict the growth of other microbes. Microbes that makes antibiotic are not susceptible to them – they have a Kevlar body suit of sorts. Let's look at two examples of antibiotics that you are probably familiar with that are produced by fungi and bacteria.

Penicillin:

- Penicillin is an antibiotic made by a fungus. Remember Alexander Fleming's famous experiment in 1929.
- Fleming had a plate of *Staphylococcus aureus* that was contaminated by some fungus.
- This fungus was later identified as *Penicillium notatum*. He observed that around the fungal colony there was a clear zone, (i.e. a zone free of bacterial growth), suggesting that the fungus produced a substance that prevented *S. aureus* from growing



- The substance turned out to be an antibiotic that works by inhibiting the bacterial cell wall enzyme, D-alanine carboxypeptidase.
- This enzyme catalyzes the formation of bonds between peptidoglycan polymers in the bacterial cell wall. If this enzyme cannot function, the cell wall is weakened, resulting in bacterial death.
- The substance was later named penicillin and heralded the birth of the golden age of antibiotics.
- The name penicillin comes from the latin penicillum for "painter's brush", apparently because the edges of the fungal growth looked like a paintbrush!

Tetracycline, Streptomycin, Daptomycin:

- The discovery that a fungus could naturally produce a bug-killing substance led to the idea that other microbes might do the same.
- *Actinomycetes* (also known as *Actinobacteria*) is a model organism of antibiotic production. Living predominantly in the soil, *Actinomycetes* are exposed to many different environmental challenges, such as competing with other bacteria for resources.
- Therefore, this little bacterium came up with a whole bunch of weapons such as tetracyclin, streptomycin and daptomycin. Think of it like a sort of weird hunger games where the victor not only removes all the competitors, but is able to multiply in doing so.
- While the use of micro-organisms as synthetic factories is growing, there are no bacteria or fungi currently used to produce antibiotics.
- Part of the issue is that the pathways to antibiotic production are highly complex and difficult to engineer in a way that will give a high product yield that can also be tolerated by the microbe producing it.
- Even with *E. coli*, one of the most well studied bacteria, scientists have not been able to adapt it for antibiotic production.

Vaccines:

Vaccines Made of Entire Pathogen:

- While antibiotics are used to treat microbial infections, vaccines are used to prevent infections. Vaccines can work by using parts of the pathogen or a whole inactive pathogen to stimulate the immune system.
- When our body meets a live pathogen during an infection, the pathogen is recognized by a primed immune system and can swiftly mount a response to eradicate the infection.
- There are many different types of vaccine and the topic is a broad one (and I'm not even going to get to adjuvants!), with new types of vaccines being researched as I write.
- **For example,** the rise of Zika virus infections around the globe and what it might do to the fetus in a pregnant woman has put global health agencies and governments on high alert to push for vaccine development.

Live Attenuated Vaccine

- The measles, mumps and rubella (MMR) vaccine consists of all three live attenuated viruses mentioned above.
- **Attenuated means** - It means that the virus (or bacteria) grows well in laboratory settings and can generate an immune response but it does not make you as sick compared to the original agent.
- Two other examples of live attenuated vaccines are Poliovirus vaccine that prevents poliomyelitis and *Bacillus C*almette-*G*uérin (BCG) vaccine that prevents tuberculosis caused by *Mycobacterium tuberculosis*.

Inactivated Vaccines

- Consisting of dead bacteria or viruses are safer than live attenuated vaccines but are weaker at boosting the immune systems and several rounds of vaccination are needed.
- **Examples** of inactivated vaccines include the *Bordetella pertussis* vaccine that prevents whooping cough.

Vaccines Made of Microbial Parts

Toxoid Vaccines

Some diseases such as tetanus and diphtheria are mainly the result of toxins produced by the micro-organisms. Tetanus, an awful condition caused by *Clostridium tetani* that enters your body if you have an injury, is the direct result of the tetanus toxin released by the bacteria. Tetanus toxin, inactivated by formalin, is the vaccine given to protect against tetanus. The tetnus toxoid vaccine is produced by growing the bacteria in culture and harvesting the toxin.

Subunit Vaccines

Consist of other bits (usually proteins) of a microbe. Determining which part of the microbe will generate the best immune response can be tricky but some have managed it. An example of a subunit vaccine is the one called acellular Pertussis vaccine against whooping cough. To confuse matters, this in fact consists of the Pertussis toxin together with filamentous haemagglutinin, fimbrial antigens and pertactin which are other microbial immunogenic proteins – quite a mix!

DNA Vaccines

These are the newest types of vaccines and are not yet approved for use in the clinic. Here, DNA encoding parts of bacteria or viruses is given to the patient. The plasmid DNA will be taken up by the patient's own cells and displayed on the cell surface to generate an immune response. The idea is to stimulate your cells to manufacture and deliver the foreign antigen to alert the immune system! And it gets better. Instead of just using plasmid DNA itself, the DNA can also be packaged within a virus or a bacterium that serves as a carrier to deliver the DNA into cells! These are called vector vaccines where the bacterial or viral vectors are the "mailman". The advantage of a DNA vaccine is that it can be rapidly modified to keep up with a mutating virus like the human immunodeficiency virus (HIV).

❖ Two main ways of destroying disease-causing microbes:

- by using microbial poisons such as antibiotics that are produced by microbes themselves
- by using vaccines to prime the body's immune system to be ready for an infection. In the next installment.

History of Biotech can be divided into three phases:

1. Ancient Biotechnology
2. Classical Biotechnology
3. Modern Biotechnology

Ancient Biotechnology (Pre-1800)

Most of the biotech developments before the year 1800 can be termed as 'discoveries' or 'developments'. If we study all these developments, we can conclude that these inventions were based on common observations about nature.

- Humans have used biotechnology since the dawn of civilization.
- After domestication of food crops (corn, wheat) and wild animals, man moved on to other new observations like cheese and curd. Cheese can be considered as one of the first direct products (or by-product) of biotechnology because it was prepared by adding rennet (an enzyme found in the stomach of calves) to sour milk.
- Yeast is one of the oldest microbes that have been exploited by humans for their benefit. The oldest fermentation was used to make beer in Sumeria and Babylonia as early as 7,000BCE.
- By 4,000BCE, Egyptians used yeasts to bake leavened bread.
- Another ancient product of fermentation was wine, made in Assyria as early as 3,500BCE.
- The Chinese developed fermentation techniques for brewing and cheese making.
- 500 BCE: In China, the first antibiotic, moldy soybean curds, is put to use to treat boils.
- Hippocrates treated patients with vinegar in 400 BCE.
- In 100BCE, Rome had over 250 bakeries which were making leavened bread.
- A.D. 100: The first insecticide is produced in China from powdered chrysanthemums.
- The use of molds to saccharify rice in the koji process dates back to at least A.D. 700.
- 13th century: The Aztecs used Spirulina algae to make cakes.
- One of the oldest examples of crossbreeding for the benefit of humans is mule. Mule is an offspring of a male donkey and a female horse. People started using mules for transportation, carrying loads, and farming, when there were no tractors or trucks.
- By the 14th century AD, the distillation of alcoholic spirits was common in many parts of the world.
- Vinegar manufacture began in France at the end of the 14th century.
- 1663: Cells are first described by Hooke.
- 1673-1723: In the seventeenth century, Antonie van Leeuwenhoek discovered microorganisms by examining scrapings from his teeth under a microscope.
- 1675: Leeuwenhoek discovers protozoa and bacteria.
- 1761: English surgeon Edward Jenner pioneers vaccination, inoculating a child with a viral smallpox vaccine.

Classical Biotechnology (1800-1945)

The Hungarian Károly Ereky coined the word "biotechnology" in Hungary during 1919 to describe a technology based on converting raw materials into a more useful product. In a book entitled Biotechnologie, Ereky further developed a theme that would be reiterated through the 20th century: biotechnology could provide solutions to societal crises, such as food and energy shortages.

- 1773-1858: Robert Brown discovered the nucleus in cells.
- 1802: The word "biology" first appears.

- 1822-1895: Vaccination against small pox and rabies developed by Edward Jenner and Louis Pasteur.
- In 1850, Casimir Davaine detected rod-shaped objects in the blood of anthrax-infected sheep and was able to produce the disease in healthy sheep by inoculation of such blood.
- 1855: The Escherichia coli bacterium is discovered. It later becomes a major research, development, and production tool for biotechnology.
- In 1868, Fredrich Miescher reported nuclein, a compound that consisted of nucleic acid that he extracted from white blood cells.
- 1870: Breeders crossbreed cotton, developing hundreds of varieties with superior qualities.
- 1870: The first experimental corn hybrid is produced in a laboratory.
- By 1875, Pasteur of France and John Tyndall of Britain finally demolished the concept of spontaneous generation and proved that existing microbial life came from preexisting life.
- 1876: Koch's work led to the acceptance of the idea that specific diseases were caused by specific organisms, each of which had a specific form and function.
- In 1881, Robert Koch, a German physician, described bacterial colonies growing on potato slices (First ever solid medium).
- In 1888, Heinrich Wilhelm Gottfried Von Waldeyer-Hartz, a German scientist, coined the term 'Chromosome.'
- In 1909, the term 'Gene' had already been coined by Wilhelm Johannsen (1857-1927), who described 'gene' as carrier of heredity. Johannsen also coined the terms 'genotype' and 'phenotype.'
- 1909: Genes are linked with hereditary disorders.
- 1911: American pathologist Peyton Rous discovers the first cancer-causing virus.
- 1915: Phages, or bacterial viruses, are discovered.
- 1919: The word "biotechnology" is first used by a Hungarian agricultural engineer.
- Pfizer, which had made fortunes using fermenting processes to produce citric acid in the 1920s, turned its attention to penicillin. The massive production of penicillin was a major factor in the Allied victory in WWII.
- 1924: start of Eugenic Movement in the US.
- The principle of genetics in inheritance was redefined by T.H. Morgan, who showed inheritance and the role of chromosomes in inheritance by using fruit flies. This landmark work was named, 'The theory of the Gene in 1926."
- Alexander Fleming discovered 'penicillin' the antibacterial toxin from the mold Penicillium notatum, which could be used against many infectious diseases. Fleming wrote, "When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacteria killer."
- 1933: Hybrid corn is commercialized.
- In 1940, a team of researchers at Oxford University found a way to purify penicillin and keep it stable.
- 1941: The term "genetic engineering" is first used by a Danish microbiologist.
- 1942: The electron microscope is used to identify and characterize a bacteriophage- a virus that infects bacteria.
- 1942: Penicillin is mass-produced in microbes for the first time.

Modern Biotechnology (1945-present)

The Second World War became a major impediment in scientific discoveries. After the end of the second world war some, very crucial discoveries were reported, which paved the path for modern biotechnology.

The origins of biotechnology culminate with the birth of genetic engineering. There were two key events that have come to be seen as scientific breakthroughs beginning the era that would unite genetics with biotechnology: One was the 1953 discovery of the structure of DNA, by Watson and Crick, and the other was the 1973 discovery by Cohen and Boyer of a recombinant DNA technique by which a section of DNA was cut from the plasmid of an E. coli bacterium and transferred into the DNA of another. Popularly referred to as "genetic engineering," it came to be defined as the basis of new biotechnology.

In Britain, Chaim Weizemann (1874–1952) developed bacterial fermentation processes for producing organic chemicals such as acetone and cordite propellants. During WWII, he worked on synthetic rubber and high-octane gas.

- 1950s: The first synthetic antibiotic is created.
- 1951: Artificial insemination of livestock is accomplished using frozen semen.
- In 1953, JD Watson and FHC Crick for the first time cleared the mysteries around the DNA as a genetic material, by giving a structural model of DNA, popularly known as, 'Double Helix Model of DNA.'
- 1954: Dr. Joseph Murray performs the first kidney transplant between identical twins.
- 1955: An enzyme, DNA polymerase, involved in the synthesis of a nucleic acid, is isolated for the first time.
- 1955: Dr. Jonas Salk develops the first polio vaccine. The development marks the first use of mammalian cells (monkey kidney cells) and the first application of cell culture technology to generate a vaccine.
- 1957: Scientists prove that sickle-cell anemia occurs due to a change in a single amino acid in hemoglobin cells
- 1958: Dr. Arthur Kornberg of Washington University in St. Louis makes DNA in a test tube for the first time.
- Edward Tatum (1909–1975) and Joshua Lederberg (1925–2008) shared the 1958 Nobel Prize for showing that genes regulate the metabolism by producing specific enzymes.
- 1960: French scientists discover messenger RNA (mRNA).
- 1961: Scientists understand genetic code for the first time.
- 1962: Dr. Osamu Shimomura discovers the green fluorescent protein in the jellyfish Aequorea victoria. He later develops it into a tool for observing previously invisible cellular processes.
- 1963: Dr. Samuel Katz and Dr. John F. Enders develop the first vaccine for measles.
- 1964: The existence of reverse transcriptase is predicted.
- At a conference in 1964, Tatum laid out his vision of "new" biotechnology: "Biological engineering seems to fall naturally into three primary categories of means to modify organisms. These are: 1. The recombination of existing genes, or eugenics. 2. The production of new genes by a process of directed mutation, or genetic engineering. 3. Modification or control of gene expression, or to adopt Lederberg's suggested terminology, euphenic engineering."
- 1967: The first automatic protein sequencer is perfected.
- 1967: Dr. Maurice Hilleman develops the first American vaccine for mumps.
- 1969: An enzyme is synthesized in vitro for the first time.
- 1969: The first vaccine for rubella is developed.
- 1970: Restriction enzymes are discovered.
- 1971: The measles/mumps/rubella combo-vaccine was formed.
- 1972: DNA ligase, which links DNA fragments together, is used for the first time.
- 1973: Cohen and Boyer perform the first successful recombinant DNA experiment, using bacterial genes.

- In 1974, Stanley Cohen and Herbert Boyer developed a technique for splicing together strands of DNA from more than one organism. The product of this transformation is called recombinant DNA (rDNA).
- Kohler and Milestein in 1975 came up with the concept of cytoplasmic hybridization and produced the first ever monoclonal antibodies, which has revolutionized diagnostics.
- Techniques for producing monoclonal antibodies were developed in 1975.
- 1975: Colony hybridization and Southern blotting are developed for detecting specific DNA sequences.
- 1976: Molecular hybridization is used for the prenatal diagnosis of alpha thalassemia.
- 1978: Recombinant human insulin is produced for the first time.
- 1978: with the development of synthetic human insulin the biotechnology industry grew rapidly.
- 1979: Human growth hormone is synthesized for the first time.
- In the 1970s-80s, the path of biotechnology became intertwined with that of genetics.
- By the 1980s, biotechnology grew into a promising real industry.
- 1980: Smallpox is globally eradicated following 20-year mass vaccination effort.
- In 1980, The U.S. Supreme Court (SCOTUS), in Diamond v. Chakrabarty, approved the principle of patenting genetically engineered life forms.
- 1981: Scientists at Ohio University produce the first transgenic animals by transferring genes from other animals into mice.
- 1981: The first gene-synthesizing machines are developed.
- 1981: The first genetically engineered plant is reported.
- 1982: The first recombinant DNA vaccine for livestock is developed.
- 1982: The first biotech drug, human insulin produced in genetically modified bacteria, is approved by FDA. Genentech and Eli Lilly developed the product. This is followed by many new drugs based on biotechnologies.
- 1983: The discovery of HIV/AIDS as a deadly disease has helped tremendously to improve various tools employed by life-scientist for discoveries and applications in various aspects of day-to-day life.
- In 1983, Kary Mullis developed polymerase chain reaction (PCR), which allows a piece of DNA to be replicated over and over again. PCR, which uses heat and enzymes to make unlimited copies of genes and gene fragments, later becomes a major tool in biotech research and product development worldwide.
- 1983: The first artificial chromosome is synthesized.
- In 1983, the first genetic markers for specific inherited diseases were found.
- 1983: The first genetic transformation of plant cells by TI plasmids is performed.
- In 1984, the DNA fingerprinting technique was developed.
- 1985: Genetic markers are found for kidney disease and cystic fibrosis.
- 1986: The first recombinant vaccine for humans, a vaccine for hepatitis B, is approved.
- 1986: Interferon becomes the first anticancer drug produced through biotech.
- 1986: University of California, Berkeley, chemist Dr. Peter Schultz describes how to combine antibodies and enzymes (abzymes) to create therapeutics.
- 1988: The first pest-resistant corn, Bt corn, is produced.
- 1988: Congress funds the Human Genome Project, a massive effort to map and sequence the human genetic code as well as the genomes of other species.
- In 1988, chymosin (known as Rennin) was the first enzyme produced from a genetically modified source-yeast-to be approved for use in food.
- In 1988, only five proteins from genetically engineered cells had been approved as drugs by the United States Food and Drug Administration (FDA), but this number would skyrocket to over 125 by the end of the 1990s.
- In 1989, microorganisms were used to clean up the Exxon Valdez oil spill.

- 1990: The first successful gene therapy is performed on a 4-year-old girl suffering from an immune disorder.
- In 1993, The U.S. Food and Drug Administration (FDA) declared that genetically modified (GM) foods are "not inherently dangerous" and do not require special regulation.
- 1993: Chiron's Betaseron is approved as the first treatment for multiple sclerosis in 20 years.
- 1994: The first breast cancer gene is discovered.
- 1995: Gene therapy, immune-system modulation and recombinantly produced antibodies enter the clinic in the war against cancer.
- 1995: The first baboon-to-human bone marrow transplant is performed on an AIDS patient.
- 1995: The first vaccine for Hepatitis A is developed.
- 1996: A gene associated with Parkinson's disease is discovered.
- 1996: The first genetically engineered crop is commercialized.
- 1997: Ian Wilmut, an Irish scientist, was successful in cloning an adult animal, using sheep as model and naming the cloned sheep 'Dolly.'
- 1997: The first human artificial chromosome is created.
- 1998: A rough draft of the human genome map is produced, showing the locations of more than 30,000 genes.
- 1998: Human skin is produced for the first time in the lab.
- 1999: A diagnostic test allows quick identification of Bovine Spongicorm Encephalopathy (BSE, also known as "mad cow" disease) and Creutzfeldt-Jakob Disease (CJD).
- 1999: The complete genetic code of the human chromosome is deciphered.
- 2000: Kenya field-tests its first biotech crop, virus-resistant sweet potato.
- Craig Venter, in 2000, was able to sequence the human genome.
- 2001: The sequence of the human genome is published in Science and Nature, making it possible for researchers all over the world to begin developing treatments.
- 2001: FDA approves Gleevec® (imatinib), a gene-targeted drug for patients with chronic myeloid leukemia. Gleevec is the first gene-targeted drug to receive FDA approval.
- 2002: EPA approves the first transgenic rootworm-resistant corn.
- 2002: The banteng, an endangered species, is cloned for the first time.
- 2003: China grants the world's first regulatory approval of a gene therapy product, Gendicine (Shenzhen SiBiono GenTech), which delivers the p53 gene as a therapy for squamous cell head and neck cancer.
- In 2003, TK-1 (GloFish) went on sale in Taiwan, as the first genetically modified pet.
- 2003: The Human Genome Project completes sequencing of the human genome.
- 2004: UN Food and Agriculture Organization endorses biotech crops, stating biotechnology is a complementary tool to traditional farming methods that can help poor farmers and consumers in developing nations.
- 2004: FDA approves the first antiangiogenic drug for cancer, Avastin®.
- 2005: The Energy Policy Act is passed and signed into law, authorizing numerous incentives for bioethanol development.
- 2006: FDA approves the recombinant vaccine Gardasil®, the first vaccine developed against human papillomavirus (HPV), an infection implicated in cervical and throat cancers, and the first preventative cancer vaccine.
- 2006: USDA grantsDow AgroSciences the first regulatory approval for a plant-made vaccine.
- 2006: The National Institutes of Health begins a 10-year, 10,000-patient study using a genetic test that predicts breast-cancer recurrence and guides treatment.
- In 2006, the artist Stelarc had an ear grown in a vat and grafted onto his arm.
- 2007: FDA approves the H5N1 vaccine, the first vaccine approved for avian flu.
- 2007: Scientists discover how to use human skin cells to create embryonic stem cells.
- 2008: Chemists in Japan create the first DNA molecule made almost entirely of artificial parts.

- 2009: Global biotech crop acreage reaches 330 million acres.
- In 2009, Sasaki and Okana produced transgenic marmosets that glow green in ultraviolet light (and pass the trait to their offspring).
- 2009: FDA approves the first genetically engineered animal for production of a recombinant form of human antithrombin.
- In 2010, Craig Venter was successful in demonstrating that a synthetic genome could replicate autonomously.
- 2010: Dr. J. Craig Venter announces completion of "synthetic life" by transplanting synthetic genome capable of self-replication into a recipient bacterial cell.
- 2010: Harvard researchers report building "lung on a chip" – technology.
- In 2010, scientists created malaria-resistant mosquitoes.
- 2011: Trachea derived from stem cells transplanted into human recipient.
- 2011: Advances in 3-D printing technology lead to "skin-printing."
- 2012: For the last three billion years, life on Earth has relied on two information-storing molecules, DNA and RNA. Now there's a third: XNA, a polymer synthesized by molecular biologists Vitor Pinheiro and Philipp Holliger of the Medical Research Council in the United Kingdom. Just like DNA, XNA is capable of storing genetic information and then evolving through natural selection. Unlike DNA, it can be carefully manipulated.
- 2012: Researchers at the University of Washington in Seattle announced the successful sequencing of a complete fetal genome using nothing more than snippets of DNA floating in its mother's blood.
- 2013: Two research teams announced a fast and precise new method for editing snippets of the genetic code. The so-called CRISPR system takes advantage of a defense strategy used by bacteria.
- 2013: Researchers in Japan developed functional human liver tissue from reprogrammed skin cells.
- 2013: Researchers published the results of the first successful human-to-human brain interface.
- 2013: Doctors announced that a baby born with HIV had been cured of the disease.
- 2014: Researchers showed that blood from a young mouse can rejuvenate an old mouse's muscles and brain.
- 2014: Researchers figured out how to turn human stem cells into functional pancreatic β cells—the same cells that are destroyed by the body's own immune system in type 1 diabetes patients.
- 2014: All life on Earth as we know it encodes genetic information using four DNA letters: A, T, G, and C. Not anymore! In 2014, researchers created new DNA bases in the lab, expanding life's genetic code and opening the door to creating new kinds of microbes.
- 2014: For the first time ever, a woman gave birth to a baby after receiving a womb transplant.
- 2014: An international team of scientists reconstructed a synthetic and fully functional yeast chromosome. A breakthrough seven years in the making, the remarkable advance could eventually lead to custom-built organisms (human organisms included).
- 2014 & Ebola: Until this year, ebola was merely an interesting footnote for anyone studying tropical diseases. Now it's a global health disaster. But the epidemic started at a single point with one human-animal interaction — an interaction which has now been pinpointed using genetic research. A total of 50 authors contributed to the paper announcing the discovery, including five who died of the disease before it could be published.
- 2014: Doctors discovered a vaccine that totally blocks infection altogether in the monkey equivalent of the disease — a breakthrough that is now being studied to see if it works in humans.
- 2015: Scientists from Singapore's Institute of Bioengineering and Nanotechnology designed short strings of peptides that self-assemble into a fibrous gel when water is added for use as a healing nanogel.

- 2015 & CRISPR: scientists hit a number of breakthroughs using the gene-editing technology CRISPR. Researchers in China reported modifying the DNA of a nonviable human embryo, a controversial move. Researchers at Harvard University inserted genes from a long-extinct woolly mammoth into the living cells — in a petri dish — of a modern elephant. Elsewhere, scientists reported using CRISPR to potentially modify pig organs for human transplant and modify mosquitoes to eradicate malaria.
- 2015: Researchers in Sweden developed a blood test that can detect cancer at an early stage from a single drop of blood.
- 2015: Scientists discovered a new antibiotic, the first in nearly 30 years, that may pave the way for a new generation of antibiotics and fight growing drug-resistance. The antibiotic, teixobactin, can treat many common bacterial infections, such as tuberculosis, septicaemia, and C. diff.
- 2015: A team of geneticists finished building the most comprehensive map of the human epigenome, a culmination of almost a decade of research. The team was able to map more than 100 types of human cells, which will help researchers better understand the complex links between DNA and diseases.
- 2015: Stanford University scientists revealed a method that may be able to force malicious leukemia cells to change into harmless immune cells, called macrophages.
- 2015: Using cells from human donors, doctors, for the first time, built a set of vocal cords from scratch. The cells were urged to form a tissue that mimics vocal fold mucosa – vibrating flaps in the larynx that create the sounds of the human voice.
- 2016: A little-known virus first identified in Uganda in 1947—Zika—exploded onto the international stage when the mosquito-borne illness began spreading rapidly throughout Latin America. Researchers successfully isolated a human antibody that "markedly reduces" infection from the Zika virus.
- 2016: CRISPR, the revolutionary gene-editing tool that promises to cure illnesses and solve environmental calamities, took a major step forward this year when a team of Chinese scientists used it to treat a human patient for the very first time.
- 2016: Researchers found that an ancient molecule, GK-PID, is the reason single-celled organisms started to evolve into multicellular organisms approximately 800 million years ago.
- 2016: Stem Cells Injected Into Stroke Patients Re-Enable Patient To Walk.
- 2016: Cloning does not cause long-term health issues, study finds
- 2016: For the first time, bioengineers created a completely 3D-printed 'heart on a chip.'
- 2017: Researchers at the National Institute of Health discovered a new molecular mechanism that might be the cause of severe premenstrual syndrome known as PMDD.
- 2017: Scientists at the Salk Institute in La Jolla, CA, said they're one step closer to being able to grow human organs inside pigs. In their latest research they were able to grow human cells inside pig embryos, a small but promising step toward organ growth.
- 2017: First step taken toward epigenetically modified cotton.
- 2017: Research reveals different aspects of DNA demethylation involved in tomato ripening process.
- 2017: Sequencing of green alga genome provides blueprint to advance clean energy, bioproducts.
- 2017: Fine-tuning 'dosage' of mutant genes unleashes long-trapped yield potential in tomato plants.
- 2017: Scientists engineer disease-resistant rice without sacrificing yield.
- 2017: Blood stem cells grown in lab for the first time.
- 2017: Researchers at Sahlgrenska Academy – part of the University of Gothenburg, Sweden – generated cartilage tissue by printing stem cells using a 3D-bioprinter.
- 2017: Two-way communication in brain-machine interface achieved for the first time.

Today, biotechnology is being used in countless areas including agriculture, bioremediation and forensics, where DNA fingerprinting is a common practice. Industry and medicine alike use the techniques of PCR, immunoassays and recombinant DNA.

Genetic manipulation has been the primary reason that biology is now seen as the science of the future and biotechnology as one of the leading industries.

Microbial biotechnology vs molecular biotechnology:

- **Biology** including **microbiology** is concerned with the study of whole organisms. Physiology is concerned with the study of the biological function of those organisms at a macro level.
- **Molecular biology** is concerned with the structure and function of the large **molecules** that make biochemistry possible.

Interferons:

Interferons are proteins synthesized in various host cells, encoded by the host genes, in response to double-stranded RNA (dsRNA). **Interferon** circulates and has the role to protect uninfected cells by inhibiting viral protein synthesis.

- Interferons (IFNs) were the first family of cytokines to be discovered. In 1957 researchers observed that if susceptible animal cells were exposed to a colonizing virus, these cells immediately become resistant to attack by other viruses.
- This resistance was induced by a substance secreted by virally-infected cells, which was named 'interferon' (IFN).
- Humans produce at least three distinct classes, IFN-a, IFN-b and IFN-g.

Biological effects,

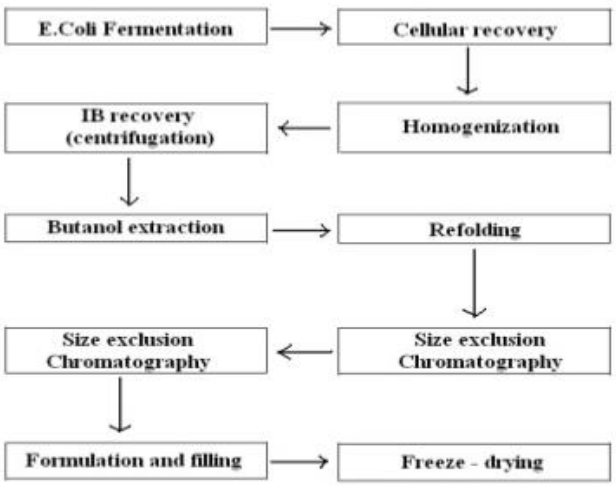
- Induction of cellular resistance to viral attack.
- Regulation of most aspects of immune function.
- Regulation of growth and differentiation of many cell types.
- Sustenance of early phases of pregnancy in some animal species.

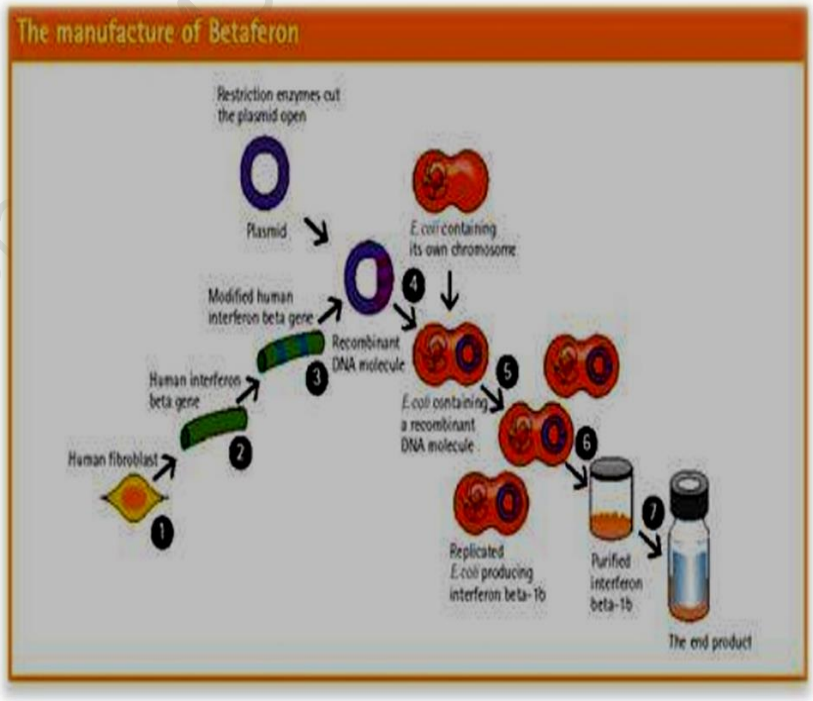
PRODUCTION OF INTERFERONS BY GENETIC ENGINEERING:

- A DNA sequence coding for the product was synthesized and inserted into *E. coli*. The recombinant product accumulates intracellularly as inclusion bodies

- Large-scale manufacture entails an initial fermentation step. After harvest, the *E. coli* cells are homogenized and the inclusion bodies recovered via centrifugation. After solubilization and refolding, the interferon is purified to homogeneity by a combination of chromatographic steps.
- The final product is formulated in the presence of a phosphate buffer and sodium chloride. It is resented as a 30 mg/ml solution in glass vials and displays a shelf- life of 24 months when stored at 2–8°C`.

Recovery OF RECOMBINANT HUMAN IFN-B





Interferon toxicity:

- ❖ Like most drugs, administration of IFNs can elicit a number of unwanted side effects.
- ❖ **Minor side effects** - Range of flu-like symptoms, e.g. fever, headache, chills.
- ❖ Serious potential side effects

  - Anorexia
  - Strong fatigue
  - Insomnia
  - Cardiovascular complication
  - Autoimmune reactions
  - Hepatic decompression

- ➢ Interferons represent an important family of biopharmaceutical products.
- ➢ They have a proven track record in the treatment of selected medical conditions, and their range of clinical applications continues to grow.
- ➢ It is also likely that many may be used to greater efficacy in the future by their application in combination with additional cytokines

PRODUCTION OF recombinant HUMAN GROWTH HORMONE (Hgh):

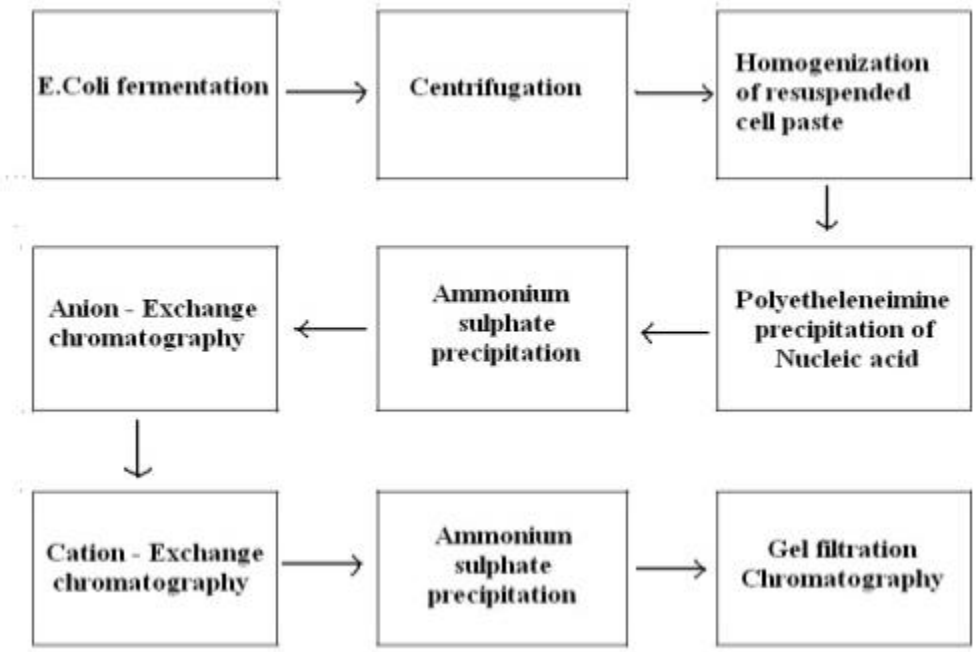
- ❖ Human growth hormone is a polypeptide hormone synthesized in the anterior pituitary. It promotes normal body growth and lactation and influences various aspects of cellular metabolism.
- ❖ Dwarfism caused by insufficient production of HGH by the pituitary gland.
- ❖ HGH can treat dwarfism – to help under sized children reach their normal height and size.
- ❖ Recombination process –

1. DNA coding for growth hormone is inserted into the plasmid.

2. Introduction of recombined plasmid into *E.coli* host cell

3. Engineered *E.coli* cell is allowed to multiply in the fermentor.

RECOVERY OF HUMAN GROWTH HORMONE:



- Cytokines constitute the single most important group of biopharmaceutical substances. As coordinators of the immune and inflammatory response, manipulation of cytokine activity can have a major influence on the body's response to a variety of medical conditions.
- Administration of certain cytokines can enhance the immune response against aϖ wide range of infectious agents and cancer cells.

DNase I Production:

A **deoxyribonuclease** (**DNase**, for short) is an **enzyme** that catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone, thus degrading DNA.

Modes of action

Some DNases cut, or "cleave", only residues at the ends of DNA molecules (exodeoxyribonucleases, a type of exonuclease). Others cleave anywhere along the chain (endodeoxy ribonucleases, a subset of endonucleases).

Some are fairly indiscriminate about the DNA sequence at which they cut, while others, including restriction enzymes, are very sequence-specific.

Some cleave only double-stranded DNA; others are specific for single-stranded molecules; and still others are active toward both.

DNase enzymes can be inhaled using a nebuliser by cystic fibrosis sufferers. DNase enzymes help because white blood cells accumulate in the mucus, and, when they break down, they release DNA, which adds to the 'stickiness' of the mucus. DNase enzymes break down the DNA, and the mucus is much easier to clear from the lungs.

Therapeutic DNase I:

- Cystic fibrosis (CF) is one of the most commonly occurring genetic diseases (1 in 2500 in northern Europe)
- Cystic fibrosis (CF), also known as mucoviscidosis, affects most critically the lungs, and also the pancreas, liver and intestine
- It is characterized by abnormal transport of chloride and sodium across an epithelium, leading to thick, viscous secretions i.e., Underlying cause is identified to the malfunction of ion transport
- Major clinical symptom is the production of viscous mucus in the respiratory track
- Patients are susceptible to frequent lung infections and some patients develop antibiotic resistance bacteria and hence, bacteria accumulate leading to a viscous mucous secretion, clogging the bronchia and bronchioles.
- Thick mucous = alginate that is secreted by the bacteria + DNA released when bacterial cells and degenerating leucocytes that accumulate in response to infection are lysed The role of DNase I can hydrolyse long polymeric DNA chains into shorter oligonucleotides and the purified enzyme can be delivered in an aerosol mist to the lungs of CF patients to prevent respiratory distress.
- The enzyme could decrease the mucus viscosity in the lungs and allow patients for easy breathing, thus reducing the severity and pain of the patient.
- This enzyme was approved for use by the US FDA in 1994

Types

The two main types of DNase found in metazoans are known as **deoxyribonuclease I** and **deoxyribonuclease II.**

Other types of DNase include micrococcal nuclease.

DNases, or deoxyribonucleases, are enzymes that specifically cleave and degrade DNA. In molecular biology, DNase (namely DNase I) is used to degrade DNA in applications such as RNA isolation, reverse transcription preparation, DNA-protein interactions, cell culture, and DNA fragmentation. Clinical uses of DNase include breaking down of mucus to clear up respiratory tracts. Other types of DNases include DNase II (or acid DNase) and shrimp DNase.

Alginate Lyase:

Alginate lyases are group of **enzymes** which catalyze depolymerization of **alginate** into oligosaccharides. **Alginate lyase** have been widely used in many applications such as in production of bioactive oligosaccharides, control of polysaccharide rheological properties, and polysaccharide structure analysis.

- Alginate lyase Alginate, a polysaccharide polymer of β-D mannuronate and α-L glucoronate, form an elastic gel, which is related to its viscosity and molecular weight.
- The excretion of alginate by mucoid strains of pseudomonas aeruginosa may infect the lungs of cystic fibrosis patient contributing significantly to the viscosity of the mucous.
- Hence treatment of cystic fibrosis depends on the DNase l therapy and depolymeriztion of the alginate which would help to clear blocked airways.
- Since the enzyme alginate lyase can liquefy viscous bacterial alginate which in addition to DNase l is good therapeutic agent of cystic fibrosis

Monoclonal antibodies production:

1. **Immunization:**

Immunization is necessary to present an antigen in a suitable form to induce the most vigorous humoural immune response to an animal. This essential step will result in the production of cells secreting antibody against your chosen antigen

2. **Hybridoma Production:**

A hybridoma is a cell line arising from one hybrid cell that is capable of secreting a monoclonal antibody specific to one epitope of your antigen permanently in culture. The hybrid cell is produced through the fusion of specific antibody producing B-cell from an immunized animal.

Production of a mouse hybrid cell:

- During the fusion process, B cells are isolated from the mouse spleen, mixed with the mouse myeloma cell line and fusion is induced with polyethylene glycol.
- The resulting hybridomas are then cultured in tissue culture medium containing Hypoxathine, Aminopterin, Thymidine (HAT), a step which kills any unfused myeloma cells that might outgrow the other weaker hybridoma cells.
- Unfused B cells have limited powers of division and will die off naturally in culture.
- Ten days after the fusion process, culture supernatant is collected and tested for the presence of the desired antibody.

3. Cloning:

- The objective of cloning the cells producing the antibody of interest is to ensure that the desired hybridoma cell line produced is obtained from a single fused cell.
- After a fusion, many different hybrid cells will be present in a single well resulting in the growth of multiple colonies in each well.
- Your specific antibody-secreting colony is therefore likely to be mixed with other cells that are either non-secreting or which are producing an antibody of undesired specificity.

4. Freezing and Thawing of cell line:

Hybridoma and myeloma cell lines are stored by freezing the cells at a controlled rate (approximately 1ºC per minute) in an appropriate cryoprotectant. This procedure allows the cell line to be preserved indefinitely.

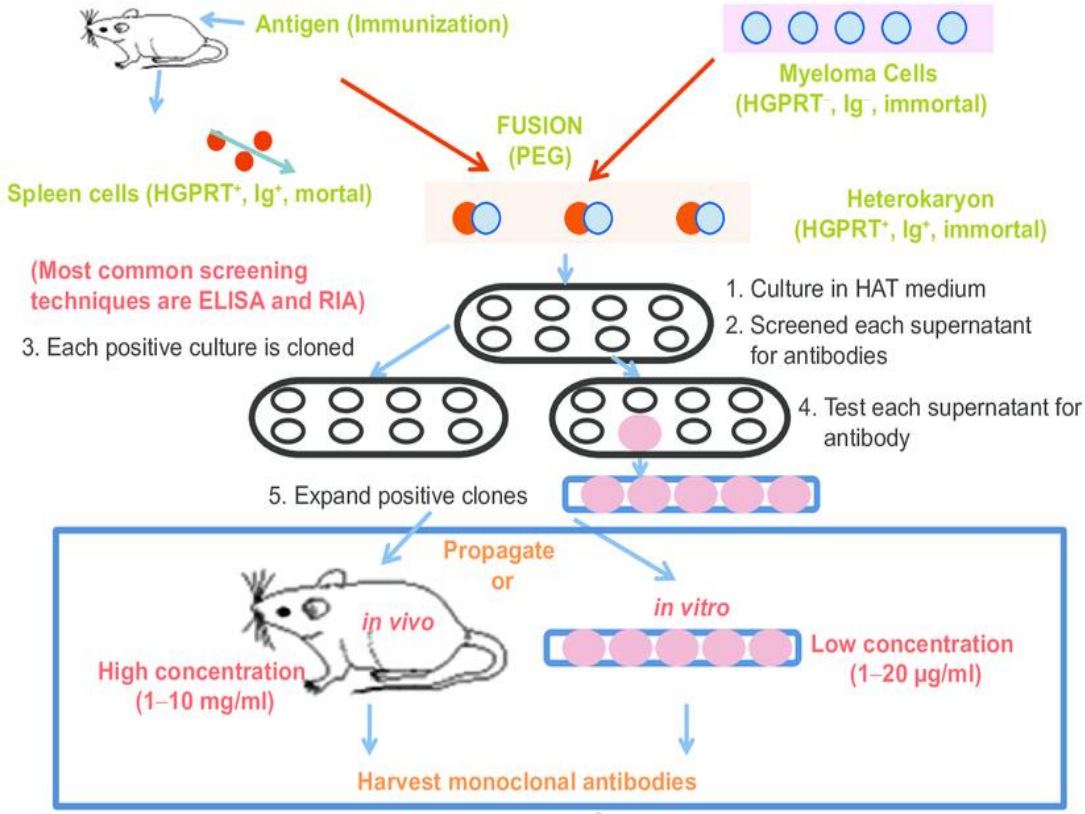
Freezing Method

- Only cells that are healthy and rapidly dividing should be frozen.
- One or two days before freezing, split the cells 1:10 into fresh medium and maintain in culture.
- On the day of freezing, count the cells.
- You need to have between 2 to $5x10^6$ cells in each freezing vial.
- Transfer the appropriate volume of cells to a sterile centrifuge tube.
- Spin at 300g for 5 minutes.
- Carefully remove as much supernatant as possible without disturbing the pellet.

- Gently resuspend the cell pellet in 1.5ml of freezing medium containing 10% Dimethylsulphoxide (DMSO, from Sigma D2650) in 90 % FCS previously heat-inactivated).
- Transfer 1.5ml of the resulting cell suspension into a freezing vial (Thermo 375418).
- Seal the vial (finger tight) to prevent liquid nitrogen from entering the vial.
- Place the sealed vials in a special freezing container (Thermo Scientific 5100-0001) which allows the cells to cool down slowly.
- Close the freezing container and place at -80ºC for at least 4 hours.
- Transfer the vials of frozen cells to liquid nitrogen.
- Extended storage of cells at -80ºC is not recommended.
- Prolonged exposure to DMSO is toxic to cells. For example, handling more than 10-20 vials at any one time will lead to extended exposure of the cells to DMSO prior to freezing.

Thawing Method

- Remove the frozen vial from the liquid N2 storage.
- Loosen the cap of the vial slightly to release the pressure inside.
- Thaw the cells in a 37ºC water bath.
- Keep the lid of the freezing vial above the surface of the water to lessen the chances of contamination.
- When the cells are almost thawed (only a small piece of ice) move the vial to the tissue culture hood.
- Wipe the outside of the vial with 70% ethanol and remove the top.
- Carefully remove the cell suspension using a sterile Pasteur pipette.
- Transfer the contents to a centrifuge tube containing 10 ml of appropriate culture médium (See Appendix I – remember myeloma medium should not contain HAT).
- Spin the cell suspension gently at 300g for 5 min.
- Carefully remove the medium without disturbing the pellet.
- Gently resuspend the cells in 10ml of fresh appropriate culture medium and place in a small T25 flask (Corning 3056).
- Take 1ml from this flask and add to 9mls of complete culture medium in another small flask. This step ensures that at least one concentration of cells is suitable for continued culture.
- Place the flasks in a 5% CO_2 incubator with their tops loosened enabling gaseous exchange to occur.



Applications of Monoclonal Antibodies

1. **Isolation and purification:** Monoclonal antibodies can be used to purify individual molecule from a mixture even when they are present in low concentration, e.g. interferon and coagulation factor VIII.
2. **Identification of cells and clones:** For example T_H, and T_C cells are identified by using anti-CD4 and anti-CD8 mAb.
3. **Diagnostic reagents:** The antigen detection kits employ various mAb tagged with detection molecules, such as fluorescent dye or enzyme to detect the specific antigens in the clinical specimen such as:

- **Detection of infections**, such as hepatitis B, serogrouping of streptococci, etc.
- **Pregnancy detection test**-by using monoclonal antibody against human chorionic gonadotropin.
- **Blood grouping** can be done by using anti-A and anti-B monoclonal antibodies.
- **Tumor detection** and imaging: By using mAb specific for tumor antigens secreted by tumor cells (e.g. prostate-specific antigen).
- **Tissue typing** for transplantation can be done by using anti-HLA monoclonal antibodies.

4. **Monitoring** proteins and drug levels in serum.

5. **Passive immunity:** For post-exposure prophylaxis against various infections, mAb targeting specific antigens of the infecting organism can be administered. Examples include-immunoglobulins against hepatitis B, rabies, and tetanus.
6. **Therapeutic use:** Monoclonal antibodies are used in the treatment of various inflammatory and allergic diseases and cancers. Monoclonal antibodies (mAbs) are useful to treat some of the cancer types.
7. **Naked mAbs** (antibodies without attached drug or radioactive material) are the most common type of mAbs used to treat cancer. So far, the US FDA has approved more than a dozen mAbs e.g. alemtuzumab, trastuzumab to treat certain cancers. Similarly, basiliximab is used to treat transplant rejection while belimumab treats systemic lupus erythematosus.
8. **Used as immunotoxin: mAb conjugated with bacterial/ chemical toxins** (e.g. diphtheria toxin) can be used to kill the target cells such as cancer cells. Here, mAb against surface receptors helps in binding to the target cells and the toxin helps in target cell killing.
9. **Used as enzymes:** Abzyme is a monoclonal antibody with catalytic activity.

Monoclonal antibodies - HIV therapeutic agents:

- Successful highly active antiretroviral therapy (HAART) has allowed the long-term effects of **HIV infection** and chronic medication toxicity to **become the primary cause of death and disability.**
- However, **HIV-positive patients** are at greater risk for **cardiovascular disease** and **cancers** due to excessive inflammation, and HAART does not eliminate HIV-related inflammation even if the patient adheres to treatment perfectly. Viral eradication, impossible at present, would solve this challenge.
- A **monoclonal antibody** strain active **against** an extensive portion of the **HIV** viral diversity could prevent (as a vaccine) or control HIV replication.
- As a result, it could reduce the **pill burden** associated with current HAART standard of care.

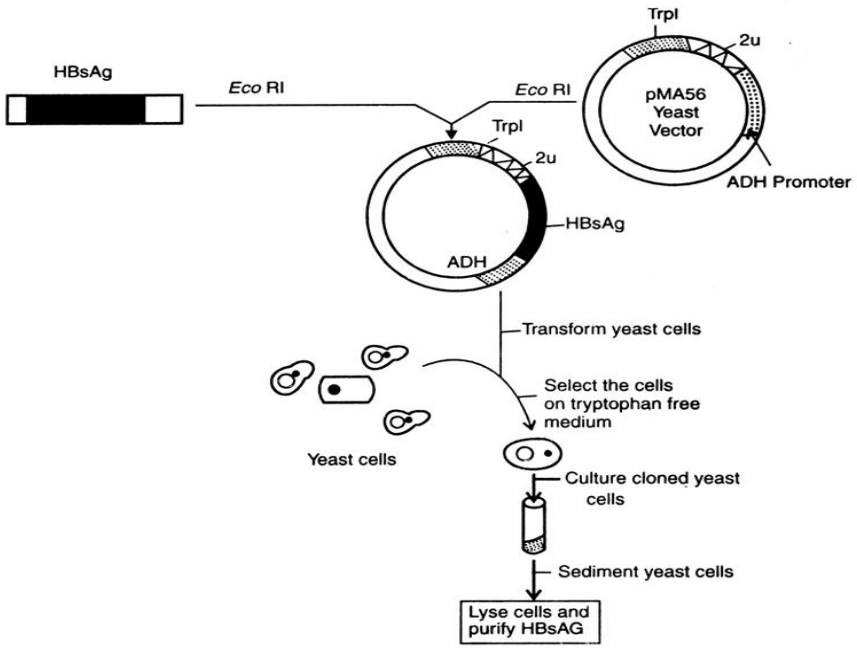
- Monoclonal antibodies are of great interest in HIV because HIV-1 has evolved several strategies to evade humoral immunity.
- One of the virus's key strategies is protecting highly conserved and important structures from antibodies generated by the human immune system.

- The monoclonal antibody 3BNC117 targets the CD4 binding site on the viral envelope of HIV-1. 3BNC117 has been shown to prevent HIV infection in animal models and suppress viremia in human subjects. It was more effective than HAART for post-exposure prophylaxis in animal models.
- A **phase 1 human** study found **3BNC117 enhances humoral immunity to HIV-1**, especially in viremic subjects. In all but 1 of the 15 subjects, 3BNC117 showed an increased neutralizing response against different autologous HIV strains.
- Its actions were less pronounced in patients who had been treated with HAART previously. The study's findings indicate that the agent's mechanism may reduce or eliminate host reservoir in combination with other strategies.
- Broad-spectrum HIV-targeting monoclonal antibodies, such as 3BNC117, may be a key approach to HIV cure in the future. Further research on effective combinations for long-term control or eventual cure is ongoing.

Subunit vaccines: - ***Second generation vaccines were developed to reduce the risks from live vaccines***

These are subunit vaccines, consisting of specific protein antigens (such as tetanus or diphtheria toxoid) or recombinant protein components (such as the hepatitis B surface antigen). They can generate T_H and antibody responses, but not killer T cell responses.

Subunit vaccines are composed of a part of the virus particle responsible for inducing protective immunity. A **subunit vaccine produced** by recombinant DNA techniques would provide an efficient and inexpensive alternative method for the control of this commercially important disease.

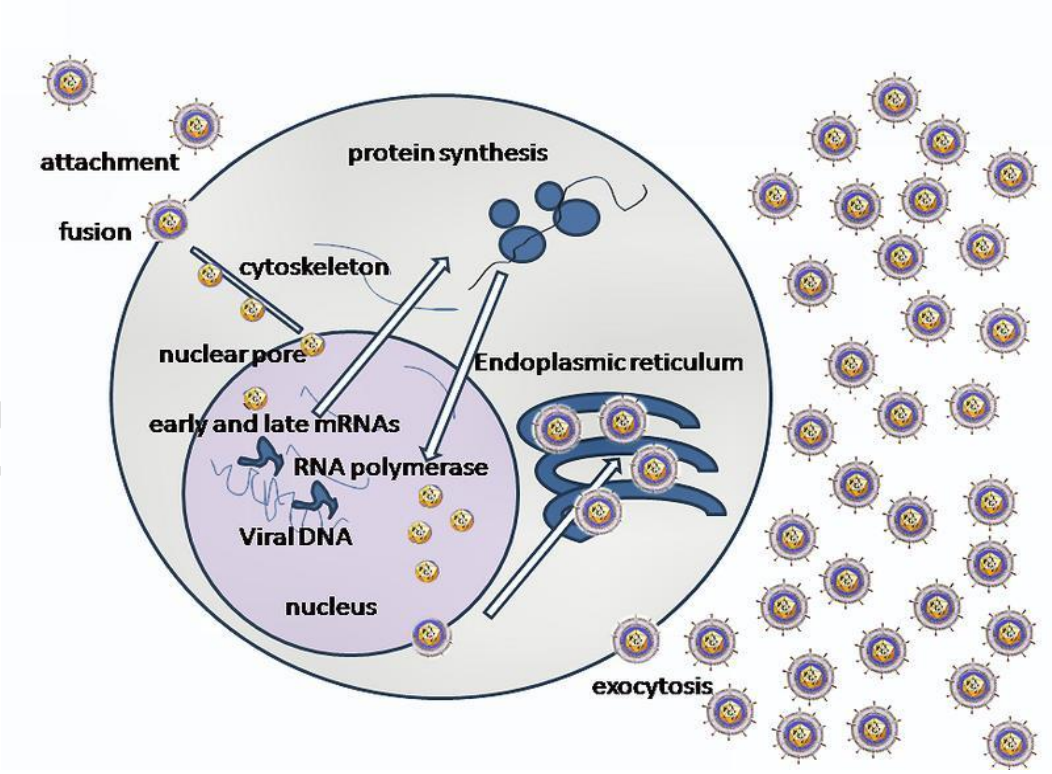


Herpes Simplex Virus Vaccines

Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2), also known as Human Herpes virus 1 and 2 (HHV-1 and HHV-2), are two members of the *Herpesviridae* family, which infect humans. Both HSV-1 and HSV-2 producing most cold sores and most genital herpes respectively are ubiquitous and contagious.

The virus can be spread when an infected person is producing and shedding the virus. Symptoms of Herpes Simplex Virus infection include mucous membranes of the nose, mouth, lips or genitals and watery blisters in the skin. Sometimes, the viruses cause very atypical or mild symptoms during infections. However, they can also cause more troublesome forms of herpes simplex. Regarded as neurotropic and neuroinvasive viruses, HSV-1 and HSV-2 can persist in the body by becoming latent and hiding from the immune system in the cell bodies of neurons.

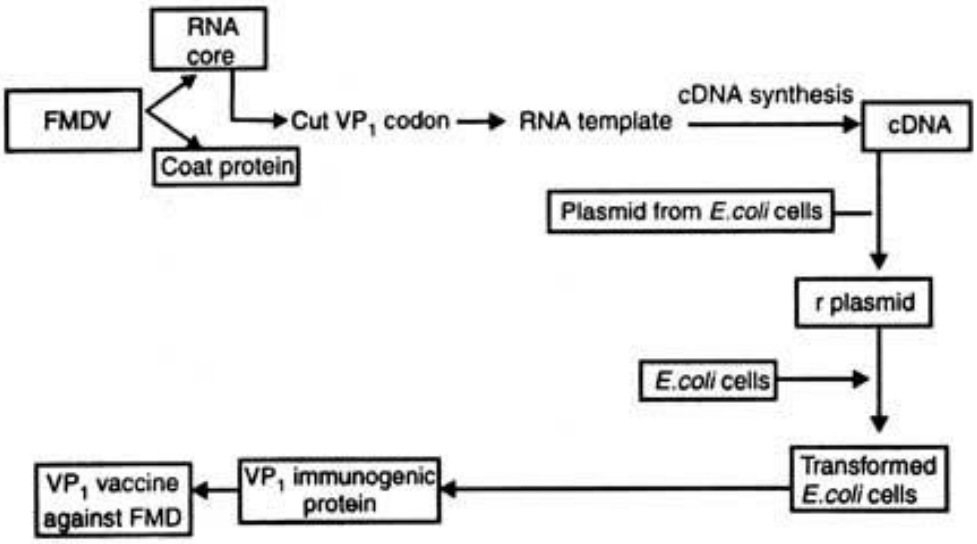
After the initial or primary infection, some infected people experience sporadic episodes of viral reactivation or outbreaks. In the outbreak, the virus in a nerve cell becomes active and is transported *via* the neuron's axon to the skin, where virus replication and shedding occur and cause new sores. In addition, it is one of the most common sexually transmitted infection viruses.



The Development of HSV Vaccine

- Multiple vaccine candidates with diverse platforms have been studied in the preclinical phase and several are being tested in clinical trials.
- The most widely used product for HSV vaccines in human clinical trials has been glycoprotein subunit vaccines.
- Glycoprotein D is expressed on the viral surface and responsible for most neutralizing antibody activity and, therefore, is a rationale target.
- There are several live-attenuated or replication-defective virus vaccine candidates in the preclinical phase. A replication-defective HSV-2 vaccine (HSV529) has entered Phase I trials for both preventive and therapeutic indications.
- A live-attenuated virus deleted in gD2 prevented skin, neural and vaginal disease in the mouse model, and also is the first construct to eliminate establishment of latency in the dorsal root ganglia.
- Novel delivery methods of glycoproteins, including lentiviral vectors expressing glycoprotein B and intranasal delivery, are being explored. Glycoprotein candidates with novel platforms are still being investigated.

Vaccine Production for Foot and Mouth Disease:



- Polypeptides which act as antigens of foot and mouth disease virus (FMDV) are produced by microorganisms which have been transformed with plasmids produced using recombinant DNA technology.

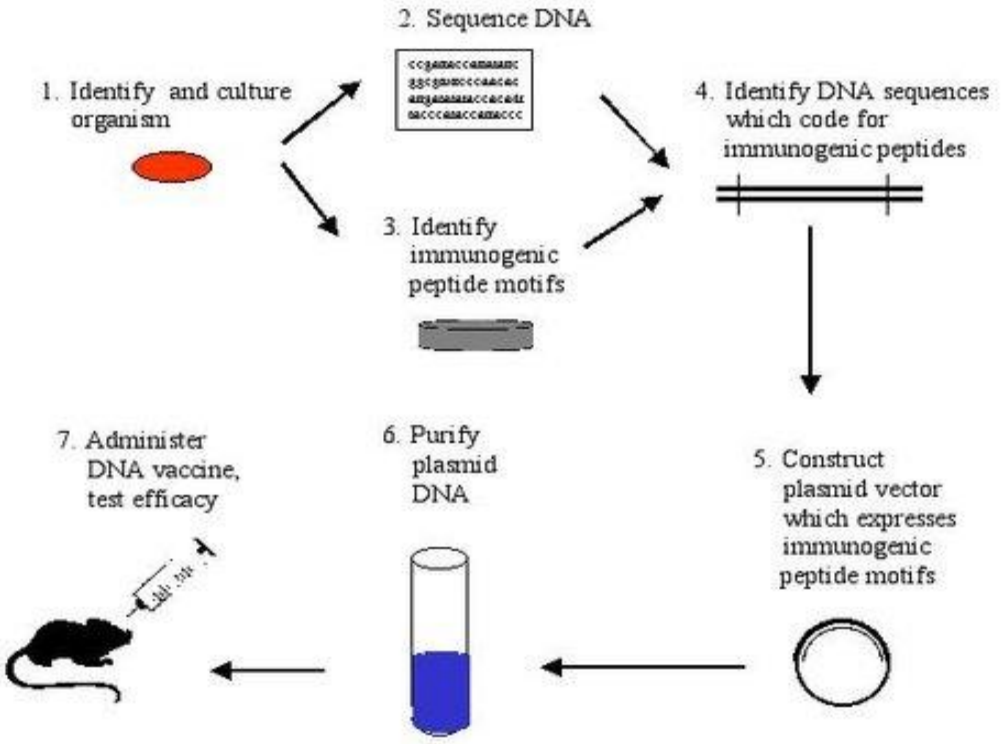
- A suitable plasmid has a gene sequence (produced by chemical synthesis or by reverse transcription) which encodes a polypeptide comprising one or more immunogenic determinants of one or more FMDV proteins, and optionally a fusion protein preferably linked via a cleavage site, the gene sequence being under the control of a promoter.
- For example, an E. Coli K12 strain was transformed with plasmid pFM_1.
- This is based on plasmid pBR322, with the DNA sequence of codons 8 to 210 of the VP, gene of FMDV type A_{12} 119ab connected to the LE' protein (from pBR322) so as to be in the same reading frame.
- This assembly is under the control of the E. Coli trp operon. The transformant E. Coli was cultured and caused to express the LE'-VP, fusion protein.
- This was isolated and shown by competitive radio immunoassay to have similar antigenic properties to purified natural VP, protein.

Vaccine for Tuberculosis:

The bacterium responsible for TB, called Mycobacterium tuberculosis (Mtb), is transmitted by people infected with pulmonary (lung) TB who release Mtb into the air through coughing, sneezing or spitting. Approximately 1/3 of the world's population carry the disease but don't have any symptoms (known as latent infection), however approximately 10% of these people will likely develop active disease during their lifetime and become capable of transmitting the bacterium.

The TB epidemic continues in spite of an available, cost-effective and broadly implemented vaccine for infants – Bacille Calmette-Guerin (BCG) – and the carefully managed use of drugs for those who do become infected through directly observed therapy (DOTs). This is because BCG vaccination is only partially effective: it provides some protection against severe forms of pediatric non-pulmonary TB, such as TB meningitis, but is unreliable against adult pulmonary TB, which accounts for most of the TB disease burden (and transmission) worldwide.

In addition, infection with Human Immunodeficiency Virus (HIV) infection can increase the likelihood of TB acquisition by up to 25-fold, and resistance to previously effective TB drug regimens is increasing.



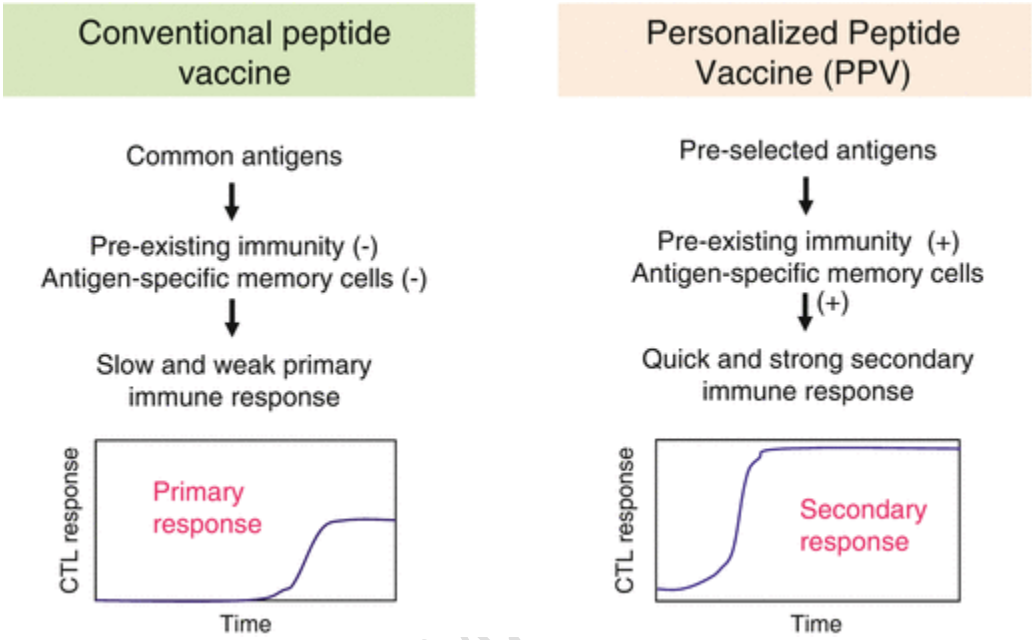
Peptide Vaccine:

A **peptide vaccine** is any **peptide** which serves to immunize an organism against a pathogen. **Peptide vaccine**s are often synthetic and mimic naturally occurring proteins from pathogens.

- ✓ The vaccine in which peptide of the original pathogen is used to immunize an organism.
- ✓ The best known example is foot and mouth disease, where protection was achieved by immunizing animals with a linear sequence of 20 amino acids.
- ✓ Synthetic peptide vaccines would have many′ advantages. Their antigens are precisely defined and free from unnecessary components which may be associated with side effects.
- ✓ They are stable and relatively cheap to manufacture. Furthermore, less quality assurance is required.′ Changes due to natural variation of the virus can be readily accommodated, which would be a great advantage for unstable viruses such as influenza.
- ✓ Synthetic peptides do not readily stimulate T cells.
- ✓ It was generally assumed that, because of their small size, peptides would behave like haptens and would therefore require coupling to a protein carrier which is recognized by T- cells.
- ✓ It is now known that synthetic peptides can be highly′ immunogenic in their free form provided they contain, in addition to the B cell epitope, T- cell epitopes recognized by T- helper cells.

Such T-cell epitopes can be provided by carrier protein molecules, foreign antigens. or within the synthetic peptide molecule itself.

- ✓ Synthetic peptides are not applicable to all viruses. This approach did not work in the case of polioviruses because the important antigenic sites were made up of 2 or more different viral capsid proteins so that it was in a concise 3-D conformation.



Advantage of peptide vaccine:

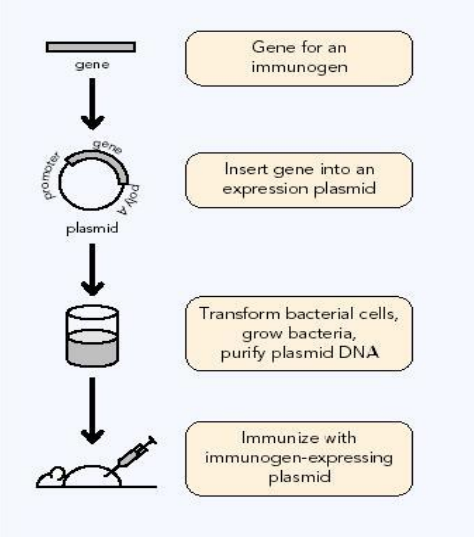
- ❖ Production and quality control simpler
- ❖ No NA or other viral or external proteins, therefore less toxic.
- ❖ Safer in cases where viruses are oncogenic or establish a persistent infection
- ❖ Feasible even if virus cannot be cultivation

Disadvantages:

- ✓ May be less immunogenic than conventional inactivated whole-virus vaccines
- ✓ Requires adjuvant
- ✓ Requires primary course of injections followed by boosters
- ✓ Fails to elicit CMI (Cell Mediated Immunity)

Genetic Immunization:

Genetic immunization represents a novel approach to **vaccination**. This technology involves transfer of a gene encoding an antigenic protein cloned in expression **vectors** to a host, leading to the induction of an immune response.



DNA vaccines

DNA vaccines are third generation vaccines. They contain DNA that codes for specific proteins (antigens) from a pathogen. The DNA is injected into the body and taken up by cells, whose normal metabolic processes synthesize proteins based on the genetic code in the plasmid that they have taken up. Because these proteins contain regions of amino acid sequences that are characteristic of bacteria or viruses, they are recognized as foreign and when they are processed by the host cells and displayed on their surface, the immune system is alerted, which then triggers immune responses.

Advantages of Nucleic Acid-Based Immunization

- No risk for infection
- Antigen presentation by both MHC class I and class II molecules
- Polarise T-cell response toward type 1 or type 2
- Immune response focused on antigen of interest
- Ease of development and production
- Stability for storage and shipping
- Cost-effectiveness
- Obviates need for peptide synthesis, expression and purification of recombinant proteins and use of toxic adjuvants

- Long-term persistence of immunogen
- *In vivo* expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications

Disadvantages of Nucleic Acid-Based Immunization:

- Limited to protein immunogens (not useful for non-protein based antigens such as bacterial polysaccharides)
- Risk of affecting genes controlling cell growth
- Possibility of inducing antibody production against DNA
- Possibility of tolerance to the antigen (protein) produced
- Potential for atypical processing of bacterial and parasite proteins

Vector Vaccines:

- ❖ Vector vaccines are liquid or freeze-dried preparations of one or more types of live micro-organisms (bacteria or viruses) that are non-pathogenic or have a low pathogenicity for the target species and in which have been inserted one or more genes encoding antigens that stimulate an immune response protective against other micro-organisms
- ❖ A live vector vaccine is a vaccine that uses a chemically weakened virus to transport pieces of the virus in order to stimulate an immune response.
- ❖ The genes used in this vaccine are usually antigen coding surface proteins from the pathogenic organism. They are then inserted into the genome of a non pathogenic organism such as Adenovirus was they are expressed on the cells surface and can elicit an immune response.

Viral vector advantages:

- •Infects human cells but some do not replicate
- •Better presentation of antigen
- •Generate T cell response

Drawbacks:

- •Can cause bad reactions
- •Can be problems with pre-exisiting immunity to virus
- •Often can only accommodate one or two antigens

UNIT II: Microbial production of commercial products Microbial production of restriction endonucleases:

Restriction enzyme:

- A **restriction enzyme**, **restriction endonuclease**, or restrictase is an **enzyme** that cleaves DNA into fragments at or near specific recognition sites within molecules known as **restriction** sites.
- These **enzymes** are found in bacteria and archaea and provide a defence mechanism against invading viruses.

PstI:

- **PstI** is a type II restriction endonuclease isolated from the Gram negative species, *Providencia stuartii*.

Providencia stuartii

- ***Providencia stuartii*** (commonly ***P. stuartii***), is a Gram negative bacillus that is commonly found in soil, water, and sewage.
- *P. stuartii* is the most common of the 5 species found in the genus Providencia, with *Providencia rettgeri*, *Providencia alcalifaciens*, *Providencia rustigianii*, *P heimbachaeP. stuartii* is the genomic source for the restriction endonuclease, PstI.
- Some other important information about *P. stuartii* is that it is motile via flagella, non-sporulating, non-lactose fermenting, catalase positive and oxidase negative.
- It can also grow in anaerobic conditions and on Simmon's Citrate Agar.

Production of PstI:

Restriction Endonucleases recognise and cut DNA at a specific palindromic base sequences, normally 4, 6 or 8 bases long, and are used selectively to cut DNA into defined fragments at sites known as 'restriction sites'

⬇

Different restriction endonucleases are obtained and purified from different species of bacteria

⬇

These enzymes are made in bacteria to degrade viral DNA

Cutting DNA with restriction enzymes can produce fragments with either blunt or sticky ends

Different restriction enzymes recognise different DNA sequences

The product of a restriction enzyme can have either sticky ends or blunt ends as shown below.

Examples of restriction endonucleases are:

- *Eco*RI - recognises the sequence 5'GAATTC'3 - sticky ends
- *Bam*HI - recognises the sequence 5'GGATCC'3 - sticky ends
- *HhaI* - recognises the sequence 5'GCGC'3 - sticky ends
- *XhoI* - recognises the sequence 5'CTCGAG'3 - sticky ends
- *HindIII* - recognises the sequence 5'AAGCTT'3 - sticky ends
- *PstI* - recognises the sequence 5'CTGCAG'3 - sticky ends
- *Sau3A* - recognises the sequence 5'GATC'3 (produces the same sticky ends as BamHI upon cutting)
- HaeIII - recognises the sequence 5'GGCC'3 - blunt ends

Function of PstI

- PstI cleaves DNA at the recognition sequence 5′-CTGCA/G-3′ generating fragments with 3′-cohesive termini.
- This cleavage yields sticky ends 4 base pairs long.
- PstI is catalytically active as a dimer.
- The two subunits are related by a 2-fold symmetry axis which in the complex with the substrate coincides with the dyad axis of the recognition sequence.
- It has a molecular weight of 69,500 and contains 54 positive and 41 negatively charged residues.

Recognition Sequence Cut Site

5'CTGCAG 3'	5'--CTGCA G--3'
3'GACGTC 5'	3'--G ACGTC—5'

- The PstI restriction/modification (R/M) system has two components: a restriction enzyme that **cleaves foreign DNA**, and a **methyltransferase** which protect native DNA strands via histone methylation.
- The combination of both provide a defense mechanism against invading viruses.
- The methyltransferase and endonuclease are encoded as two separate proteins and act independently.
- In the PstI system, the genes are encoded on opposite strands and hence must be transcribed divergently from separate promoters.
- The transcription initiation sites are separated by only 70 base pairs.
- A delay in the expression of the endonuclease relative to methylase is due to the inherent differences of the two proteins.
- The endonuclease is a dimer, requiring a second step for assembly, whereas the methylase is a monomer.

Applications of PstI:

- **PstI** is a useful **enzyme** for DNA cloning as it provides a selective system for generating hybrid DNA molecules.
- Its use is not limited to molecular cloning; it is also **used** in
  - **restriction site** mapping
  - genotyping
  - Southern blotting
  - **restriction** fragment length polymorphism (RFLP)
  - SNP (Single Nucleotide Polymorphism).

Production of Indigo Dye by Microbes:

- **Indigo dye** is an organic compound with a distinctive blue color
- Colonies of the bacterium *Escherichia coli*, genetically modified to **produce** the **dye indigo** (blue).
- In addition, introduction of the gene for the enzyme xylene oxidase, which is encoded in the TOL plasmid, can convert tryptophan to indoxyl, which then spontaneously oxidizes to **indigo**
- A commercially important blue pigment that is used to dye both cotton and wool
- Isolated from plant but is currently synthesized chemically
- Ability to produced indigo from bacteria opens the possibility of developing an efficient and economical commercial microbial process for its production.

Biosynthesis of Indigo

• A number of bacteria, most notably Pseudomonas species have the ability to use a variety of organic compound such as naphthalene, toluene, xylene etc.

• The genes encoding the enzymes for these degradation of organic compounds are located on large naturally occurring plasmids.

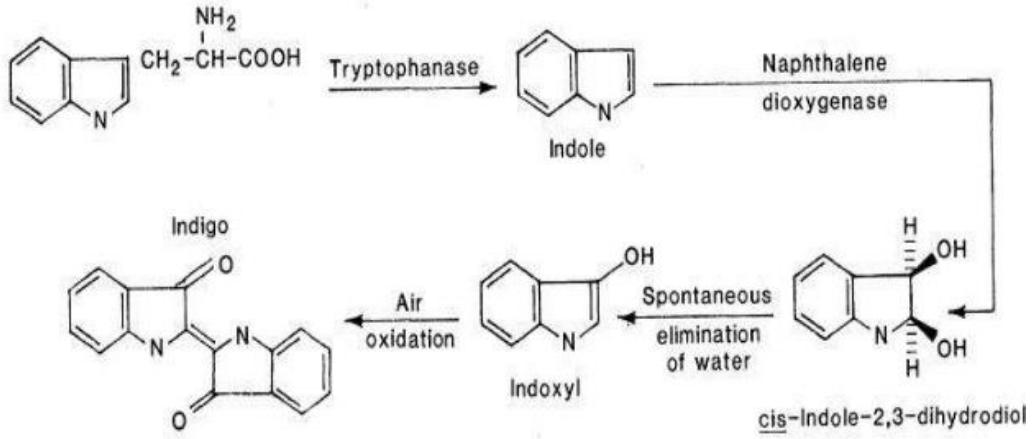
• Plasmid NAH7 has two separate and distinct operons that allow pseudomonas to grow on naphthalene as the sole carbon source.

• As a first step, NAH7 was digested and ligated with Hind III in plasmid PBR322 and introduced in *E.coli.*

• *E. coli* contains the enzyme tryptophanase, E. coli, the cells have the ability to synthesize indigo from tryptophan.

Steps of microbial indigo production

- Conversion of tryptophan in the growth medium to indole by the enzyme tryptophanase, which is produced by the *E. coli* host cell
- Oxidation of indole to cis-indole-2,3-dihydrodiol by naphthalene dioxygenase, which is encoded by the DNA that was cloned from the NAH7 plasmid
- Spontaneous elimination of water
- Air oxidation to form indigo
- Thus, the combination of enzymes from two different pathways and two different organisms resulted in the synthesis of an unexpected compound, the dye indigo
- In addition, introduction of the gene for the enzyme xylene oxidase, which is encoded in the TOL plasmid, can convert tryptophan to indoxyl, which then spontaneously oxidizes to indigo.



Commercial Importance of Indigo

- Used to make hair dyes
- Used for textile dyeing and printing
- Indigo is as a dye for cotton yarn
- Production of denim cloth for blue jeans
- Small amounts are used for dyeing wool and silk
- Indigo carmine or indigo, is an indigo derivative which is also used as a colorant
- It is also used as a food colorant.

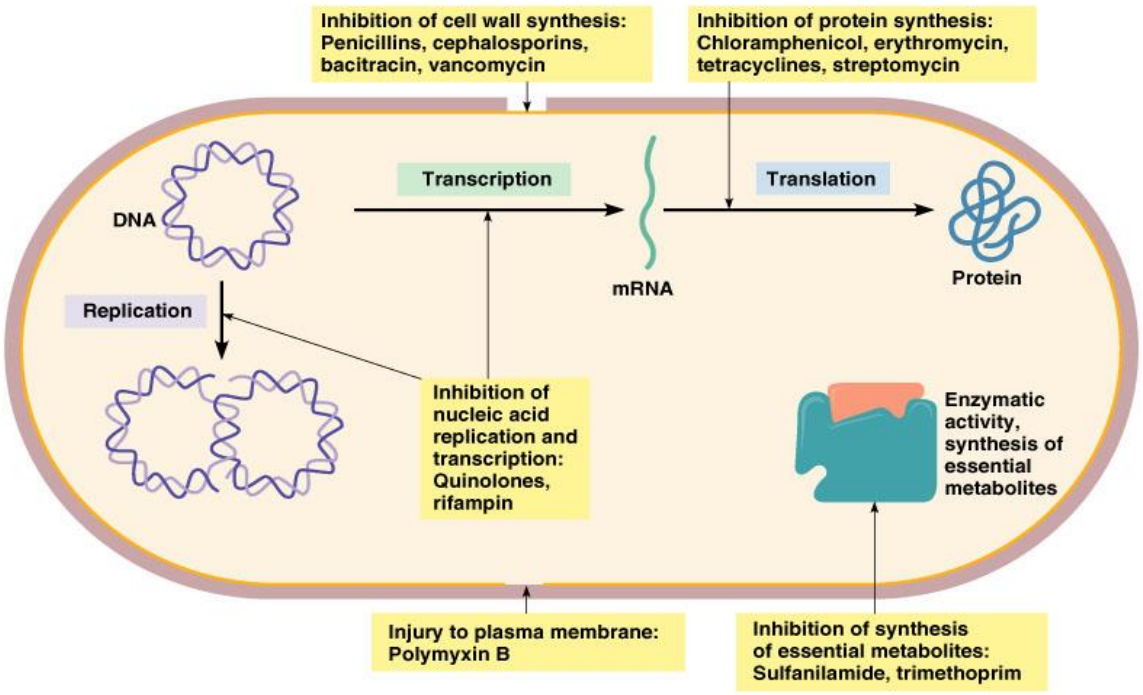
Antibiotic/Antimicrobial Compounds:

- Antibiotic: Chemical produced by a microorganism that kills or inhibits the growth of another microorganism
- Antimicrobial agent: Chemical that kills or inhibits the growth of microorganisms

Microbial Sources of Antibiotics:

Microorganism	Antibiotic
Gram-Positive Rods	
Bacillus subtilis	Bacitracin
Bacillus polymyxa	Polymyxin
Actinomycetes	
Streptomyces nodosus	Amphotericin B
Streptomyces venezuelae	Chloramphenicol
Streptomyces aureofaciens	Chlortetracycline and tetracycline
Streptomyces erythraeus	Erythromycin
Streptomyces fradiae	Neomycin
Streptomyces griseus	Streptomycin
Micromonospora purpureae	Gentamicin
Fungi	
Cephalosporium spp.	Cephalothin
Penicillium griseofulvum	Griseofulvin
Penicillium notatum	Penicillin

Modes of Antimicrobial Action:



Industrial production techniques

1. **Fermentation**

- Industrial microbiology can be used to produce antibiotics via the process of fermentation, where the source microorganism is grown in large containers (100,000–150,000 liters or more) containing a liquid growth medium.
- Oxygen concentration, temperature, pH and nutrient levels must be optimal, and are closely monitored and adjusted if necessary.
- As antibiotics are secondary metabolites, the population size must be controlled very carefully to ensure that maximum yield is obtained before the cells die.
- Once the process is complete, the antibiotic must be extracted and purified to a crystalline product.
- This is easier to achieve if the antibiotic is soluble in organic solvent. Otherwise it must first be removed by ion exchange, adsorption or chemical precipitation.

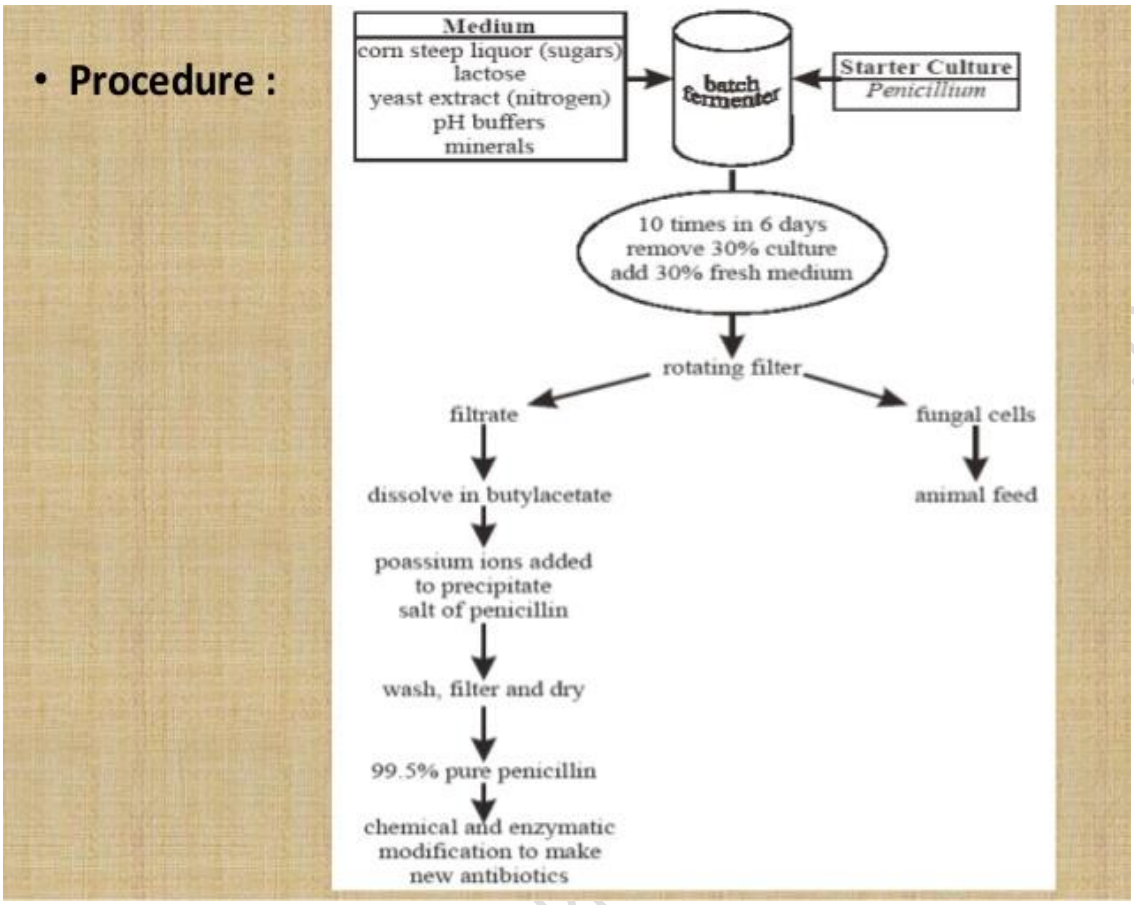
2. **Semi-synthetic**

- A common form of antibiotic production in modern times is semi-synthetic.

- Semi-synthetic production of antibiotics is a combination of natural fermentation and laboratory work to maximize the antibiotic.
- Maximization can occur through efficacy of the drug itself, amount of antibiotics produced, and potency of the antibiotic being produced.
- Depending on the drug being produced and the ultimate usage of said antibiotic determines what one is attempting to produce.
- An example of semi-synthetic production involves the drug ampicillin. A beta lactam antibiotic just like penicillin, ampicillin was developed by adding an addition amino group (NH_2) to the R group of penicillin.
- This additional amino group gives ampicillin a broader spectrum of use than penicillin. Methicillin is another derivative of penicillin and was discovered in the late 1950s, the key difference between penicillin and methicillin being the addition of two methoxy groups to the phenyl group.
- These methoxy groups allow methicillin to be used against penicillinase producing bacteria that would otherwise be resistant to penicillin.

3. Synthetic

- Not all antibiotics are produced by bacteria; some are made completely synthetically in the lab.
- These include the quinolone class, of which nalidixic acid is often credited as the first to be discovered.
- Like other antibiotics before it the discovery of nalidixic acid has been chalked up to an accident, discovered when George Lesher was attempting to synthesize chloroquine.
-



Uses of Antibiotics:

- Antitumor antibiotics
- Food preservative antibiotics
- Antibiotics used in animal feed and veterinary medicine
- Antibiotics for control of human and plant diseases
- Antibiotics as tools in molecular biology

Biopolymer: Xanthan gum:

- **Xanthan gum**is a polysaccharide with many industrial uses, including as a common food additive
- It is an effective thickening agent and stabilizer to prevent ingredients from separating
- It can be produced from simple sugars using a fermentation process, and derives its name from the species of bacteria used, *Xanthomonas campestris*.

Introduction:

- Xanthan gum is a natural extra cellular polysaccharide which is produced by the fermentation of glucose/sucrose by Xanthomonas campestris
- It is soluble in cold water & exhibits high pseudoplastic flow
- Polysaccaride is resistant to enzymatic digestion

- Its viscosity is highly stable over a wide pH and temperature
- Its Molecular formula is C 35 H 44 O29

Manufacturing Process

The manufacturing process for both Xantham Gum and Gellan Gum are very similar:-

- **Xanthan gum** is produced by **aerobic submerged fermentation** using the ***bacterium Xanthomonas campestris***, a micro-organism which is found naturally on cabbages.
- **Gellan gum** is produced by the same process using the bacterium ***Pseudomonas elodea***.

Xanthomonas campestris is produced from a pure culture of bacterium by an aerobic submerged in fermentation process.

⬇

The steps of manufacture of *Xanthomonas campestris* is as follows A well aerated medium containing nitrogen¬ source, glucose and various trace elements is chosen. Some times in some industries Corn syrup is chosen as the medium the medium must contain good amount of nutrition required by the bacteria

⬇

The medium is inoculated with *Xanthomonas¬ campestris*. Now the inoculated medium is transferred to an agitator. This is where the bacteria is incubated.

⬇

In this sugary environment the bacterium replicates rapidly and forms around trillion identical cells in 48 hours/ after few days the bacteria have used all the carbohydrates present in the medium/broth and produce a polysaccharide.

⬇

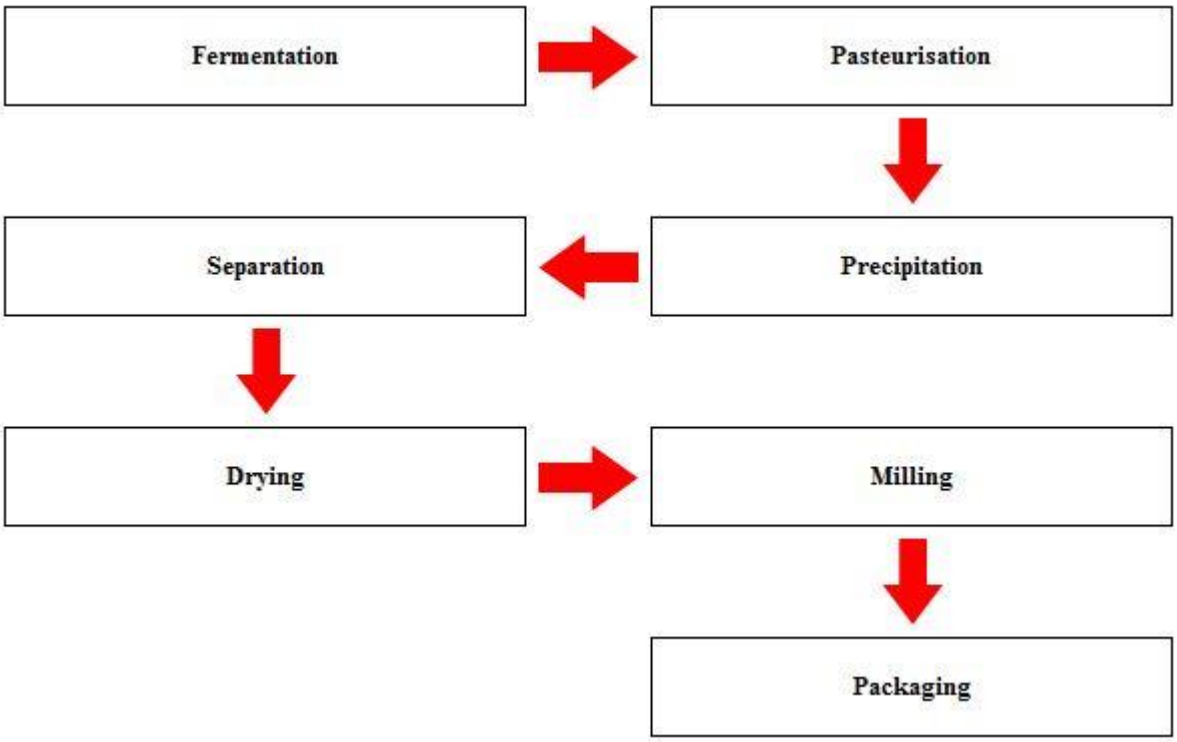
This polysaccharide is called xanthan gum

⬇

When the final fermentation process is finished thebroth is pasteurized to kill all the bacteria present in the broth & xanthan gum is recovered by precipitation with isopropyl alcohol.

⬇

The recovered xanthan gum is then hydrated to remove water and is dried, milled and finally packed. The xanthan gum is now ready to be sold for¬ commercial uses.



Applications of Xanthan Gum:

- In foods, xanthan gum is common in salad dressings and sauces.
- It helps to prevent oil separation by stabilizing the emulsion, although it is not an emulsifier.
- Xanthan gum also helps suspend solid particles, such as spices.
- Xanthan gum helps create the desired texture in many ice creams.
- Toothpaste often contains xanthan gum as a binder to keep the product uniform.
- Xanthan gum also helps thicken commercial egg substitutes made from egg whites, to replace the fat and emulsifiers found in yolks. It is also a preferred method of thickening liquids for those with swallowing disorders, since it does not change the color or flavor of foods or beverages at typical use levels.
- In gluten-free baking, xanthan gum is used to give the dough or batter the stickiness that would otherwise be achieved with gluten. In most foods it is used at concentrations of 0.5% or less. Xanthan gum is used in wide range food products, such as sauces, dressings, meat and poultry products, bakery products, confectionery products, beverages, dairy products, others.
- In the oil industry, xanthan gum is used in large quantities to thicken drilling mud. These fluids carry the solids cut by the drilling bit to the surface.
- In cosmetics, xanthan gum is used to prepare water gels. It is also used in oil-in-water emulsions to enhance droplet coalescence

Biopolymer – Polyhydroxyalkanoates (PHA) Production:

- **Polyhydroxyalkanoates** or **PHAs** are polyesters produced in nature by numerous microorganisms, including through bacterial fermentation of sugars or lipids
- When produced by bacteria they serve as both a source of energy and as a carbon store. More than 150 different monomers can be combined within this family to give materials with extremely different properties.
- These plastics are biodegradable and are used in the production of bioplastics.
- They can be either thermoplastic or elastomeric materials, with melting points ranging from 40 to 180 °C.

Biosynthesis

Biosynthesis PHA, a culture of a micro-organism such as *Cupriavidus necator* is placed in a suitable medium and fed appropriate nutrients so that it multiplies rapidly.

⇩

Once the population has reached a substantial level, the nutrient composition is changed to force the micro-organism to synthesize PHA.

⇩

The yield of PHA obtained from the intracellular granule inclusions can be as high as 80% of the organism's dry weight.

⇩

The biosynthesis of PHA is usually caused by certain deficiency conditions (e.g. lack of macro elements such as phosphorus, nitrogen, trace elements, or lack of oxygen) and the excess supply of carbon sources

⇩

Polyesters are deposited in the form of highly refractive granules in the cells.

Depending upon the microorganism and the cultivation conditions, homo- or copolyesters with different hydroxyalkanic acids are generated.

⇩

PHA granules are then recovered by disrupting the cells. Recombinant *Bacillus subtilis* str. pBE2C1 and *Bacillus subtilis* str. pBE2C1AB were used in production of polyhydroxyalkanoates (PHA) and it was shown that they could use malt waste as carbon source for lower cost of PHA production.

⇩

PHA synthases are the key enzymes of PHA biosynthesis. They use the coenzyme A - thioester of (r)-hydroxy fatty acids as substrates.

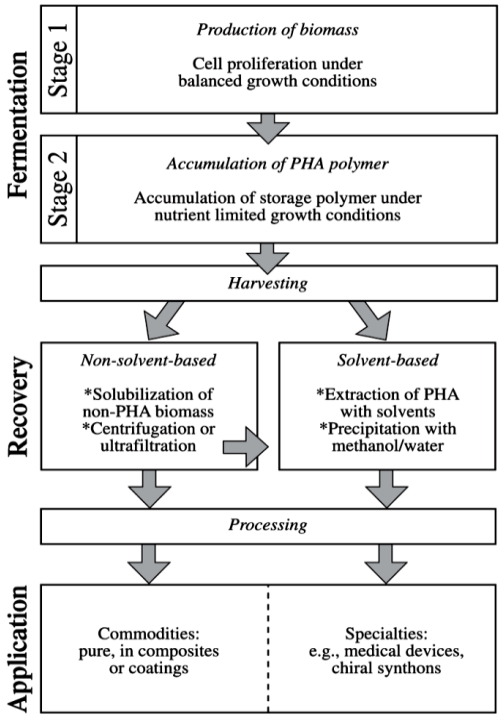
⇩

The two classes of PHA synthases differ in the specific use of hydroxy fatty acids of short or medium chain length.

⇩

The resulting PHA is of the two types:

- Poly (HA SCL) from hydroxy fatty acids with short chain lengths including three to five carbon atoms are synthesized by numerous bacteria, including *Cupriavidus necator* and *Alcaligenes latus* (PHB).
- Poly (HA MCL) from hydroxy fatty acids with medium chain lengths including six to 14 carbon atoms, can be made for example, by *Pseudomonas putida*.



Applications:

Polyhydroxyalkanoates have a great variety of characteristics, and their sustainability, biodegradability, and biocompatibility mean that many industries would benefit from utilizing them further. Here are some applications in which PHAs have become very useful:

- Single use packaging for foods, beverages, consumer products, etc
- Medical applications like sutures, bone marrow scaffolds, bone plates, etc
- Agricultural foils and films

Production of Alcohol:

Yeasts are the main fermentor and **alcohol** producer in the **production** of wine, beer and other **alcohol** drinks. The main yeast species used is Saccharomyces cerevisiae. It ferments the sugars, coming from different sources, e.g., grapes for wine, barley for beer, to **alcohol** and carbon dioxide.

Production process

1. **Feedstock**

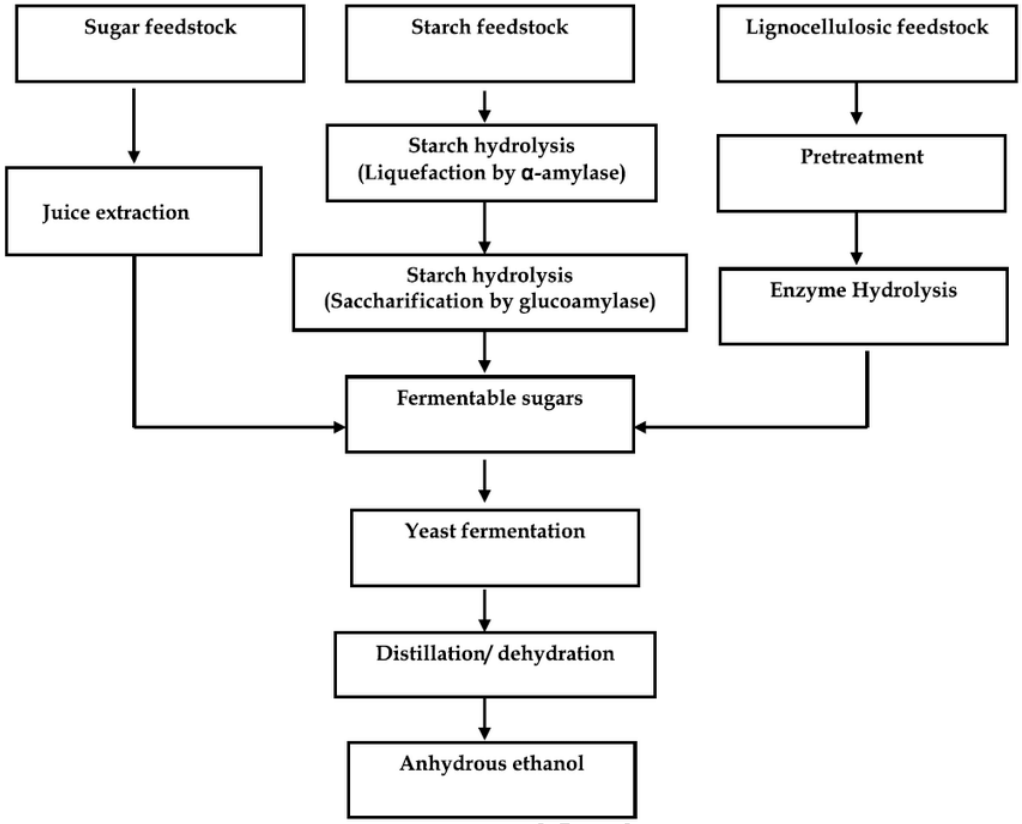
The feedstock for ethanol production can be any material containing appreciable amounts of sugar or substances that can be converted to sugar. Conventional production uses sugar (from sugar cane and sugar beet), starch (from corn, wheat or potatoes) or other polysaccharides. The production process of second generation ethanol, also called cellulosic alcohol, uses cellulosic feedstock (e.g. from agricultural residues) which require further pretreatment.

2. **Metabolic conversion**

- In a fermentation process sugar (glucose, fructose or other monosaccharides) is converted to ethanol by microbes (mostly varieties of the yeast Saccharomyces cerevisiae), which are inoculated to the feedstock.
- The monosachharides originate either directly from disaccharides, which are broken up via invertase enzymes, or from starch which is hydrolysed with amylase enzymes.
- In addition to ethanol, water and carbon dioxide are prdouced also.
- The glucose-to-ethanol reaction is represented by the equation below:

$$C_6H_{12}O_6 + 2\ ADP + 2\ Pi \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ H_2O + 2\ ATP$$

- Common processes produce a fermentation broth with concentration of 5% - 10% ethanol per volume, as ethanol itself is toxic to the microorganisms. More advanced facilities are able to increase the concentration up to 20% due to the use of adapted and specialized yeasts.



Applications:

- Alcoholic Drinks
- Industrial methylated spirits
- Use of ethanol as a fuel
- Ethanol as a solvent
- Methanol as a fuel
- Methanol as an industrial feedstock

Lactic Acid Fermentation:

- **Lactic acid fermentation** is a metabolic process by which glucose and other six-carbon sugars (also, disaccharides of six-carbon sugars, e.g. sucrose or lactose) are converted into cellular energy and the metabolite lactate, which is lactic acid in solution.
- It is an anaerobic fermentation reaction that occurs in some bacteria and animal cells, such as muscle cells

Process of Production:

Lactic acid produced two ways

1. In ***homolactic fermentation***, one molecule of glucose is ultimately converted to two molecules of lactic acid.
2. ***Heterolactic fermentation***, in contrast, yields carbon dioxide and ethanol in addition to lactic acid, in a process called the phosphoketolase pathway.

❖ **Homofermentative process**

Homofermentative bacteria convert glucose to two molecules of lactate and use this reaction to perform substrate-level phosphorylation to make two molecules of ATP:

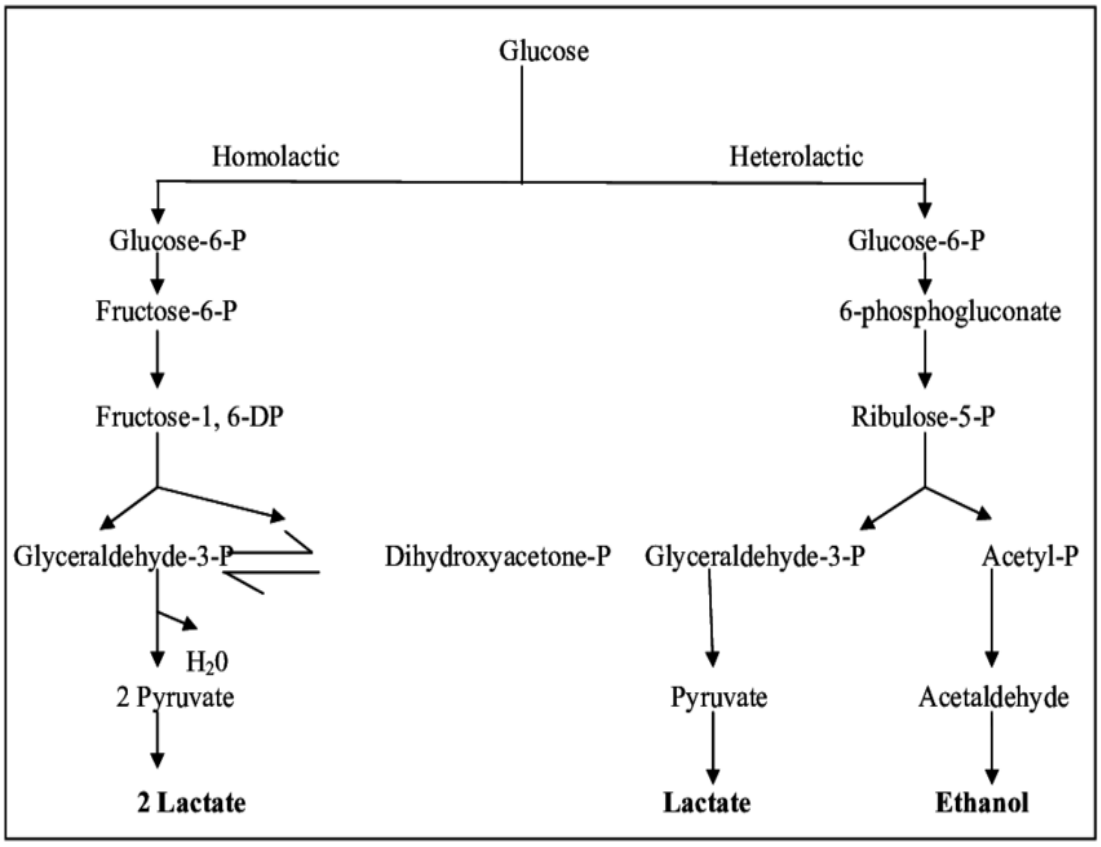
Glucose + 2 ADP + 2 P_i → 2 lactate + 2 ATP

❖ **Heterofermentative process**

Heterofermentative bacteria produce less lactate and less ATP, but produce several other end products:

Glucose + ADP + P_i → lactate + ethanol + CO_2 + ATP

Examples include *Leuconostoc mesenteroides*, *Lactobacillus bifermentous*, and *Leconostoc lactis*.



Uses of Lactic acid:

- This medication has 2 types of ingredients (emollient, keratolytic) that work together to treat or prevent dry, rough, scaly, itchy skin (such as that caused by eczema, keratosis, xerosis).
- Dry skin is caused by a loss of water in the upper layer of the skin.
- Emollients are substances that soften and moisturize the skin and decrease itching and flaking.
- Emollients/moisturizers work by forming an oily layer on the top of the skin that traps water in the skin. Petrolatum, lanolin, mineral oil, and dimethicone are common emollients.
- Lactic acid, salicylic acid, and urea are keratolytics.
- They increase moisture in the skin by softening/dissolving the horny substance (keratin) holding the top layer of skin cells together.
- This helps the dead skin cells fall off and helps the skin keep more water in. Higher strengths of urea are used to treat corns, callous, and some nail problems (e.g., ingrown nails).
- Urea is also used to help remove dead tissue in some wounds to help wound healing.

Streptomycin:

Streptomycin is an aminoglycoside antibiotic produced by the soil actinomycete ***Streptomyces griseus***. It acts by binding to the 30S ribosomal subunit of susceptible organisms and disrupting the initiation and elongation steps in protein synthesis. It is bactericidal due to effects that are not fully understood.

1. **Introduction:**

- Streptomycin is a bactericidal antibiotic drug belonging to class aminoglycosides.
- Used against TB
- Derived from actinobacterium *Streptomyces griseus*.
- Used against gram negative bacteria especially.
- Dihydrostrepomycin prepared by hydrogenation of streptomycin with platinum as catalyst & is commercially more successful.

2. **Chemical composition:**

- Chemically, it contains 3 sugars derived from glucose with C, N, O & H elements.
- Chemical formula – C21H39N7O12

3. **Medium:**

- Medium is a nutritive substance in which cultures are grown for scientific purposes.
- The culture medium for streptomycin consists of

1. Carbon source: starch, dextrin, glucose, glycerol & other economically available material.

2. Nitrogen source: natural agricultural by-products, soybean meal, corn steep liquor, cotton seed flour, casein hydrolyte, or yeast & its extract. Inorganic N salts like ammonium sulphate & ammonium nitrates are also used.

3. Animal oils, vegetable oils and mineral oils are also used.

4. **Culture:**

- *S.griseus* spores maintained in soil stocks or lyophilized in carrier are inoculated into sporulation medium which builds up mycelial inoculum.

5. **Fermentation process**:

- Spores of *S.griseus* are inoculated into a medium to establish a culture with high mycelial biomass for introduction into inoculum tank, using inoculum to initiate the fermentation process.
- Yield in production vessel responds to high aerationυ & agitation conditions. Other conditions involve
  - Temperature range 25-30°C
  - pH range 7-8
  - Time 5-7 days
- The fermentation process for production of Streptomycin involves 3 phases.

PHASE 1:

- Initial fermentation phase and there is little production of streptomycin.
- Rapid growth with production of mycelial biomass.
- Proteolytic enzymatic activity of S.griseus releases NH3 from soya meal, raising the pH to 7.5
- Characterized by release of ammonia.

- Carbon nutrients of soya meal are utilized for growth. Glucose is slowly utilized with slight production of Streptomycin.

PHASE 2:

- Little production of mycelia.

- Glucose added to the medium & the NH3 released from soya meal are consumed.

- pH remains fairly constant ranging between 7.6 to 8.

PHASE 3:

- Final phase of fermentation.

- Depletion of carbohydrates from medium.

- Streptomycin production ceases & bacterial cells begin to lyse.

- Ammonia from lysed cells increases the pH.

6. Recovery & purification:

- Mycelium is separated from broth by filtration & streptomycin is recovered.
- Recovery process – broth is acidified, filteredυ & neutralized. Then its subjected to column containing cation exchange resin to adsorb Streptomycin from the broth & column is washed with water & streptomycin eluted with HCl before concentration in vacuo almost to dryness.
- The streptomycin is dissolved in methanol & filtered.
- Acetone is used in filterate to precipitate the antibiotic.
- Percipitate is washed with acetone & dried in vacuo.
- Purification is done by dissolving in methanol to form pure S. chloride complex. Further by, adsorbing it onto activated charcoal & eluting with acid alcohol.

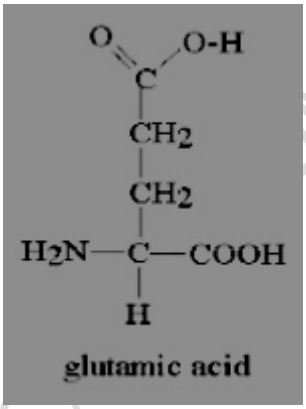
7. **uses**

- Treatment of diseases
  - 1. Tuberculosis
  - 2. Plague
  - 3. Veterinary medicine against gram negative bacteria.
- Pesticideυ & fungicide.
- Cell culture.
- Protein purification.

Glutamic Acid

Glutamic acid is an amino acid used to form proteins. In the body it turns into glutamate. This is a chemical that helps nerve cells in the brain send and receive information from other cells. It may be involved in learning and memory. It may help people with t hypochlorhydria (low stomach acid) or achlorhydria (no stomach acid).

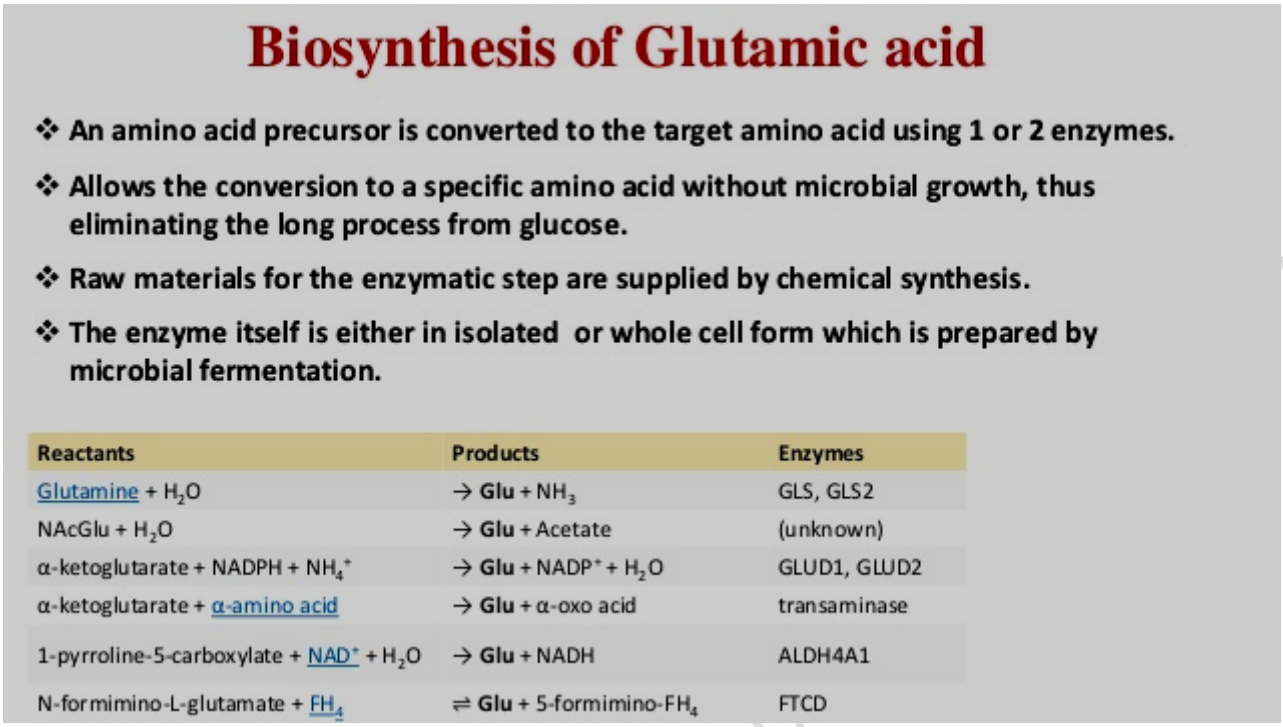
- Glutamic acid is an α-amino acid that used in¬ biosynthesis of proteins.
- It contains an α-amino group which is in the¬ protonated −NH3+.
- An α-carboxylic acid group which is in the¬ deprotonated −COO. And a side chain carboxylic acid.
- Polar negatively charged (at physiological pH), aliphatic¬ amino acid.
- It is non-essential in humans, meaning the body can¬ synthesize it.



glutamic acid

Uses of Glutamic acid:

- Food Production:
  - As flavor enhancer, to improve flavor.
  - As nutritional supplement.
- Beverage: As flavor enhancer:
  - in soft drink and wine
- Cosmeticsθ As Hair restorer:
  - in treatment of Hair Loss.
  - As Wrinkle: in preventing aging.
- Agriculture/Animal Feed:
  - As nutritional supplement: in feed additive to enhance nutrition.
- Other Industries:
  - As intermediate: in manufacturing of various organic chemicals.



Methods of Production of Glutamic acid:

- The manufacturing process of glutamic acid by fermentation comprises: -
  - **a. fermentation,**
  - **b. crude isolation,**
  - **c. purification processes.**
- There are 3 types of fermentation are used:
  - **(1) Batch Fermentation.**
  - **(2) Fed-batch Fermentation.**
  - **(3) Continuous Fermentation.**

1) Batch Fermentation:

- Widely use in the production of most of amino acids.
- Fermentation is a closed culture system which contains anϖ initial, limited amount of nutrient.
- A short adaptation time is usually necessary (lag phase) before cells enter the logarithmic growth phase (exponential phase).
- Nutrients soon become limited and they enter the stationary phase in which growth has (almost) ceased.
- In glutamic acid fermentations, production of the acid normally starts in the early logarithmic phase and continues through the stationary phase.

- For economical reasons the fermentation time should be as short as possible with a high yield of the amino acid at the end.
- A second reason not to continue the fermentation in the late stationary phase is the appearance of contaminant- products.
- The lag phase can be shortened by using a higher concentration of seed inoculum.
- The seed is produced by growing the production strain in flasks and smaller fermenters.

(2) Fed-batch fermentation:

- Batch fermentations which are fed continuously, or intermittently, with medium without the removal of fluid.
- In this way the volume of the culture increases with time.
- The residual substrate concentration may be maintained at a very low level.
- This may result in a removal of catabolite repressive effects and avoidance of toxic effects of medium components.
- Oxygen balance.
- The feed rate of the carbon source (mostly glucose) can be used to regulate cell growth rate and oxygen limitation, especially when oxygen demand is high in the exponential growth phase.

(3) Continuous fermentation:

- In continuous fermentation, an open system is set up.
- Sterile nutrient solution is added to the bioreactor continuously.
- And an equivalent amount of converted nutrient solution with microorganisms is simultaneously removed from the system.

Process of Production:

Natural product such as sugar cane is used.

⬇

Then, the sugar cane is squeezed to make molasses.

⬇

The heat sterilizes raw material and other nutrients are put in the tank of the fermenter.

⬇

The microorganism (*Corynebacterium glutamicum*) producing glutamic acid is added to the fermentation broth.

⬇

The microorganism reacts with sugar to produce glutamic acid.

⬇

Then, the fermentation broth is acidified and the glutamic acid is crystallized.

- **Separation and purification**: After the fermentation process, specific method is require to separate and purify the amino acid produced from its contaminant products, which include:
    - ✓ Centrifugation.
    - ✓ Filtration.
    - ✓ Crystallisation.
    - ✓ Ion exchange.
    - ✓ Electrodialysis.
    - ✓ Solvent extraction.
    - ✓ Decolorisation.
    - ✓ Evaporation.
- The glutamic acid crystal is added to the sodium hydroxide solution and converted into monosodium glutamate (MSG).
- MSG is more soluble in water, less likely absorb moisture and has strong umami taste.
- The MSG is cleaned by using active carbon, which has many micro holes on their surface.
- The clean MSG solution is concentrated by heating and the monosodium glutamate crystal is formed.
- The crystal produce are dried with a hot air in a closed system.
- Then, the crystal is packed in the packaging and ready to be sold.

Lipase Enzyme:

A **lipase** is any **enzyme** that catalyzes the hydrolysis of fats (lipids). **Lipases** are a subclass of the esterases. **Lipases** perform essential roles in digestion, transport and processing of dietary lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms.

- ❖ Lipases are also called as Glycerol ester hydrolases
- ❖ They are a subclass of esterases
- ❖ It splits fats into mono or di- glycerides and fatty acids.
- ❖ They are extracellular enzymes
- ❖ Mainly produced by Fungi Eg: *Aspergillus, Mucor, Rhizopus, Peniciilum* etc
- ❖ Bacteria producing lipases include species of *Pseudomonas, Achromobacter* and *Staphylococcus*.
- ❖ Yeasts like *Torulopsis* and *Candida* are also commercially used.

Applications

- ❖ Primarily marketed for therapeutic purposes as digestive enzymes to supplement pancreatic lipases.
- ❖ Since free fatty acids affect the odor and taste of cheese, and the cheese ripening process is affected by lipases, microbial affects during the aging process can be due to lipase action.
- ❖ In the soap industry, lipases from *Candida cylindraceae* are used to hydrolyze oils.

Methods of Enzyme Production:

1. Semisolid Culture
2. Submerged Culture

1. Semisolid Culture:

The enzyme producing culture is grown on the surface of a suitable semi-solid substrate (Moistened Wheat or Rice Bran with nutrients)

⬇

Preparation of Production Medium – Bran is mixed with solution containing nutrient salts.

⬇

pH is maintained at a neutral level. Medium is steam sterilized in an autoclave while stirring.

⬇

The sterilized medium is spread on metal trays upto a depth of 1-10 centimeters.

⬇

Culture is inoculated either in the autoclave after cooling or in trays.

⬇

High enzyme concentration in a crude fermented material.

Advantages of Semi-solid culture:

- It involves comparatively low investment
- Allows the use of substrate with high dry matter content. Hence it yields a high enzyme concentration in the crude fermented material.
- To cultivate those moulds which cannot grow in the fermenters due to wall growth.
- Allows the moulds to develop into their natural state

Disadvantages of Semi-solid culture:

- Requires more space and more labour
- Involves greater risk of infection
- Difficult to introduce automation in such systems

2. **Submerged Culture**

Fermentation equipment used is the same as in the manufacture of antibiotics.

It's a cylindrical tank of stainless steel and it is equipped with an agitator, an aerating device, a cooling system and various ancillary equipment (Foam control, pH monitoring device, temperature, oxygen tension etc).

Good growth is not enough to obtain a higher enzyme yield.

Presence of inhibitors or inducers should also be checked in the medium.

Example – Presence of Lactose induces the production of β- galactosidase.

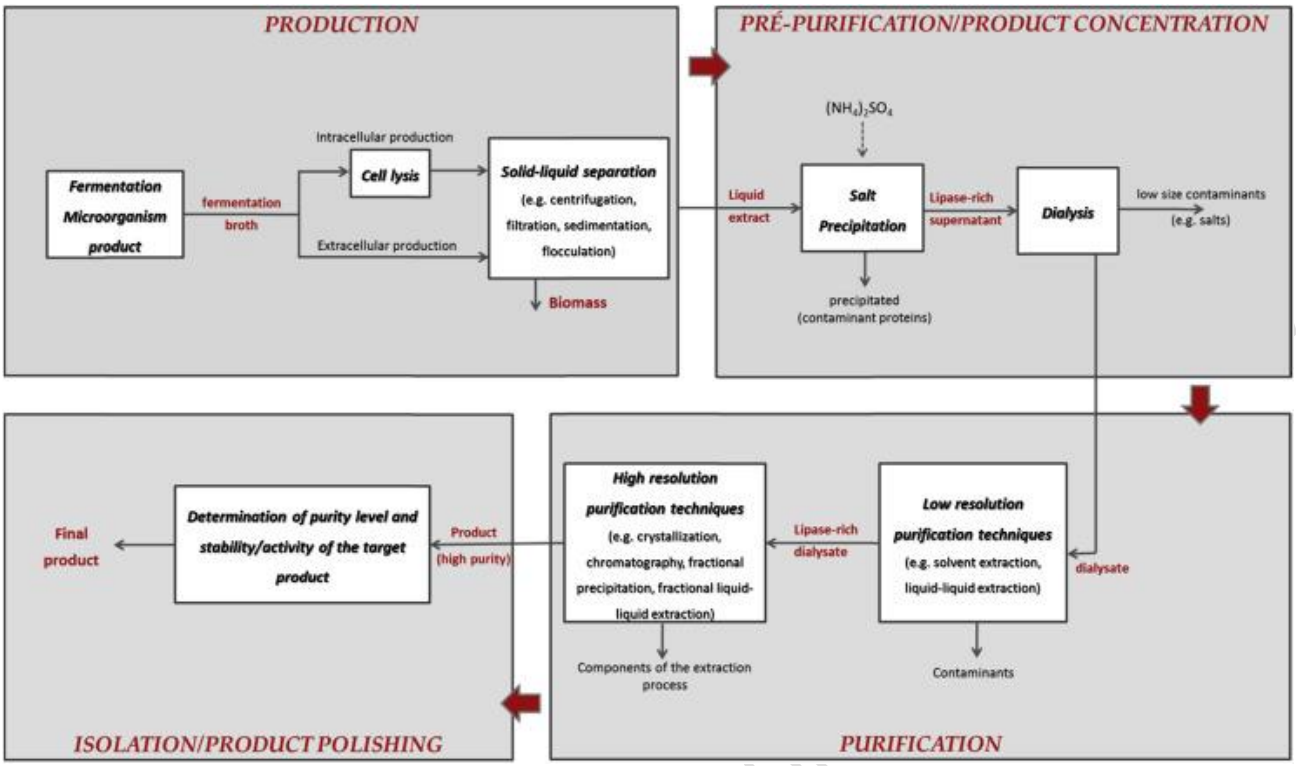
As the inducers are expensive, constitutive mutants are used which do not require an inducer.

Glucose represses the formation of some enzymes (α-amylases).

Thus the glucose concentration is kept low.

Either the glucose can be supplied in an incremental manner or a slow metabolizable sugar (Lactose or metabolized starch) Certain surfactants in the production medium increases the yield of certain enzymes.

Non- ionic detergents (eg. Tween 80, Triton) are frequently used.



Advantages of Submerged culture:

- Requires less labor and space
- Low risk of infection
- Automation is easier

Disadvantage of Submerged Culture:

- Initial investment cost is very high.

Riboflavin (Vitamin B2):

Riboflavin, also known as vitamin B_2, is a vitamin found in food and used as a dietary supplement. Food sources include eggs, green vegetables, milk and other dairy product, meat, mushrooms, and almonds. Some countries require its addition to grains

Only a limited number of **bacteria** are known to **produce vitamin B12**, three of which—Pseudomonas denitrificans, Bacillus megaterium, and Propionibacterium freudenreichii—are **used** for commercial **production**

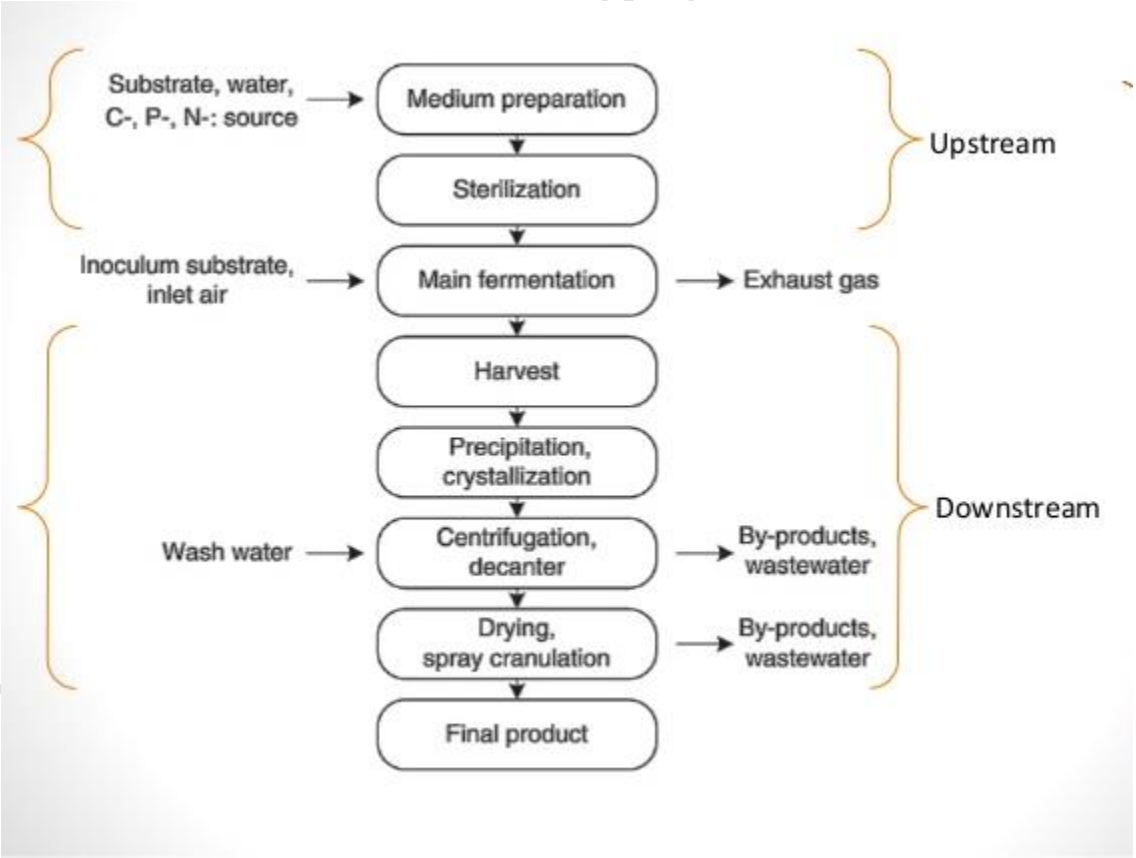
Uses of Riboflavin:

- **Vitamin B2** helps break down proteins, fats, and carbohydrates.
- It plays a vital role in maintaining the body's energy supply.

- Riboflavin helps convert carbohydrates into adenosine triphosphate (ATP).
- The human body produces ATP from food, and ATP produces energy as the body requires it.

Production Process:

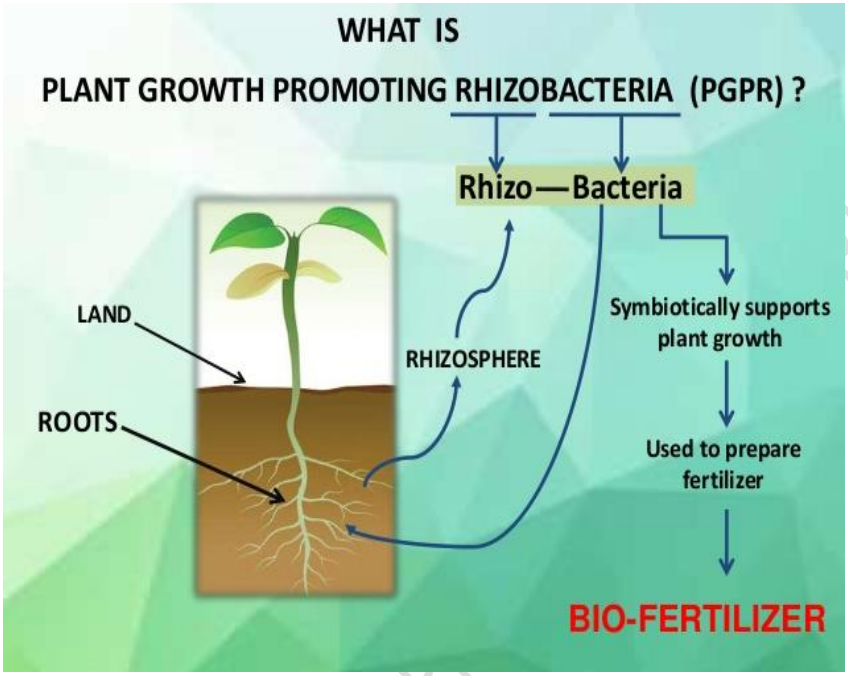
- In this case study, a batch process using *E. Ashbyii* with a capacity of around 1000 tons/year is analyzed.
- Upstream processing consists of preparation of medium and associated continuous counter-current sterilization.
- Feed components are: 70% glucose syrup, yeast and malt extract, sunflower oil, sulfuric acid, and concentrated salt solution at room temperature.
- Fermentation is operated batch-wise with 10% inoculum ratios.
- Downstream processing starts with harvesting followed by crystallization, centrifugation (decanter), and final drying (spray dryer)
- The requested purity of riboflavin is 70%. The residual 30% consists of salts and biomass.
- The product is obtained as dry powder or as granulate.



UNIT III: Production of PGPR, biofertilizers and biocontrol agents:

PGPR:

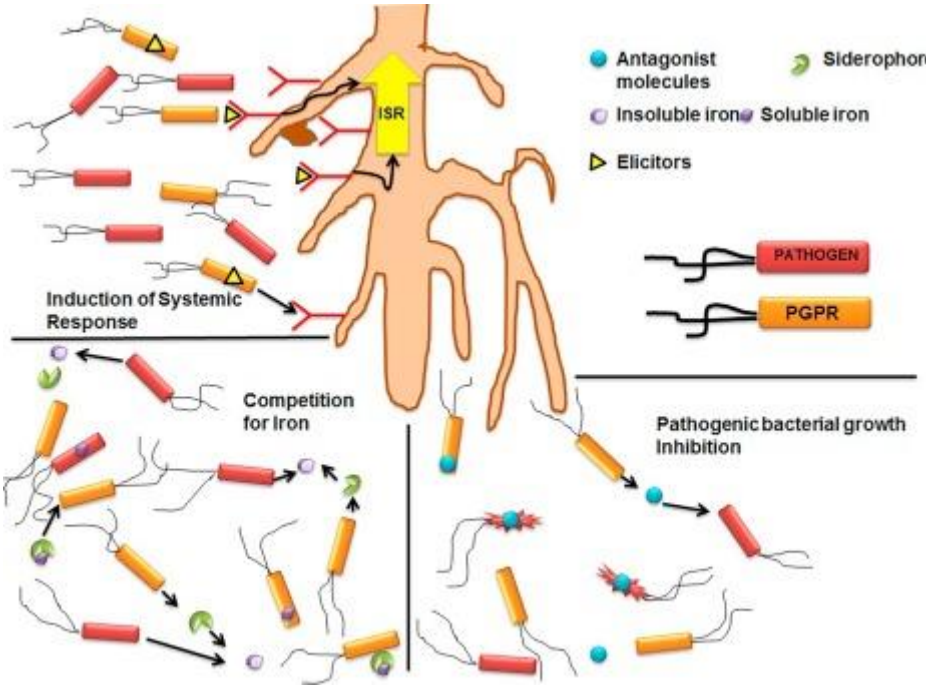
Plant growth-promoting rhizobacteria (**PGPR**) are a heterogeneous group of beneficial root-associated bacteria that improve plant health and/or growth by diverse mechanisms.



Mechanisms of action

- PGPRs enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well characterized.
- Direct mechanisms of plant growth promotion by PGPRs can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPRs to reduce the harmful effects of plant pathogens on crop yield.
- PGPRs have been reported to directly enhance plant growth by a variety of mechanisms:
  - fixation of atmospheric nitrogen transferred to the plant,
  - production of siderophores that chelate iron and make it available to the plant root,
  - solubilization of minerals such as phosphorus, and
  - synthesis of phytohormones.
- Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPRs has also been reported.
- PGPR strains may use one or more of these mechanisms in the rhizosphere.

- Molecular approaches using microbial and plant mutants altered in their ability to synthesize or respond to specific phytohormones have increased understanding of the role of phytohormone synthesis as a direct mechanism of plant growth enhancement by PGPRs.
- PGPR that synthesize auxins and cytokinins or that interfere with plant ethylene synthesis have been identified.

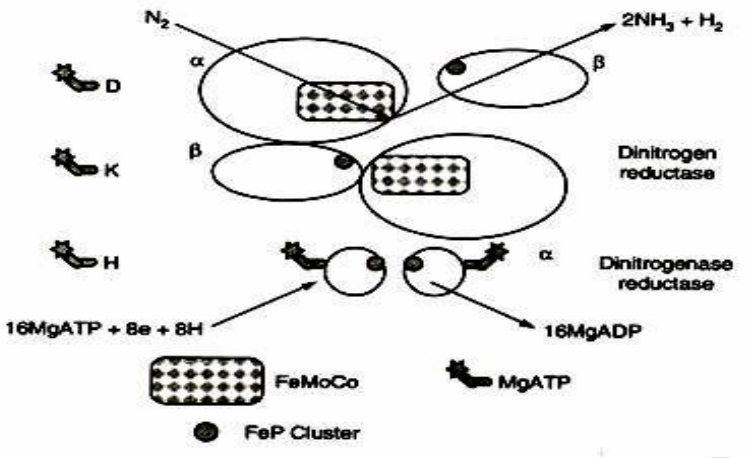


Genetic engineering of nitrogenase gene cluster (Nif):

Structure and Operation of Nitrogenase:

Active nitrogenase can be reconstituted by the addition of purified Mo-Fe and Fe proteins of different microorganisms. For examples, proteins of *Klebsiella pneumoniae* and *Bacillus polymyxa* and those of blue-green algae and photosynthetic bacteria have been combined to reconstitute active nitrogenases, capable of reducing acetylene to ethylene.

During catalysis by nitrogenase, protons and nitrogen compete for electrons. Therefore, in an atmosphere containing nitrogen, hydrogen evolution occurs simultaneously with ammonia formation. This evolution of hydrogen diverts 25-35% of the total reductants available for the nitrogenase reaction, which is regarded as an intracellular wastage of energy in the overall process of nitrogen fixation, and the reaction can be summarized



Nitrogenase contains the two proteins molybdoferredoxin and azoferredoxin. This MoFe cofactor is unique to nitrogen fixation and distinct from the Mo-pterin cofactor of other Mo proteins (e.g., nitrate reductase, xanthine oxidase).

Organization of Nif Genes:

- Nitrogen fixation is carried out by **three groups of genes**.
- These are;
    - Nod gene (responsible for nodule formation),
    - Nif gene (responsible for nitrogen fixation) and
    - Hup gene (responsible for nitrogen uptake).
- All these three types of genes are present in a group on a single chromosome. This makes their copying and transfer mechanism simple for genetic engineering purposes.
- Though the mechanism of nodule formation is complex nod gene is responsible for nodule formation as well as host recognization and specificity. However, a few genes located on plasmids can produce nodules. Plasmid of *R. leguminosarum* is less than 10 kb even then it has property to recognize host and nodule formation.

Nod gene:

Most of the biological nitrogen fixing bacteria contains a large plasmid called mega-plasmid. In several functions it is similar to Ti plasmid and contains genes responsible for auxin and cytokinin production. Excess production of these plant growth regulators helps in nodule formation. According to Rosenberg (1981) several special genes are present along with nod genes. Such plasmids are absent in non-symbiotic bacteria.

A nod gene is a group of genes containing Nod A, B, C, D genes having 8.5 kb length. These genes form polypeptides of different lengths (196, 197, 402, 211 amino acid). Nod genes of different rhizobium species have almost 70% homologies which are called common Nod genes.

Nif genes:

This gene is responsible for nitrogen fixation and present in the genome of symbiotic and non symbiotic nitrogen fixing bacteria. In symbiotic bacteria Rhizobium, it is present near nod genes on the megaplasmid, while in non-symbiotic cyanobacteria it is present on the main DNA. Initially Nif gene has been transferred in *E. coli.*

In higher plants, chloroplast is a cell organelle which might have been originated from prokaryotes, therefore attempt are made to transfer Nif gene into chloroplast. Easy availability of ATP and $NADPH_2$ in chloroplast also makes them ideal recipient for this gene transfer.

Most of the cereal plants are monocots and any such effort to transfer such Nif gene will revolutionize the yield, economics and environmental pollution. However, there are many difficulties in transferring, integration and expression of a prokaryotic gene into a monocot.

Hup gene:

Gene responsible for nitrogen uptake is Hup gene. In symbiotic bacteria this gene recycles the hydrogen produced during nitrogen fixation as shown in the Figure. Hydrogen produced at different steps is assimilated in the reduction of nitrogen. In most of the legumes 30-50% energy (in the form of ATP) is spent on hydrogen liberation. This results in loss in capacity of nitrogen fixation. If this hydrogen can be recycled by nitrogenase enzyme we can save a lot of energy, and this can be carried out by improved Hup gene.

Klebsiella pneumoniae strain M5 a1 (Enterobacteriaceae) is a free living bacteria which has been studied extensively for genetics of nitrogen fixation. This bacterial genome is quite similar to that of *E. coli* and ***Salmonella typhimurium***. Therefore most of the techniques of genetic engineering can be applied to *Klebsiella.*

Nif Gene Organization in Klebsiella:

Several mutants of *Klebsiella* were developed by growing the bacteria on medium containing a mutagen, methyl-nitro nitro-so-guanidine. Different mutants obtained were used in transformation, transduction to map the Nif gene (Table). This provided the information that Nif gene is downstream to histidine operator (Fig.). On the basis of this Nif gene of *Klebsiella* was transferred in E. coli on the basis of homology in the plasmid and not in genomic DNA. Nif gene organization of *Klebsiella* is similar to that of *Azotobacter, Asospirillum, Clostridium* and prokaryotic blue-green algae.

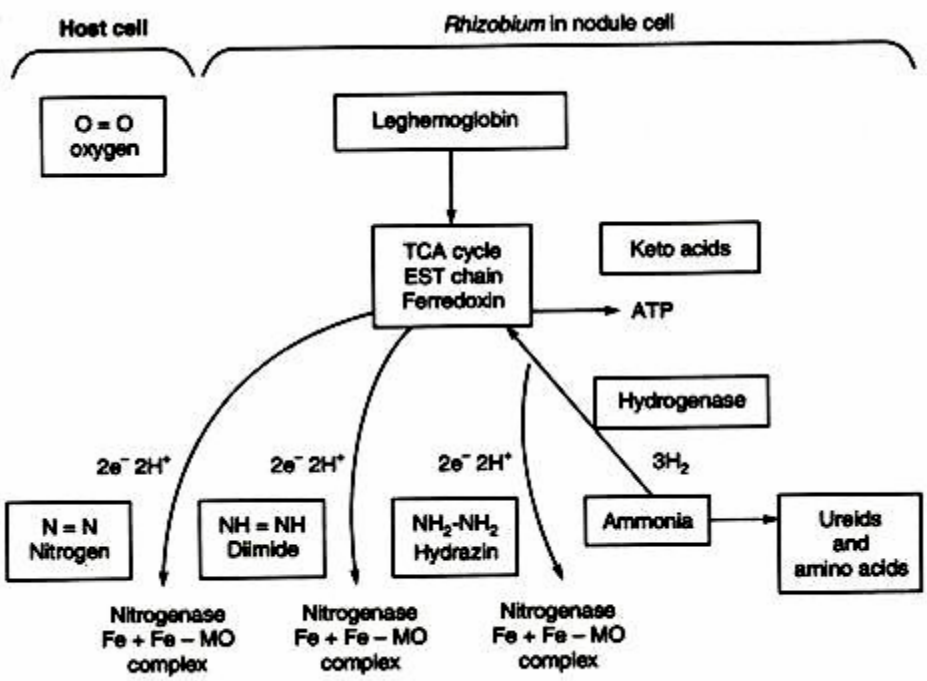


Fig. Hydrogen evolution and utilization.

Table .. Description and product of *Nif* genes of *Klebsiella*.

Nif H	Structural gene 35kd protein, a subunit of nitrogenase reductase.
Nif D	Structural gene 56kd, β subunit of nitrogenase.
Nif K	Structural gene 60kd, a subunit of nitrogenase, helps in transcription of Nif H-D and -K.
Nif E and N	46 and 50 kd protein formation; helps in formation of Fe-Mo co-factor and protein for Nif B.
Nif M and S	Formation of 18 kd protein which activates nitrogenase reductase.
Nif F	17 kd protein, function of electron transport factor.
Nif Q and V	Function unknown.
Nif A and L	Important role in expression of other *Nif* genes, product unknown.
Nif J	120 kd structural gene, important for activity of nitrogenase.

Regulation of Nitrogen Fixation:

All the nif genes in Klebsiella are clustered and coordinately regulated (Fig. 11.5). E. coli to which the nif genes of Klebsiella have been transferred can fix N_2. In both the original Klebsiella and the E. coli nitrogenase is expressed only in the absence of both O_2 and NH_3 in the growth medium. The nif genes are regulated by the nifLA operon. The nitrogen regulators NtrC (= GlnG), and NtrB determine whether or not the nifLA operon is expressed (depending on the presence of ammonia or organic nitrogen).

In the absence of ammonia or organic nitrogen the NtrC protein is phosphorylated by the NtrB protein. NtrC-P then binds to the upstream region of the nifLA operon and activates transcription. NtrA (= GlnF = RpoN = σ54) is the nitrogen sigma factor, which is needed for expression of the nifLA operon and the nif structural genes. NtrA is an alternative sigma factor used by RNA polymerase to recognize many genes involved in nitrogen metabolism which are not recognized by the standard sigma factor.

The nif A gene encodes a protein required for switching on all of the nif genes except the regulatory genes nifLA themselves. If nif A protein is made, its function is to activate the other nif genes. The nifL gene is required for O_2 repression. In the absence of NifL protein, nitrogenase is made in the presence of O_2 (but is inactivated by O_2). When oxygen is present, the nifL protein binds to nif A and prevents it from activating the other nif genes.

The greater study of the infectious process and the genes involved and the physiological aspects will lead to better nitrogen use by plants grown on nitrogen-poor soils in agriculture and can reduce the requirement for chemical fertilizers.

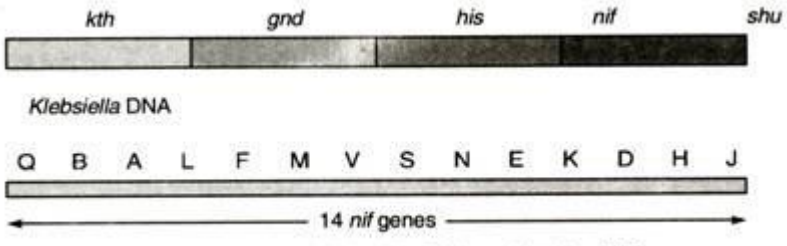


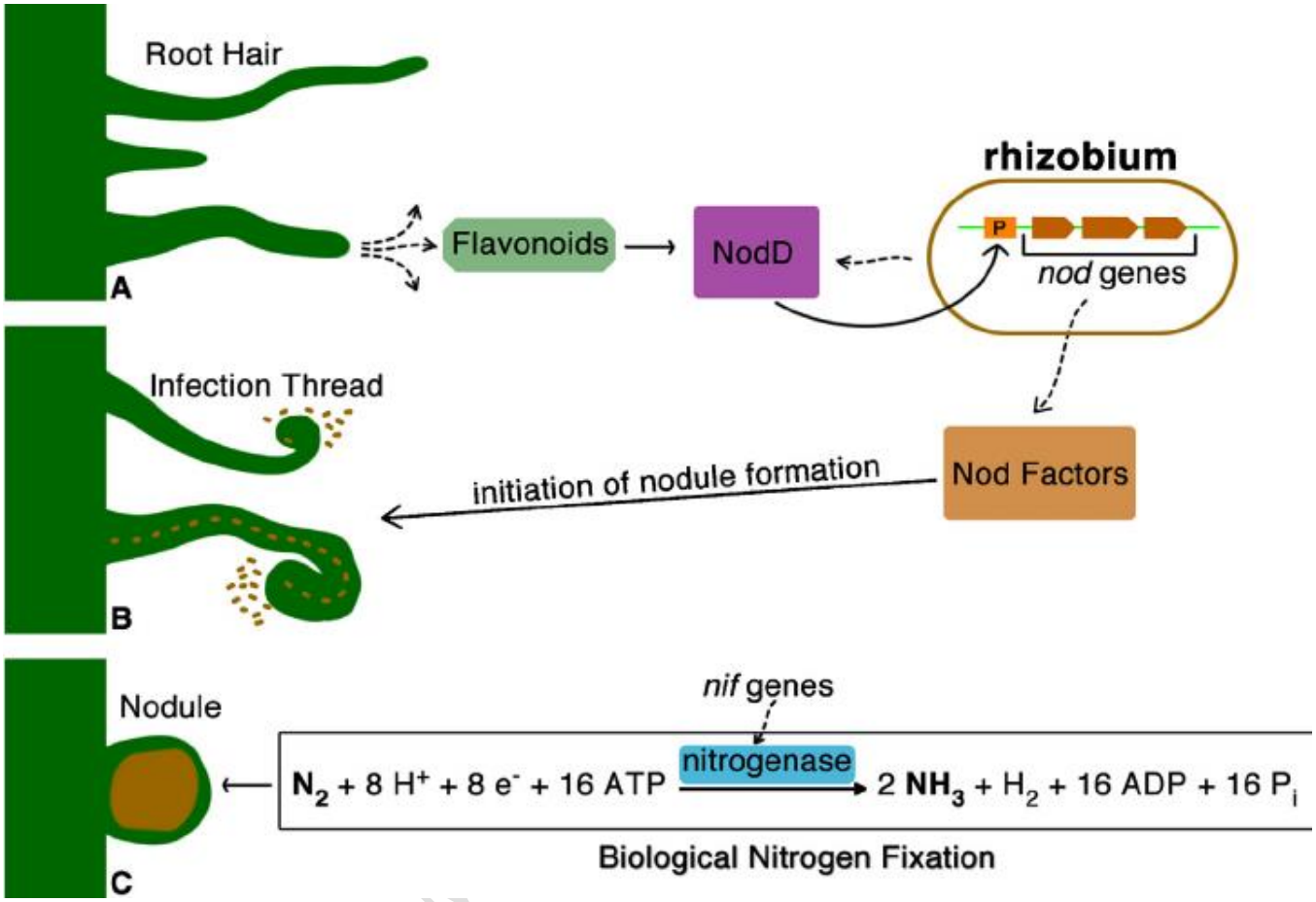
Fig. Nif genes on *Klebsiella* DNA and details of Nif genes.

Hydrogenase:

Hydrogenase is an enzyme those catalyses the reversible oxidation of molecular hydrogen, as shown below: Hydrogen uptake is coupled to the reduction of electron acceptors such as oxygen, nitrate, sulfate, carbon dioxide, and fumarate.

Nodulation:

Nodulation is a symbiotic interaction between soil bacteria and plant hosts, most notably between rhizobia and legumes. This interaction is important for plant hosts, since it enables them to access atmospheric nitrogen made available by the bacteria.



BIOFERTILIZERS – DEFINITION:

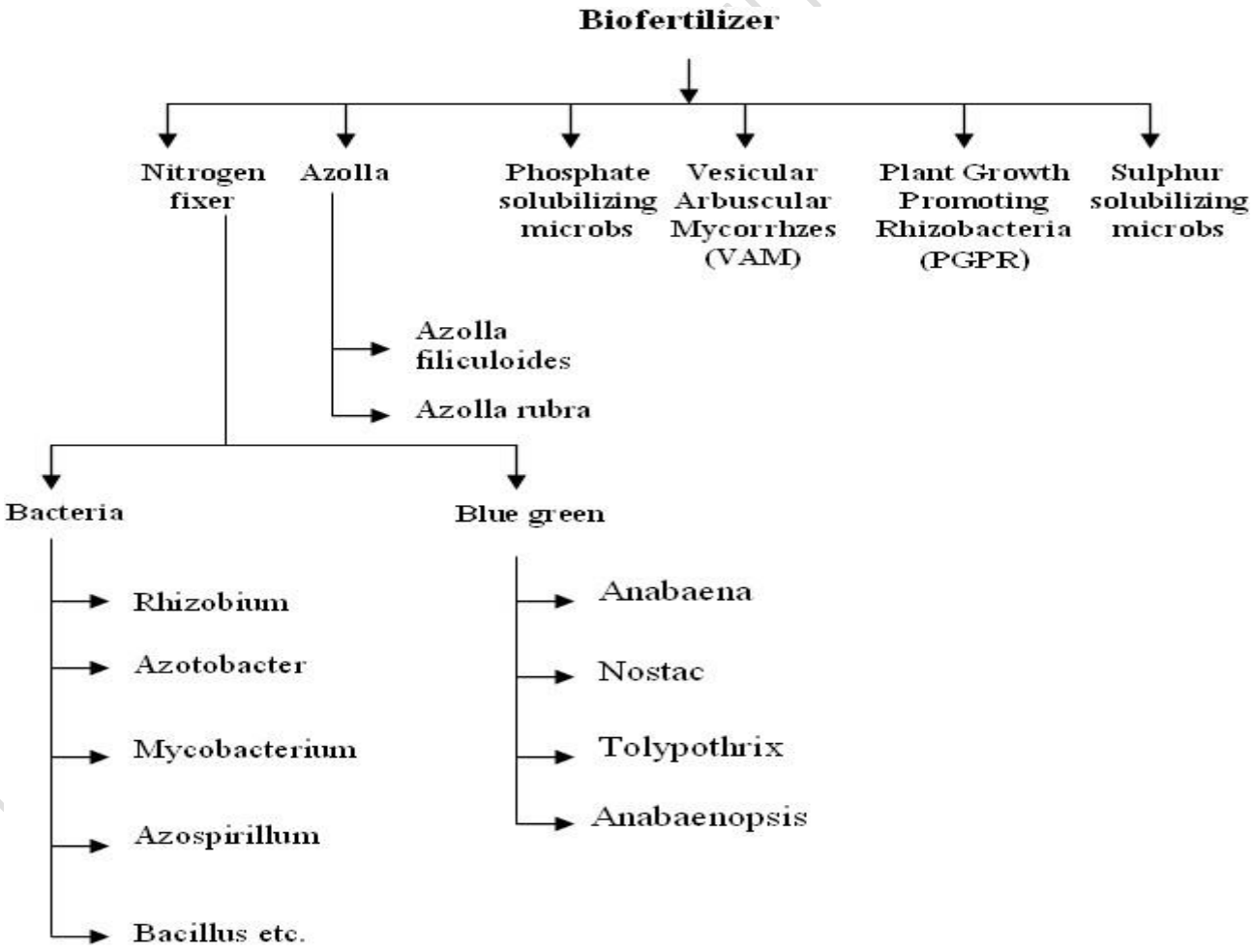
Biofertilizer is a Natural organic fertilizer known that helps to provide all the nutrients required by the plants and helps to increase the quality of the soil with a natural microorganism environment.

- The use of chemical fertilizers and pesticides has caused tremendous harm to the soil, food & fiber products and environment
- Biofertilizers, an environmentally friendly fertilizer now used in most advanced countries
- Biofertilizers are organisms that enrich the nutrient quality of soil
- The main sources of Biofertilizers are bacteria, fungi, and cynobacteria
- The most striking relationship that these have with plants is symbiosis, in which the partners derive benefits from each other.

ADVANTAGES OF BIO-FERTILIZERS

- Cost effective.
- Supplement to fertilizers.
- Eco-friendly (Friendly with nature).
- Reduces the costs towards fertilizers use, especially regarding nitrogen and phosphorus
- Bio-fertilizers improve soil texture and yield of plants
- They do not allow pathogens to flourish
- They do not cause any harm to the environment.

TYPES OF BIOFERTILIZERS:



Nitrogen Biofertilizers:-

- ✈ This type of biofertilizer helps the soil to correct its nitrogen level
- ✈ Nitrogen is an essential component for plant growth but plants need it in a limited amount
- ✈ Different soils have different requirement for nitrogen that is why it depends on the cultivated crops that which type of nitrogen biofertilizer should be used.
- ✈ Azotobacter is a biofertilizer which provides the required amount of nitrogen to the plant from the soil.

Phosphorus Biofertlizers:-

- ❖ Phosphorus is also a limiting factor and plants need it in particular amount
- ❖ Phosphorus biofertlizers help the soil to correct the phosphorus level
- ❖ As nitrogen biofertilizers depend on the cultivating crops, phosphorus biofertlizers do not depend on the cultivating crops
- ❖ Rhizobium is the phosphorus biofertilizer.

Compost Biofertlizers:-

- ➢ Compost biofertilizers are animals' wastes which are degraded by the bacteria and used as the best naturally occurring biofertlizers
- ➢ They not only protect the plants from diseases nut also help them to grow in a healthy environment

Azotobacter Biofertlizers:-

- ✓ Azotobacter is a bacterium that lives freely in the environment and has the ability to fix the atmospheric nitrogen into soil
- ✓ It is used in the cultivation of many important crops because it is the important source of providing nitrogen to the plants
- ✓ It enables the plant to germinate and grow without being effected from any harmful microbes.

Phosphate Solubilizers:-

- ☆ Phosphate Solubilizers have the ability to dissolve the fixed phosphate and convert it in the form which can be utilized by the plants

- ☆ They produce enzymes, hormones and organic acids
- ☆ These components make possible the solubilization of insoluble phosphate so that it can easily be used by the plants

Cyanobacteria

INTRODUCTION:

- ❖ Cyanobacteria are photosynthetic bacteria formerly known as *blue-green algae*
- ❖ Most are found in the soil and in freshwater and saltwater environments
- ❖ The majority of species are unicellular, but some may remain linked and form filaments
- ❖ Cyanobacteria, which are autotrophic, serve as important fixers of nitrogen in food chains
- ❖ In addition, cyanobacteria, a key component of the plankton found in the oceans and seas, produce a major share of the oxygen present in the atmosphere, while also serving as food for fish
- ❖ Some species of cyanobacteria coexist with fungi to form **lichens**

Type of toxins

- ❖ Cyanobacteria can produce several toxins, but two types of toxins are of particular concern and are tested for: Microcystins and Anatoxin-A.
- ❖ Microcystins are a group of hepatoxins (toxins that affect the liver). Microcystins are very stable and do not break down quickly in water.
- ❖ Anatoxin A is a potent neurotoxin (toxins that affect the nervous system) which can cause lethargy, muscle aches, confusion, memory impairment, and, at sufficiently high concentrations, death.

EXAMPLES:

Cyanobacteria are those bacteria that obtain their energy through photosynthesis just like plants. Some examples are *Nostoc, Oscillatoria, Gloeocapsa*.

ISOLATION:

Culture studies The fresh field samples were subjected to enrichment cultures in culture tubes and agar petriplates in sterile medium. The visible cyanobacterial masses were collected and washed in sterile water. A small quantity of the cyanobacterial aggregate was put in 10 m1 of sterile culture

medium and used as initial inoculum. The isolation was carryout by the streak and spread plate methods. Subculturing was done repeatedly, whereby pure cultures of some species (unicyanobacterial isolates) were obtained. The cultures were periodically examined under research microscope for assessing their pure nature. Liquid cultures were maintained in conical flasks and solid cultures in agar plates / slants.

Algalization:

It was Japanese workers (Watanabe and coworkers) who developed techniques for mass cultivation of blue-green algae to be used as biofertilizer in paddy fields. Venkataraman (1961) coined the term *'algalization'* to denote the process of application of blue-green algal culture in field as biofertilizer. He initiated algalization technology in India and demonstrated the way how this technology could be transferred to farmer level who hold small lands

The main objectives of the programme were

- *(i)* to develop low cost indigenous technology for mass production of cyano bacteria,
- *(ii)* to isolate regional specific fast growing and better N_2 - fixing strains,
- *(iii)* to develop starter inoculum,
- *(iv)* to demonstrate the farmers in field, and
- *(v)* to study the benefits on both economy and ecology. As a result of these studies cyanobacterial biofertilizer was found very useful, especially for small and marginal farmers of the country with the view point of both economy and ecology.

The Blue-Green Algae (Cyanobacteria):

Role of the blue-green algae *(e.g. Aulosira, Anabaena, Cylindrospermum, Nostoc, Plectonema, Tolypothrix)* in the paddy fields was realized much earlier. In water-logging condition, the cyanobacteria multiply, fix atmospheric N_2 and release it into the surroundings in the form of amino acids, proteins and other growth promoting substances. Recent works done at Central Rice Research Institute, (Cuttack), Indian Council of Agricultural Research (New Delhi) and other centres, *e.g.* Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi are of much importance.

Contribution of nitrogen by some of the nodulated legumes when used as green manure/cover crop.

	N_2 fixing system	Amount of N contributed (q/ha)
1.	*Green manure legumes :*	
	Sesbania aculeata – Rhizobium	70-120
	Leucaena leucocephala - Rhizobium	500-600
	Beans (broad beans / lupines /soybean / lentil, etc.) – *Rhizobium*	60-210
	Fodders *(Trifolium / Medicagol Melilotus,* etc.) - *Rhizobium*	100-300
2.	*Cover crop legumes :*	
	Lablab purpureus – Rhizobium	240
	Glycine jawanica – Rhizobium	210
3.	*Non-legumes :*	
	Casuarina equisitifolia - Frankia	100
	Alnus – Frankia	30-300
4.	*Others :*	
	Azolla - Anabaena	25-190
	Grasses - *Azospirillium*	15-100

Mass cultivation of cyanobacterial biofertilizers:

- For outdoor mass cultivation of cyanobacterial biofertilizers, the regional specific strains should be used.
- Mixture of 5 or 6 regional acclimatized strains of cyanobacteria, *e.g.* species of *Anabaena*, *Aulosira*, *Cylindrospermum*, *Gloeotrichia*, *Nostoc*, *Plectonema*, *Tolypothrix* are generally used for starter inoculum.
- The following four methods are used for mass cultivation :
  - *(i)* cemented tank method.,
  - *(ii)* shallow metal troughs method,
  - *(iii)* polythene lined pit method, and
  - *(iv)* field method. The polythene lined pit method is most suitable for small and marginal

farmers to prepared algal biofertilizer. In this method, small pits are prepared in field and lined with thick polythene sheets. Mass cultivation of cyanobacteria is done by using any of the four methods under the following steps:

(i) Prepare the cemented tanks, shallow trays of iron sheets or polythene lined pits in an open area. Width of tanks or pits should not be more than 1.5 m. This will facilitate the proper handling of culture.

(ii) Transfer 2 -3 Kg soil (collected from open place for lm ^{2} area of the tank) and add 100 g of superphosphate. Water the pit to about 10 cm height. Mix lime to adjust the pH 7. Add 2 ml of insecticide *e.g.* malathion to protect the culture from mosquitoes. Mix well and allow to settle down soil particles.

(iii) When water becomes clear, sprinkle 100 g of starter inoculum on the surface of water.

(iv) When temperature remains between 35-40° during summer, optimum growth of cyanobacteria is achieved. Always maintain the water level to about 10 cm during this period,

(v) After drying, the algal mat will get separated from the soil and forms flakes. During summer about 1 kg pure algal mat per m^2 area is produced. These are collected, powdered, kept in sealed polythene bags and supplied to the farmers.

(vi) The algal flakes can be used as starter inoculum if the same process is repeated.

Field Applications:

Seed Treatment	Prepare a solution of 125 gm. aof jaggery (gurh) in one litre of water and mix one packet (200gm.) Phosphate Solubalizing Bacteria to form a slurry. Sprinkle this slurry over the seeds required for one acre of crop, mix thoroughly and shade dry the seeds before sowing
Seedling treatment	Prepare a thick mixture of 2 pockets (400 gm) of Phosphate Solubalizing Bacteria in 20-25 litre of water in big flat container. Dip the roots of seedlings in it for 1/2 hr and then transport them in field.
Soil Treatment	Mix 5 packets (1000gm) of culture powder in 50 kg. of F.Y.M./Vermicompost/Nadep compost/Soil spread in one acre field at the time of last ploughing before sowing or with the first irrigation.

Azolla:

Azolla is an aquatic heterosporous fern which contains an endophytic cyanobacterium, *Anabaena azollae*, in its leaf cavity. The significance of *Azolla* as biofertilizer in rice field was realized in Vietnam. Recently, it has become very popular in China, Indonesia, Philippines, India and Bangladesh

A total of six species of *Azolla* are known so far *viz*., *A. caroliniana, A. filiculoides, A. mexicana, A. microphylla, A. nilotica, A. pinnata* and *A. rubra.* Out of these *A. pinnate* is commonly found in India. The global collections of several species of *Azolla* are maintained at CRRI (Cuttack). Within the leaf cavity filaments of *Anabaena azollae* are present. Dr. P.K. Singh, at CRRI has done an outstanding work on mass cultivation of *Azolla* and its use as biofertilizer in rice and other crop fields.

Mass* cultivation of *Azolla

Microplots ($20m^2$) are prepared in nurseries in which sufficient water (5-10 cm) is added. For good growth *of Azolla*, 4-20 Kg P_2O_5/ha is also amended. Optimum pH (8.0) and temperature (14-30°C) should be maintained. Finally, microplots are inoculated with fresh *Azolla* (0.5 to 0.4 Kg/ m^2). An insecticide (furadon) is used to check the attack of insects. After three, week of growth mat formed by *Azolla* is harvested and the same microplot is inoculated with fresh *Azolla* to repeat the cultivation.

Azolla mat is harvested and dried to use as green manure. There are two methods for its application in field: (a) incorporation of *Azolla* in soil prior to rice plantation, and *(b)* transplantation of rice followed by water draining and incorporation of *Azolla*. However, reports from the IRRI (Philippines) reveal that growing of *Azolla* in rice field before rice transplantation increased the yield equivalent to that obtained from 30Kg N/ha as urea or ammonium phosphate.

Moreover, *Azolla* shows tolerance against heavy metals viz. As, Hg, Pb, Cu, Cd, Cr, etc. It tolerates low concentration but at high levels a setback in biochemical pathways is caused. *A. pinnata* absorbs heavy metals into cell walls and vacuoles through evolution of specific metal resistant enzymes. Therefore, heavy metal resistant species such as *A. pinnata* can also be incorporated as green manure in rice field near the polluted areas where heavy metal concentration is between 0.01 and 1.5 mg/liter. Due to development of chemical industries and discharge of effluents into water bodies, heavy metal concentration is gradually increasing day by day. Industries where work of electroplanting, fertilizers, tanning etc. are done, they act as a chief source for soil and water pollution. For example, disturbed vegetation in aquatic system around Damodar river valley in India has received a great attention.

FIELD APPLICATION

Seed Treatment	Prepare a solution of 125 gm. aof jaggery (gurh) in one litre of water and mix one packet (200gm.) Phosphate Solubalizing Bacteria to form a slurry. Sprinkle this slurry over the seeds required for one acre of crop, mix thoroughly and shade dry the seeds before sowing
Seedling treatment	Prepare a thick mixture of 2 pockets (400 gm) of Phosphate Solubalizing Bacteria in 20-25 litre of water in big flat container. Dip the roots of seedlings in it for 1/2 hr and then transport them in field.
Soil Treatment	Mix 5 packets (1000gm) of culture powder in 50 kg. of F.Y.M./Vermicompost/Nadep compost/Soil spread in one acre field at the time of last ploughing before sowing or with the first irrigation.

Use of Azolla

- Azolla covering water surface **reduce light penetration of soil surface**, resulting in the depreciation in the germination of weeds (70% of the weed). Thus growth of azolla reduces aquatic weeds in flooded rice fields.
- The integrated use of azolla with rice and fish farming has been developed. The integrated approach can enhance a farmer's income while reduce the use of pesticide and fertilizers and consequently environmental pollution.
- **It can fix atomospheric nitrogen, carry out photosynthesis** and uptake nutrients from its surrounding environment through its root system.
- It has wide range of use including **fodder for dairy cattle, pigs, chicken, ducks and fish.**
- The application **of azolla as biofertilizer on agriculture crops,** in order to provide a natural source of crucial nutrients nitrogen, can be very beneficial for the future.
- Due to fact that rice paddy field from an ideal environment for azolla.
- **Improve the nutritional status of the soil.**
- **Azolla has been used as green manure.**
- Improve yields by 15-20 per cent.
- Azolla can be used **as an animal feed a human food, a medicine and water purifier.**

- It may also be used for the **production of hydrogen fuel** the **production of biogas the control of mosquitoes** and the reduction of ammonia volatilization which accompanies the application of chemical nitrogen fertilizer.

NITROGEN FIXERS:

Bacteria that change nitrogen gas from the atmosphere into solid nitrogen usable by plants are called nitrogen-fixing bacteria. These bacteria are found both in the soil and in symbiotic relationships with plants.

- ☆ **Nitrogen-fixing bacteria** are microorganisms present in the soil or in plant roots that change nitrogen gases from the atmosphere into solid nitrogen compounds that plants can use in the soil
- ☆ Examples: *Rhizobium sp. Frankia, Azolla*

***Rhizobium* - ISOLATION IDENTIFICATION CHARACTERIZATION AND APPLICATION**

Rhizobium:

- ❖ They are Gram-negative
- ❖ motile
- ❖ non-sporulating rods

ISOLATION OF *Rhizobium:*

Yeast Extract Mannitol Agar (YEMA) plates were prepared and sterilized by autoclaving

⬇

Legume plants (Groundnut) were carefully uprooted and washed under running water to remove the adhesive soil particles

⬇

Healthy unbroken pink nodules were selected

⬇

Surface Sterilization of root nodules by 0.1 % mercuric chloride and 3-5% hydrogen peroxides

⬇

The nodules were immersed in sterilizing agents for 4-5 minutes and then washed with sterile distilled water.

⬇

Then they are washed in 70% ethyl-alcohol followed by washing with sterile distilled water again

⬇

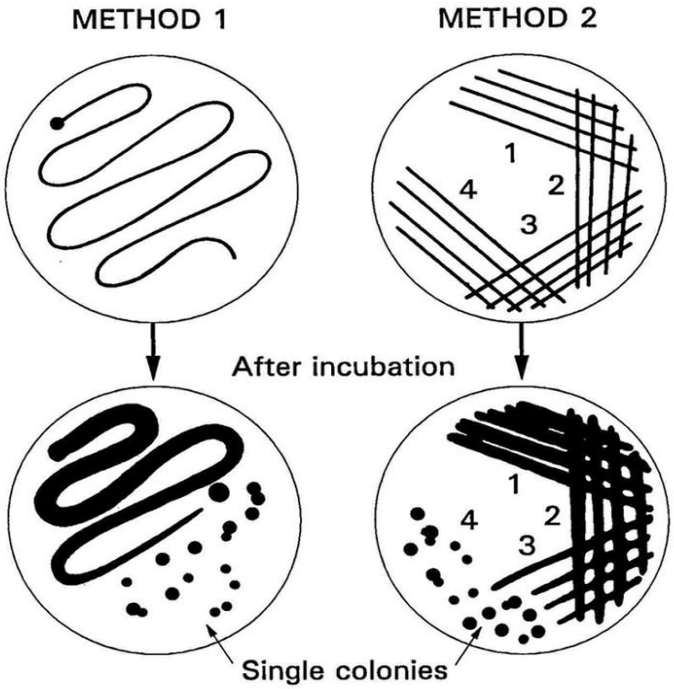
Crushed nodular extract (1 ml) suspension was diluted with 9 ml of sterile distilled water (10^{-1}) making the dilution from 10^{-2} to 10^{-8}

⬇

Suspension (0.1 ml) of nodular extract was inoculated into sterile YEMA plates

⬇

Inoculated Petri plates were incubated for 4-7 days in an incubator at 37°C.



IDENTIFICATION AND CHARACTERIZATION OF *Rhizobium*

CELLULAR	
Staining	Gram-negative
Morphology	Rods 0.5-0.9 x 1.2-3.0 um. Commonly pleomorphic under adverse growth conditions
Motility	Motile by one polar or subpolar flagellum or two to six peritrichous flagella
Specialized structures	Fimbriae have been described on a few strains Usually contain granules of poly-B- hydroxyl butyrate which are refractile by phase-contrast microscopy. Non spore forming
Division	

COLONIAL	
Solid	Colonies are circular, convex, semi translucent, raised and mucilaginous, usually 2-4 mm in diameter

surface within 3-5 days on yeast-mannitol-mineral salts agar.

Liquid Pronounced turbidity develops after 2 or 3 days in agitated broth

CLASSIFICATION OF *Rhizobium:*

Rhizobium is not part of a species because it is not a species. *Rhizobium* is the name of a genus of bacteria that fix nitrogen. Inside the genus is a multitude a species. Its name comes from Greek (Riza = Root and Bios = Life). Rhizobim's bacteria that are part of its genus are all aerobic bacteria.

Domain: Eubacteria

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Alpha Proteobacteria

Order: Rhizobiales:

Family: Rhizobicacea

Genus: Rhizobium

Examples of *Rhizobium sp.*

R. alamii, *R. alkalisoli*, *R. cellulosilyticum*, *R. gallicum*, *R. indigoferae*, *R. leguminosarum*, *R. leucaenae*,*R. loessense*, *R. lupini*, *R. mesoamericanum*, *R. nepotum*, *R. oryzae*, *R. petrolearium*, *R. phaseoli*, *R. pisi*

MASS PRODUCTION OF *Rhizobium* BIOFERTILIZER

Methods of Cultivation:

Following are the steps of mass cultivation of *Rhizobium:*

Sterilize the growth medium and inoculate with broth of mother culture prepared in advance

Incubate for 3-4 days at 30 - 32°C

↓

Test the cultures for its purity and transfer to a large fermenter, wait for 4-9 days for bacterial growth

(for good bacterial growth make the device for its aeration)

↓

Allow to grow the bacteria either in a large fermenter containing broth or in small flasks as per demand

⬇

Check the quality of broth

⬇

Blend the broth with sterile carrier *e.g.* peat, lignite, farmyard manure and charcoal powder

⬇

Pack the culture in polyethylene bags and keep at 25°C

⬇

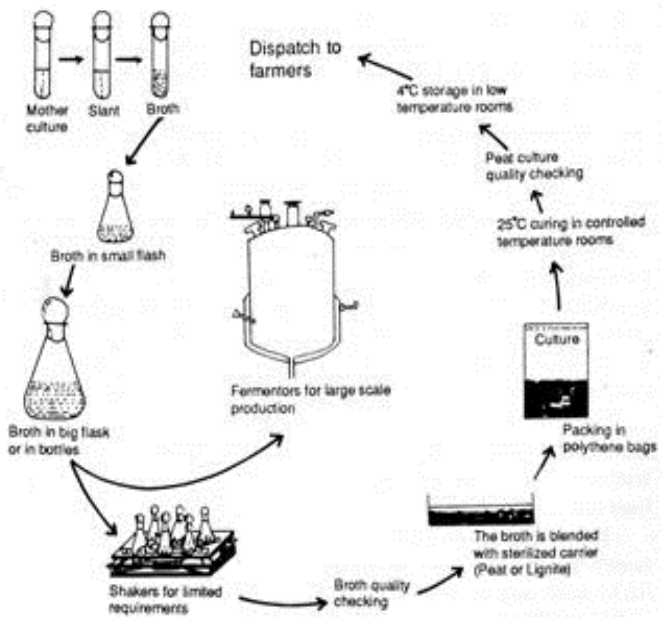
Check the quality of carrier culture

⬇

Store at 4°C in a controlled-temperature room

⬇

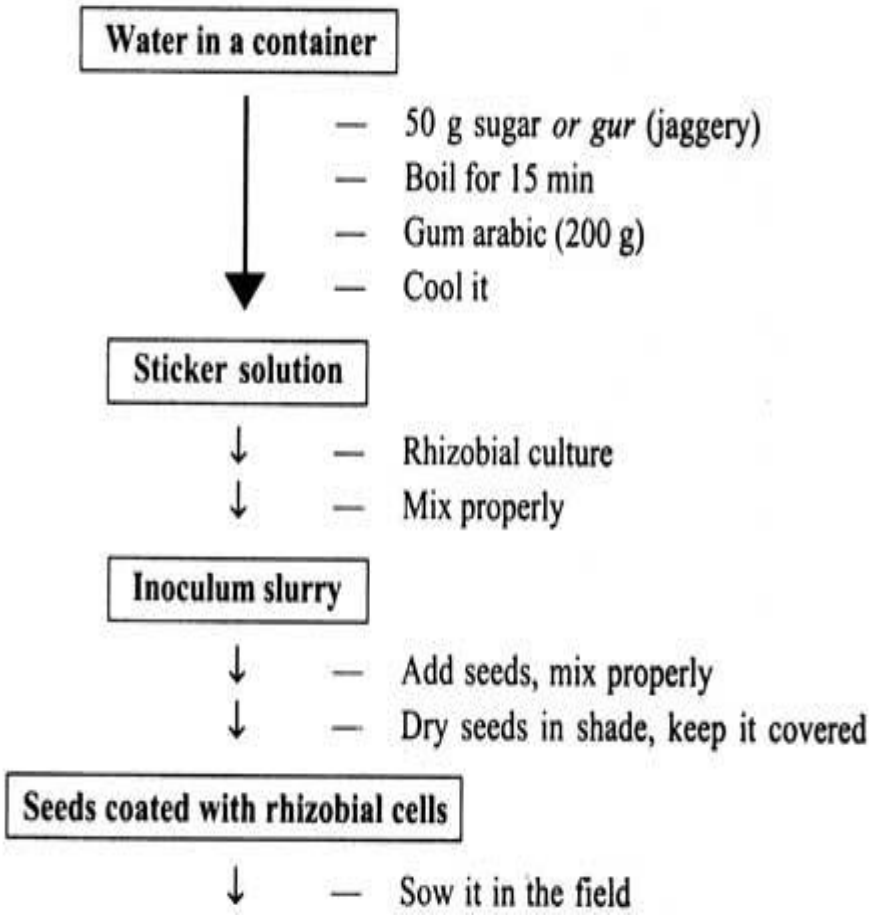
Supply to farmers



METHODS OF SEED INOCULATION WITH RHIZOBIAL CULTURE:

- ✪ Dissolve 10 per cent sugar or *gur* (jaggery) in water by boiling it for some time
- ✪ Leave the content to cool down. Gum arabic solution (10%) may also be added to the solution
- ✪ This serves as sticker for *Rhizobium* cells to seeds
- ✪ Mix this carrier based culture of *Rhizobium* to form the inoculum slurry
- ✪ For one hectare, 400 g charcoal based culture would be sufficient for mixing the seeds

- ✪ Transfer the inoculum slurry on seeds and mix properly
- ✪ The number of rhizobial cells/ seed should be between 10^5 to 10^6
- ✪ Spread the seeds in shade for drying on cement floor or plastic sheets



APPLICATION OF BIOFERTILIZERS:

1. Seed treatment or seed inoculation
2. Seedling root dip
3. Main field application

Seed treatment

- ✪ One packet of the inoculant is mixed with 200 ml of rice kanji to make a slurry
- ✪ The seeds required for an acre are mixed in the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dried for 30 minutes
- ✪ The shade dried seeds should be sown within 24 hours
- ✪ One packet of the inoculant (200 g) is sufficient to treat 10 kg of seeds.

Seedling root dip

- ☆ This method is used for transplanted crops
- ☆ Two packets of the inoculant is mixed in 40 litres of water
- ☆ The root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 minutes and then transplanted

Main field application

- ❖ Four packets of the inoculant is mixed with 20 kgs of dried and powdered farm yard manure and then broadcasted in one acre of main field just before transplanting.

APPLICATION OF *Rhizobium* BIOFERTILIZER:

- ➢ It is a low cost and easy technique
- ➢ It can be used by small and marginal farmers
- ➢ It is free from pollution hazards and increase soil fertility
- ➢ Secrete growth promoting substances like IAA, IBA, NAA, aminoacids, proteins, vitamins, etc. They add sufficient amount of organic matter in soil
- ➢ The bioferilizers increase physico-chemical properties of soils such as soil structure, texture, water holding capacity, cation exchange capacity and pH by providing several nutrients and sufficient organic matter
- ➢ It fix nitrogen from air to plant

MECHANISM OF BIOLOGICAL NITROGEN FIXATION

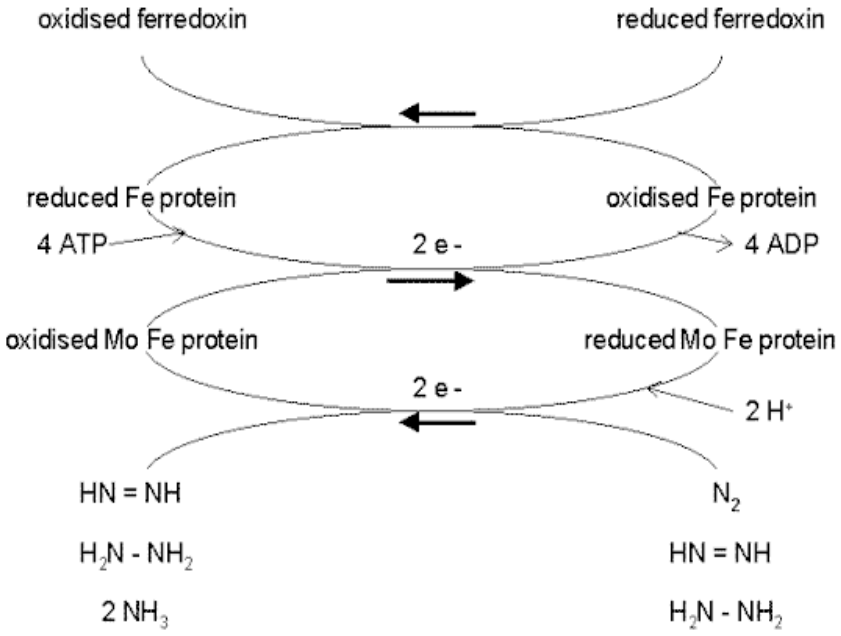
Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

$$N_2 + 8H+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi$$

This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an enzyme complex termed **nitrogenase**. This enzyme consists of two proteins - an iron protein and a molybdenum-iron protein, as shown below.

The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to $H_2N\text{-}NH_2$, and this in turn is reduced to $2NH_3$.

Depending on the type of microorganism, the reduced ferredoxin which supplies electrons for this process is generated by photosynthesis, respiration or fermentation.



Formation of a root nodule

Multiple interactions are involved in the formation of root nodules:

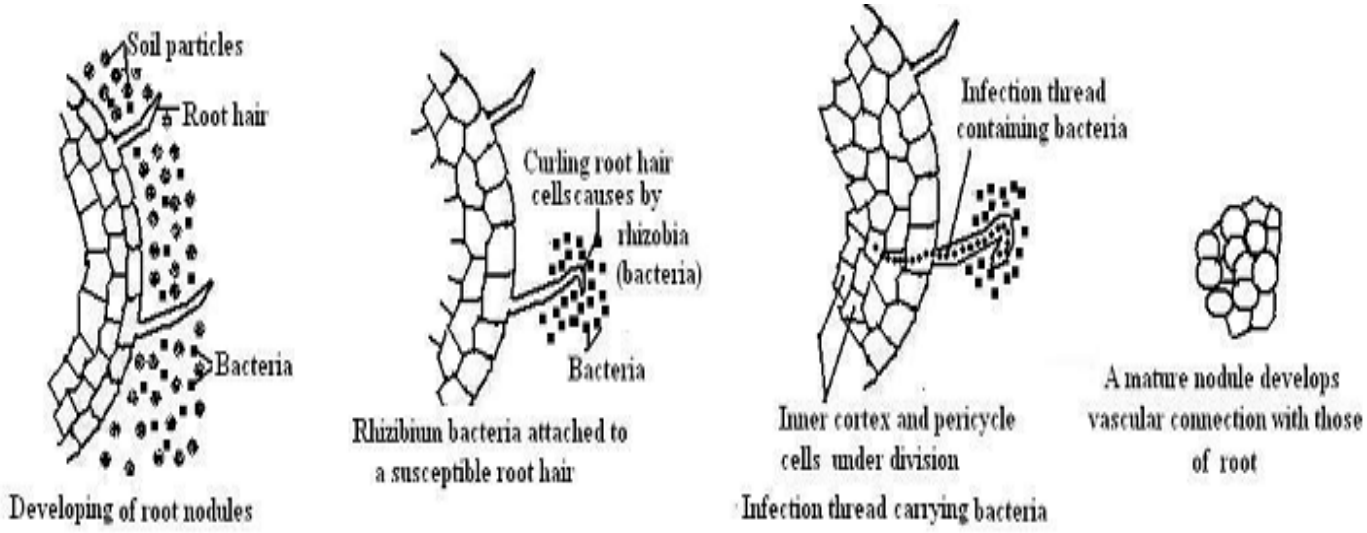
1) The Rhizobium bacteria divide and form colonies. These get attached to the root hairs and epidermal cells.

2) The root hairs get curled and are invaded by the bacteria.

3) This invasion is followed by the formation of an infection thread that carries the bacteria into the cortex of the root. The bacteria get modified into rod-shaped bacteroides.

4) As a result, the cells in the cortex and pericycle undergo division, leading to the formation of root nodules.

5) The nodules finally get connected with the vascular tissues of the roots for nutrient exchange.



Non-Symbiotic N_2-Fixing Bacteria:

The non-symbiotic nitrogen fixing bacteria do not require a host plant. In **1891, Winogradsky** observed that when soil was exposed to the atmosphere, the nitrogen content of the soil was recorded to be increased.

- Anaerobic bacterium ***Clostridium pasteurianum*** was found responsible for such **an increase of the nitrogen content** in soil
- In 1901, Beijerinck proved that there were also free-living aerobic bacteria, ***Azotobacter chroococcum*** that **could fix atmospheric nitrogen**
- Another bacterial group, **Granulobacter (purple colour)** obtains **nitrogen directly from the atmosphere**
- Aerobic soils of tropical climatic regions, the acid tolerant N_2-fixer ***Azotobacter beijerinckia*** is most abundant ***Azospirillum spp***. also **fixes N_2-non-symbiotically** and **help to many crops for their growth** and **yield**

Azospirillum:

- *Azospirillum*, a free-living nitrogen-fixing bacteria closely associated with grasses
- Gram negative
- Motile bacteria

- ✈ Microaerophilic
- ✈ Non-fermentative
- ✈ Secretes some fungicides, enzymes but in minute amount
- ✈ Belonging to the order ***Rhodospirillales***
- ✈ Associated with roots of monocots, including important crops, such as wheat, corn and rice

Azospirillum	
Scientific classification	
Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alphaproteobacteria
Order:	Rhodospirillales
Family:	Rhodospirillaceae
Genus:	***Azospirillum***
Type species	
Azospirillum lipoferum	
Species	
A. brasilense, *A. canadense*, *A. doebereinerae*, *A. fermentarium*, *A. formosense* *A. halopraeferens*, *A. humicireducens*, *A. irakense*, *A. largimobile*, *A. lipoferum* *A. melinis*, *A. oryzae*. *A. picis*, *A. rugosum*	

Isolation of *Azospirillum*

The roots are separated from the plants and thoroughly washed in running tap water

🡻

Then, they are transferred into 1 L flasks containing 0.5 L of sterile tap water and shaken for 30 minutes

🡻

The procedure is repeated three times, after which the same procedure is repeated with distilled water three times

🡻

The washed roots are moved to a sterile Petri dish and are cut into minuscule pieces with scissors pretreated with alcohol and burnt in the flame of an alcohol lamp

🡻

The root pieces are transferred into tubes containing 6mL of semi liquid selective media

🡻

The tubes are incubated at 30^{o}C or at 37^{o}C for 3-5 days

🡻

0.1 mL of culture liquid is transferred to the tubes containing fresh medium and incubated for 5-7 days

🡻

On semiliquid medium, azopirilla form a special subsurface growth ring

⬇

The tubes with special microaerophilic growth are inspected, and the microbial growth ring is observed under the microscope

⬇

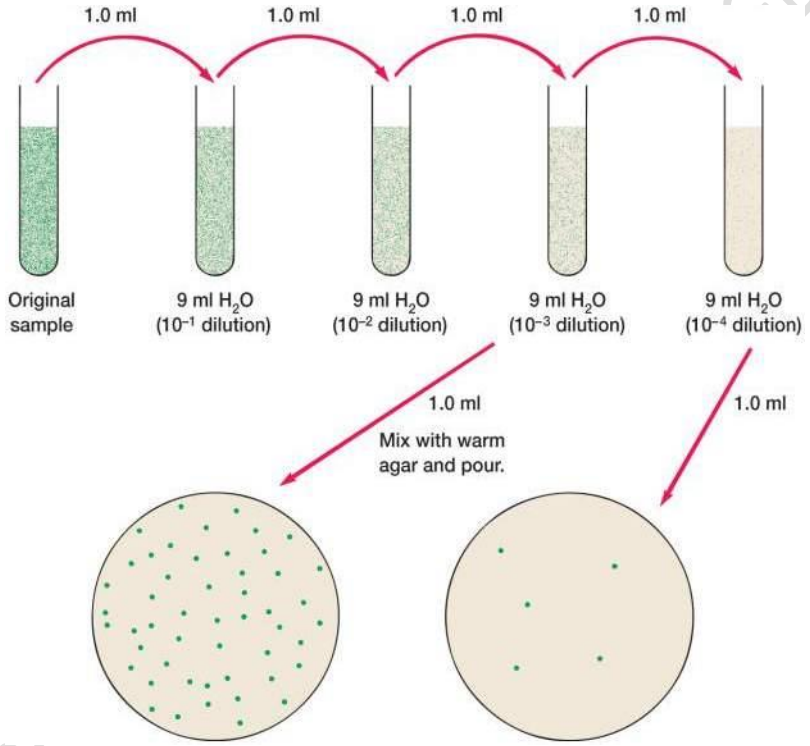
The samples with special helical movement are checked and selected

⬇

Dilutions of the inoculum are plated on the same agar medium and incubated for 3-5 days

⬇

The isolated colonies are selected



IDENTIFICATION:

- Preliminary identification is made with the immunodiffusion method
- The strains forming precipitation bands are selected
- Physiological and biochemical tests, immunochemical analysis, and 16S rRNA gene sequence analysis are used for the identification of isolated cultures

COLONY APPEARANCE:

CELLULAR	
Staining	Gram-negative to Gram-variable.
Morphology	Plump, slightly curved and straight rods, about 1.0 um in diameter and 2.1-3.8 um in length, often with pointed ends.
Motility	Motile in liquid media by a single polar flagellum. On solid media at 30`C numerous lateral flagella of shorter wavelength are also formed.
Specialized structures	Intracellular granules of poly-B-hydroxybutyrate present. Enlarged, pleomorphic forms may occur in old, alkaline cultures or under conditions of excess oxygen
Division	

COLONIAL		
Solid surface: Colonies on potato agar are typically light or dark pink, often wrinkled and non-slimy.		
Liquid	:	Turbidity

MASS CULTIVATION OF *Azospirillum:*

Sterilize the growth medium and inoculate with broth of mother culture prepared in advance

Incubate for 3-4 days at 30 - 32°C

↓

Test the cultures for its purity and transfer to a large fermenter, wait for 4-9 days for bacterial growth (for good bacterial growth make the device for its aeration)

↓

Allow to grow the bacteria either in a large fermenter containing broth or in small flasks as per demand

↓

Check the quality of broth

↓

Blend the broth with sterile carrier *e.g.* peat, lignite, farmyard manure and charcoal powder

↓

Pack the culture in polyethylene bags and keep at 25°C

↓

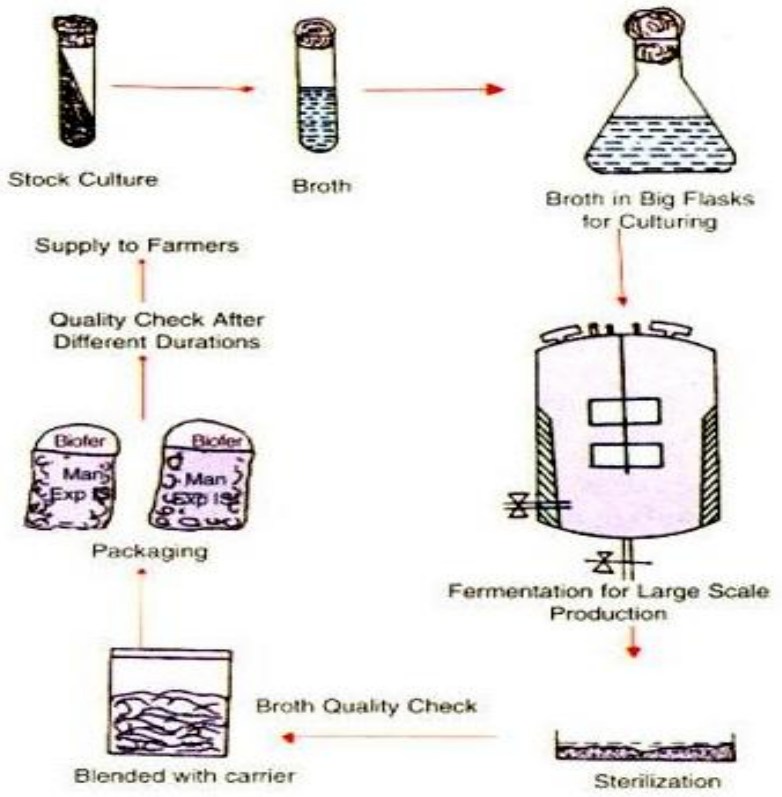
Check the quality of carrier culture

↓

Store at 4°C in a controlled-temperature room

↓

Supply to farmers



APPLICATION OF BIOFERTILIZERS

1. Seed treatment or seed inoculation
2. Seedling root dip
3. Main field application

Seed treatment

One packet of the inoculants is mixed with 200 ml of rice kanji to make a slurry. The seeds required for an acre are mixed in the slurry so as to have a uniform coating of the inoculants over the seeds and then shade dried for 30 minutes. The shade dried seeds should be sown within 24 hours. One packet of the inoculants (200 g) is sufficient to treat 10 kg of seeds.

Seedling root dip

This method is used for transplanted crops. Two packets of the inoculant is mixed in 40 litres of water. The root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 minutes and then transplanted.

Main field application

Four packets of the inoculant is mixed with 20 kgs of dried and powdered farm yard manure and then broadcasted in one acre of main field just before transplanting.

MECHANISM OF NITROGEN FIXATION:

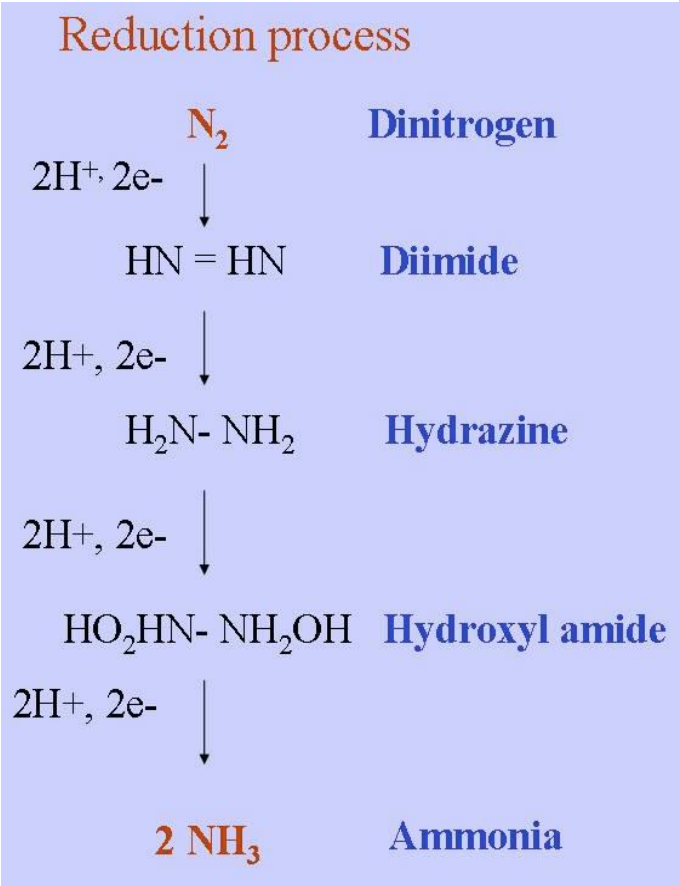
Reduction takes place on the surface of the enzyme

- Six electrons are required to reduce one mole of N to two moles of ammonia.

$N_2 + 8H^+ + 8e^- + 16\ ATP \text{-------} 2\ NH_3 + 16\ ADP + 2H^+ + 16pi$

It is postulated that, atoms of N2 are separated torch charge in the valency of metal ion (mo) bound to the enzyme involved in reduction of N2. For every electron transfer, 4 ATP moles are required.

- **Hydrogenase** -Uptake hydrogenase (HUP+) converts the release d hydrogen during N2 fixation, and cycled back the Hydrogen for energy generation by this they contribute 9-10 %ATP requirement for N2 fixation process



Azotobacter

- *Azotobacter* is a genus of usually **motile**
- **Oval** or **spherical bacteria** that form thick-walled cysts and may produce large quantities of **capsular slime**
- They are **aerobic**
- **Free-living soil microbes** which play an important role in the **nitrogen cycle**
- **Binding atmospheric nitrogen**, which is **inaccessible to plants**, and **releasing** it in the **form of ammonium ions into the soil** (nitrogen fixation)
- **Diazotrophs** (Diazotroph: "Di": two + "A": without + "Zoo": life + "Troph": pertaining to food or nourishment. "Azote": Nitrogen (French). Named by French chemist and biologist Antoine Lavoisier, who saw it as the part of air which cannot sustain life)
- Used by humans for the production of **biofertilizers**, **food additives**, and some **biopolymers**
- The first representative of the genus, ***Azotobacter chroococcum***
- **Discovered** and described in **1901** by the **Dutch microbiologist** and **botanist Martinus Beijerinck**
- *Azotobacter* species are **Gram-negative bacteria**

- Found in **neutral** and **alkaline soils**

Scientific classification	
Domain:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pseudomonadales
Family:	Pseudomonadaceae/Azotobacteraceae
Genus:	***Azotobacter*** Beijerinck, 1901

Species
Azotobacter agilis
Azotobacter armeniacus
Azotobacter sp. AR
Azotobacter beijerinckii
Azotobacter chroococcum
Azotobacter sp. DCU26
Azotobacter sp. FA8
Azotobacter nigricans
Azotobacter paspali
Azotobacter salinestris
Azotobacter tropicalis
Azotobacter vinelandii

ISOLATION OF *Azotobacter:*

Ashby's medium:

Mannitol	20.0 g
K_2HPO_4	0.2 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
K_2SO_4	0.1 g
$CaCO_3$	5.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Dissolve mannitol, $MgS0_4$. $7H_20$, NaCl, K_2S0_4 and $CaC0_3$ in 200 ml distilled water. Dissolve K_2S0_4 separately in 100 ml distilled water (to prevent precipitation) in another flask. Mix both solutions and make up the volume to 1000 ml. Sterilize at 15 lbs (121°C) for 15 minutes and use.

Or

Waksman medium No.77 (N-free Mannitol Agar Medium for *Azotobacter*)

Mannitol	:	10.0 g
Ca CO3	:	5.0 g
K2HPO4	:	0.5 g
Mg SO4.7H2O	:	0.2 g
NaCl	:	0.2 g
Ferric chloride	:	Trace
MnSO4.4H2O	:	Trace
N-free washed Agar	:	15.0 g
pH	:	7.0
Distilled Water	:	1000 ml

Pour Ashby's medium into sterile Petri plates and allow them to solidify.

2. Sieve the soil through 2 mm sieve, weigh two 10 g samples, keep one sample in an oven over night at 150°C. Weigh this sample to find out the percentage of moisture in soil.

3. Add the other 10 g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.

4. Make serial dilutions of this sample though sterile water blanks as mentioned under bacteria.

5. Add 1 ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28°C for 3-4 days.

COLONY APPEARANCE: *Azotobacter* colonies appear as flat, soft, mucoid and milky colonies.

MASS PRODUCTION OF *Azotobacter*:

i. **Mother culture:**

A pure growth of any organism on a small scale is called as a mother culture

Mother culture is always prepared in a conical flask of 500 or 1000 ml

Capacity and then this mother culture is used for further production

For this purpose, one liter conical flasks are taken to which 500 ml of broth of nitrogen free medium

is added and these flasks are then plugged with non-absorbent cotton, sterilized in an auto slave for 15-20 minutes at 75 lbs pressure for 15 minutes

Flasks are then inoculated with mother culture with the help of inoculating needle aseptically

The flasks are transferred to shaker and shaking is done for 72-90 hours so as to get optimum growth of bacteria in broth. Bacteria are multiplied by binary method i.e. cell division

After about 90 days, the number of per milliliters comes to about 100 crores

Total growth of bacteria in this broth means starter culture or mother culture, which should carefully be done, since further purity of biofertilizer or quality of biofertilizer depends upon how mother culture is prepared

ii. Production on a large scale:

Azotobacter is multiplied on a large scale by two ways viz. **Fermenter** and **Shaker**

The fermenter is **most automatic** and **accurate method** of multiplication of any micro-organism

<u>In this First method</u>, the medium is taken in a fermenter and then sterilized. **After this pH of the medium is adjusted and 1% mother culture is added**

In order to get an **optimum growth of the *Azotobacter* required temperature and oxygen supply is adjusted** so that concentrated broth is made

This concentrated **broth of the culture** is then **mixed with a carrier** previously sterilized and **bio-fertilizers are prepared**

Depending upon the demand and **supply suitable fermenter** is selected

<u>In the 2nd method</u> i.e. shake method, a suitable medium is prepared transferred to conical flask of suitable capacity

⬇

These flasks are then sterilized in an autoclave at 15 lbs pressure for 15 minutes

⬇

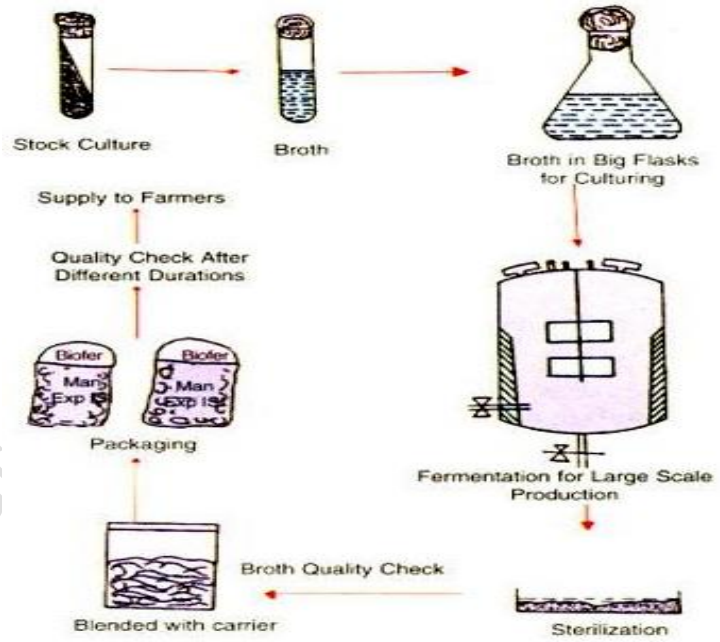
Each flask is inoclulated with 10 ml mother culture and they are transferred to shaker for multiplication where they are kept for 72-90 hours

⬇

This broth is mixed with a suitable carrier previously sterilized

⬇

Thus biofertilizer is prepared, filled in plastic bags and stored in cool place.

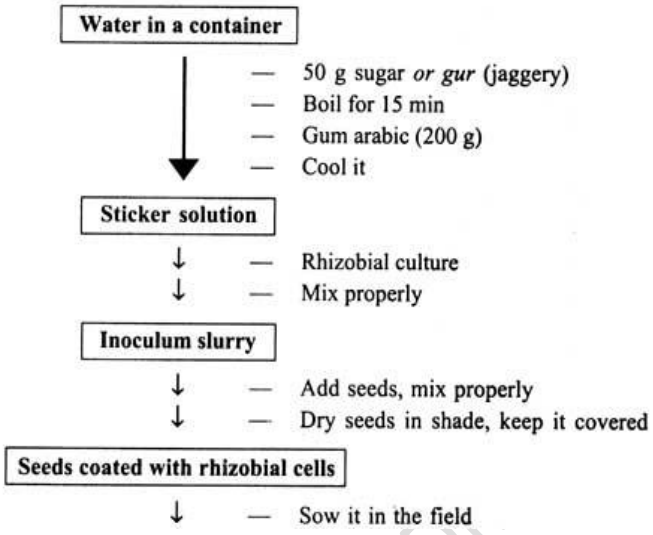


FIELD APPLICATIONS:

a. **Seed inoculation:**

- ✈ On the basis of efficiency of *Azotobacter*, other micro-organisms present in the soil, benefits obtained from biofertilizer and expenditure it has been fixed to use Azotobacter - bio-fertilizer at the rate of 250 g biofertilizer for 10-15 kg
- ✈ If one knows this proportion then take a definite quantity of seed to be inoculated

- ✈ The required quantity of fresh biofertilizer is secured and slurry is made by adding adequate, quantity of water
- ✈ This slurry is uniformly applied to seed, seed is then dried in shed and sown
- ✈ Some stickers are used in order to adher biofertilizer to seeds. Viz. Jaggery or gum arebia.



b. Seedling inoculation:

- ❖ This method of inoculation is used where seedlings are used to grow the crop
- ❖ In this method, seedlings required for one acre are inoculated using 4-5 packets (2-2.5 kg)
- ❖ For this, in a bucket adequate quantity of water is taken and biofertilizer from these packets is added to bucket and mixed properly
- ❖ Roots or seedlings are then dipped in this mixture so as to enable roots to get inoculums. These seedlings are then transplanted e.g. Tomato, Rice, Onion, Cole, Crops, flowers.

c. Self inoculation or tube inoculation:

- ➢ In this method 50 litres of water is taken in a drum and 4-5 kg of Azotobacter biofertilizer is added and mixed properly
- ➢ Sets are required for one acre of land are dipped in this mixture. Potato tubers are dipped in the mixture of biofertilizer and planting is done.

d. Soil application:

This method is mostly used for fruit crops, sugarcane, and trees. At the time of planting fruit tree 20 g of biofertilizer mixed with compost is to be added per sappling, when trees became matured the same quantity of biofertilizer is applied.

***Advantages of* Azotobacter:**

1. Azotobacter contributes moderate benefits

2. Azotobacter is heaviest breathing organism and requires a large amount of organic carbon for its growth.

3. It is poor competitor for nutrients in soil and hence its growth promoting substances, fungistatic substances.

4. It can benefit crops by Nitrogen fixation, growth promoting substances, fungi static substances.

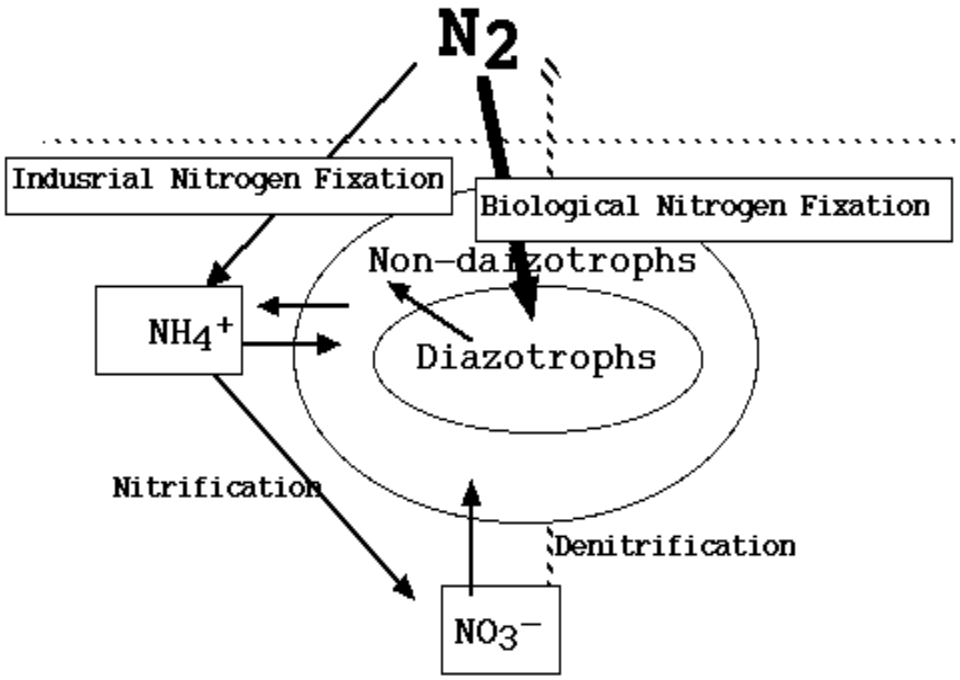
5. Azotobacter is less effective in soils with poor organic matter content.

6. It improves seed germination and plant growth

7. It thrives even in alkaline soils.

8. Azotobacter is tolerant to high salts

NITROGEN FIXATION / METABOLISM:



Nitrogen cycle in biosphere

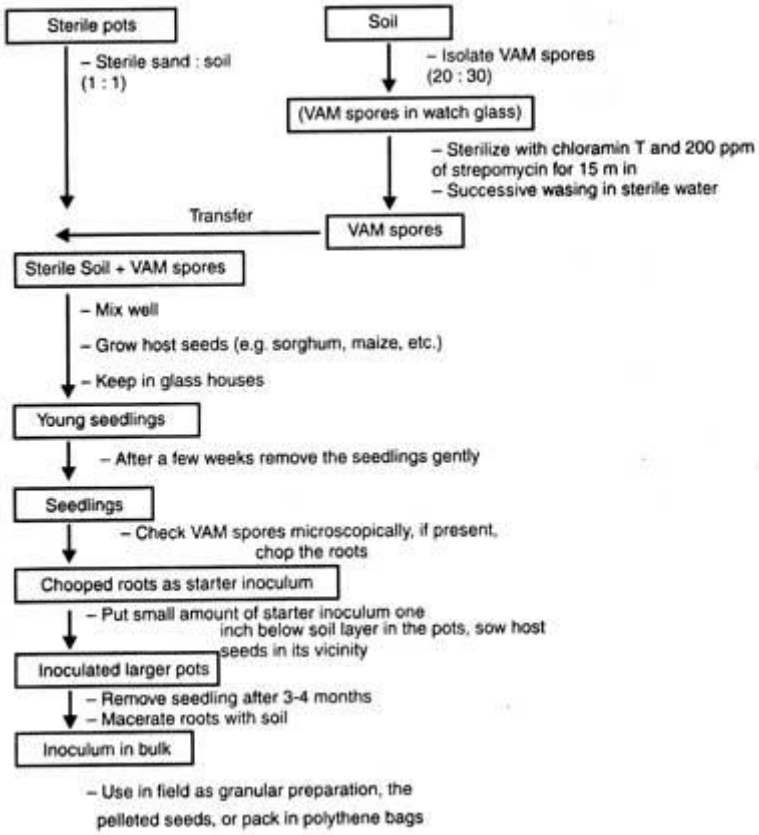
Diazotrophs = Nitrogen fixing organisms

- *Azotobacter* has generated a good deal of interest in the scientific community because of their unique mode of metabolism, by which they can fix nitrogen aerobically
- The cells' uniquely high respiration rates allow the normally oxygen-sensitive nitrogenase to experience limited oxygen exposure
- *Azotobacter* is also capable of producing a protein which protects the nitrogenase from sudden oxygen-provoked stress
- Another individualistic trait of *Azotobacter* is their ability to synthesize not just one, but three nitrogenases
- Specific genes are used to synthesize each nitrogenase. *Azotobacter*'s cells are large rods, at least 2 microns in diameter
- They can live singly, in chains, or in clumps, and may or may not be mobile by flagella
- Their resting stage is spent as a thick-walled cyst, which protects the organism from harsh climates

VAM

Vesicular arbuscular mycorrhizae (VA mycorrhizae, or **VAM**) are the most abundant of a group of symbiotic fungi that infect plant roots. ... These tubes originally grow from fungal spores, extending short distances (on the order of millimeters) into the soil in search of the roots of host plants

Mass Production of VAM:



Applications of Mycorrhizae :

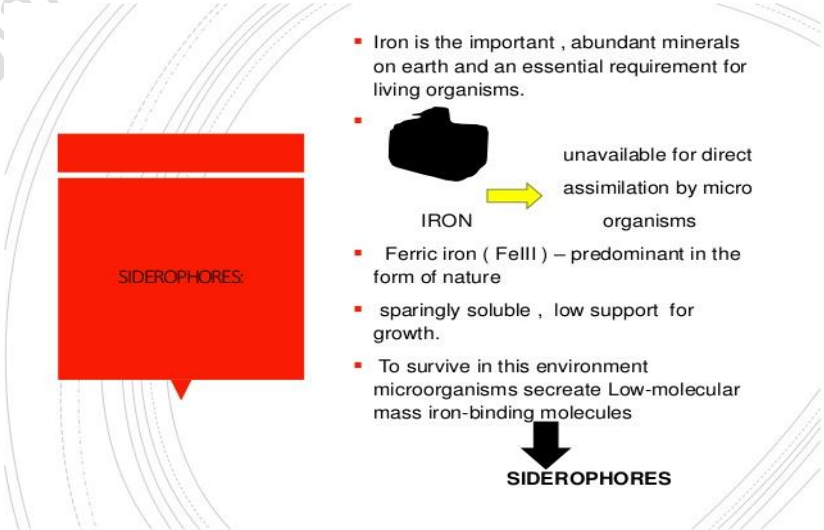
- Increase nutrient uptake of plant from soil.
- P nutrition and other elements: N, K, Ca, Mg, Zn, Cu, S, B, Mo, Fe, Mn, Cl Increase diversity of plant.
- Produce uniform seedling. Significant role in nutrient recycling. More tolerant to adverse soil chemical constraints which limit crop production.
- Increase plant resistance to diseases and drought. Stimulate the growth of beneficial microorganisms. Improve soil structure.
- Stable soil aggregate – hyphal polysaccharides bind and aggregate soil particles.
- Increases absorption of phosphate by crops. uptake of zinc also increases.
- Increases uptake of water from soil. Increases uptake of sulphur from the soil
- Increases the concentration of cytokinins and chloroplast in plants.
- They protect plants during stress condition.

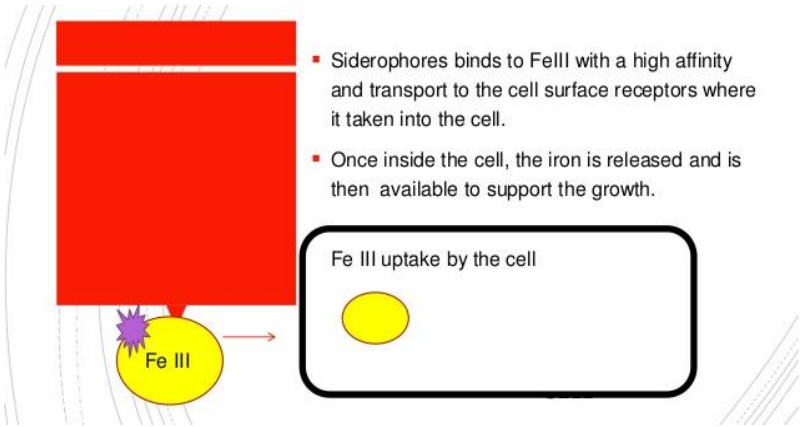
Biocontrol of Pathogens:

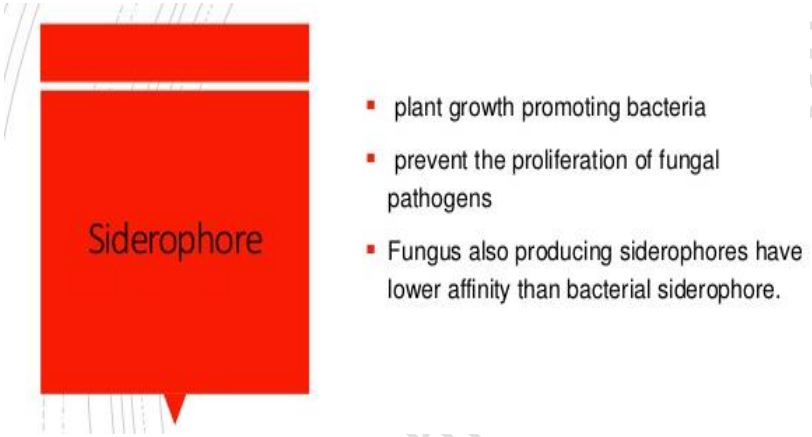
- **Biocontrol of pathogens** using **siderophores**, **antibiotics**, **enzymes**
- Plant growth promoting **bacteria** can act as a **biocontrol** agent to suppress **pathogens**
- They produce a variety of substances that limit the damage by phytopathogens.

Biocontrol Agent - Siderophore: Introduction

- Phytopathogens – organisms parasitic on plant host.
- Serious agricultural problems
- Reduce crop yields from 25% to 100%
- Most bacterial diseases, plant may be asymptamatic , cause rapid outbreak
- Cause entire crop damage and difficult to϶ eradicate and costly.
- Chemical agents are hazardous to humans, animals and difficult to remove from ecosytem.
- For ‘’ecofriendly’’ biological agents are used.
- Developing transgenic plants that are resistant to phytopathogens.
- Plant growth promoting bacteria can act as a biocontrol agent to suppress pathogens .
- They produce a variety of substances that limit the damage by phytopathogens. eg. Siderophores, antibiotics, enzymes.







Structure of Siderophore:

- ❖ Three functional or iron binding groups
- ❖ Each functional group presents two atoms of oxygen (or) two nitrogen atoms that bind iron.
- ❖ Bidentate
- ❖ Trivalent ferric iron accommodate three groups ⟶ six co-ordinate complex
- ❖ In some exceptions the functional groups are hydroxamates (CONOH) or catecholates (chelating)
- ❖ Siderophores have different functional group
- ❖ **Bacteria VS Fungi**: Fungi have hydroxymates
- ❖ Bacteria have catecholates
- ❖ Catecholates have tight binding more than hydroxymates
- ❖ Bacterial siderophores called pseudobactin has been bind to FeIII
- ❖ Pseudobactin synthesis inhibit fungal growth and other phytopathogens
- ❖ Defective gene identified:
  - 1. lack of fluorescence under UV light
  - 2. Inability to grow in the presence of bipyridyl, that molecule hidden many iron molecules in the medium.

Biocontrol agent – Antibiotics:

Introduction:

- ❖ Plant growth promoting bacteria synthesis antibiotics and prevent proliferation of phytopathogens .
- ❖ eg: Pseudomonads - agrocin 84, agrocin 434, 2,4,-diacetyl phloroglucinol ,oomycin, pyrrolnitrin
- ❖ Biocontrol activity of bacteria improved by enhancing the biosynthesis of antibiotics than the normal level.
- ❖ Single bacterium can supress the normal phytopathogens and use limited nutrients from the plant

1. Controlled Gene:

- pseudomonas – antibiotic synthesis - controlled by protein - global transcriptional regulator.
- By modifying this regulator, enhance antibiotic synthesis
- Example: pythium ultimum fungus

modifying pseudomonas flourescens RNA polymerase sigma factor

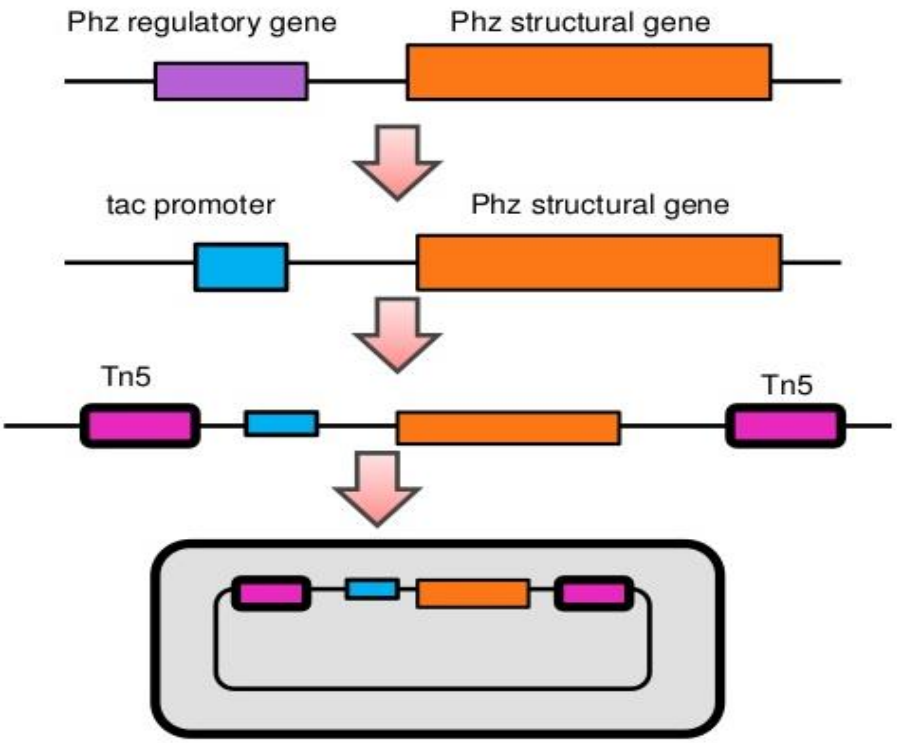
2. Inactivation of Pqq

- Pqq genes - biosynthesis of pyrroloquinoline quinone
- P.flourescens CHAO stimulated the production of antibiotic pyoluteorin.

3. Phz gene:

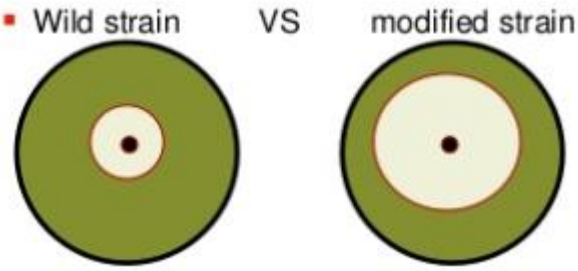
- Phz gene responsible for antibiotic synthesis – phenazine – 1 – carboxylic acid
- Phz gene
  - 1) regulatory gene
  - 2) strucutral gene
- regulatory gene – control the expression of seven biosynthetic gene
- phz regulatory gene was replaced by tac promoter
- P. fluorescens does not utilize lactose as a carbon source ➡ so absence of lac repressor ➡ tac promoter expressed continously

- inserted into a derivative of Transposon Tn5 adjacent to kannamycin resistant gene on a plasmid.
- Tn5 facilitates the integration of DNA into the chromosomal of host cell.
- Tn5 designed – so that it does not easily pass from the biocontrol strain to other bacteria from the environment.



Selection and screening:

- Wild type – does not synthesis phz
- Modified – able to synthesis phz
- P.ultimum in a solid medium
- Inoculate P.flourescens
- Larger zone of lysis in modified strain



Crowngall disease:

- Crown gall disease caused by *Agrobacterium tumefaciens*
- Found in almond trees, stone fruit trees, and peach trees.
- Controlled by Agrobacterium radiobacter by antibiotic synthesis agrocin 84
- Agrocin resistant strains of agrobacterium tumefaciens can develop if the plasmid responsible for the gene was accidentally transferred
- So, plasmid responsible for transmission is deleted.

Biocontrol agent of Enzymes:

Introduction:

- PGPB produce enzymes such as Chitinase, ß- 1,3- glucanase, protease, lipase that degrade fungal cell wall
- some have antifungal activity and have degrading fungal cell wall eg: chitinase , Beta- glucanase
- *E. agglomerans* significantly decreased the damage to cotton plants
- some Tn5 mutants were unable to protect plants against the fungal pathogen
- Indicating fungal was a active element
- Some bacteria enzymes can lyse fungal cell wall indicating the presence of chitinase and ß-glucanase are encoded in a single gene.

Isolate the genes

⬇

Transfer them to PGPB

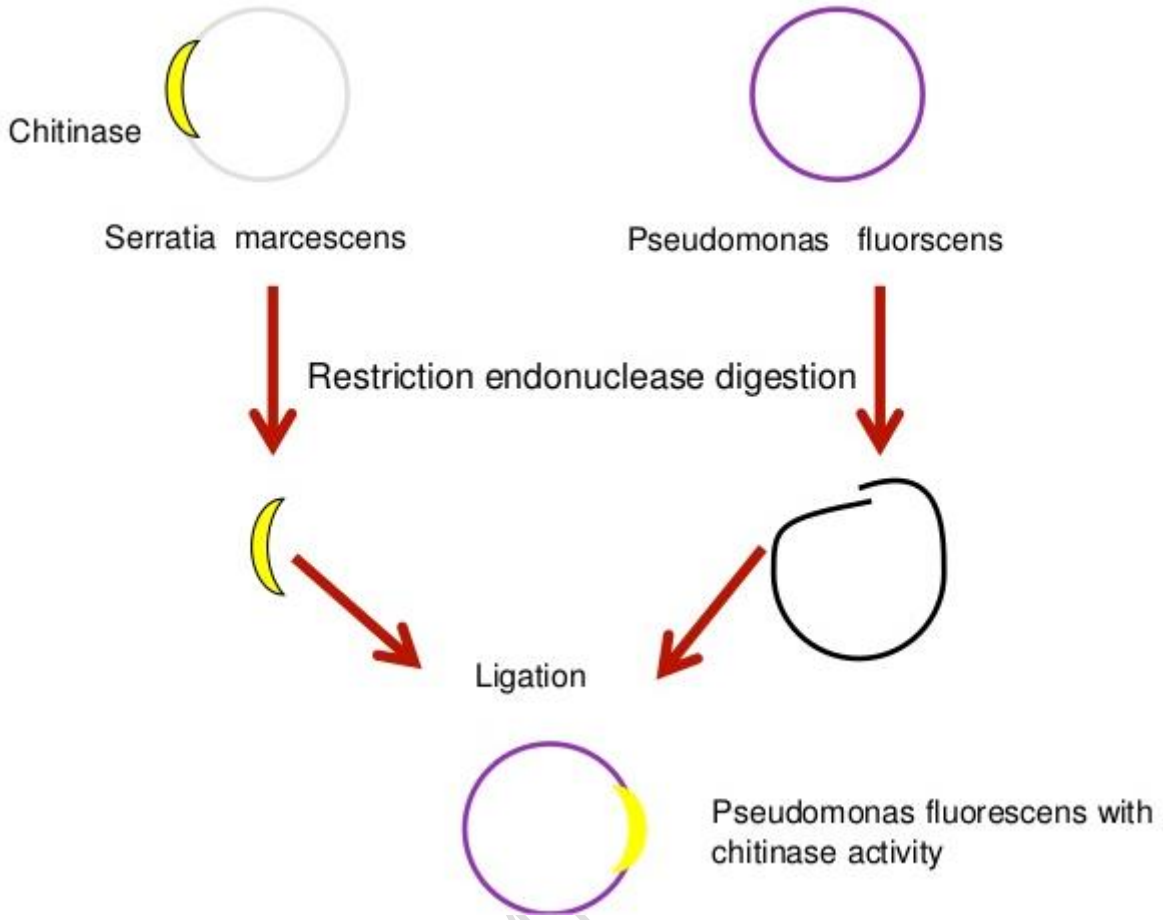
⬇

Constructing strains

⬇

Fungal cell wall degraded

Bacteria	enzyme	Fungi
Burkholderia cepacia	ß- 1,3- glucanase	Rhizoctonia solani Sclerotium rolfsii Pythium ultimum
Enterobacter agglomerans	chitinase	Rhizoctonia solani
Pseudomonas flourescenes from Serratia marcescens	Antifungal , chitinase	R. solanii



Ice nucleation:

Bacterial **ice-nucleation** proteins. Bacterial **ice-nucleation** proteins **is** a family of proteins that enable Gram-negative **bacteria** to promote **nucleation** of **ice** at relatively high temperatures (above -5C). These proteins are localised at the outer membrane surface and can cause **frost** damage to many plants.

Production

To systematically create the ice-minus strain of *P. syringae*, its ice-forming gene must be isolated, amplified, deactivated and reintroduced into *P. syringae* bacterium. The following steps are often used to isolate and generate ice-minus strains of *P. syringae*:

1. Digest *P. syringae*'s DNA with restriction enzymes.
2. Insert the individual DNA pieces into a plasmid. Pieces will insert randomly, allowing for different variations of recombinant DNA to be produced.

3. Transform the bacterium *Escherichia coli* (*E.coli*) with the recombinant plasmid. The plasmid will be taken in by the bacteria, rendering it part of the organism's DNA.
4. Identify the ice-gene from the numerous newly developed *E. coli* recombinants. Recombinant *E. coli* with the ice-gene will possess the ice-nucleating phenotype, these will be "ice-plus".
5. With the ice nucleating recombinant identified, amplify the ice gene with techniques such as polymerase chain reactions (PCR).
6. Create mutant clones of the ice gene through the introduction of mutagenic agents such as UV radiation to inactivate the ice gene, creating the "ice-minus" gene.
7. Repeat previous steps (insert gene into plasmid, transform *E. coli*, identify recombinants) with the newly created mutant clones to identify the bacteria with the ice-minus gene. They will possess the desired ice-minus phenotype.
8. Insert the ice-minus gene into normal, ice-plus *P. syringae* bacterium.
9. Allow recombination to take place, rendering both ice-minus and ice-plus strains of *P. syringae*.

The ice nucleating nature of *P. syringae* incites frost development, freezing the buds of the plant and destroying the occurring crop. The introduction of an ice-minus strain of *P. syringae* to the surface of plants would reduce the amount of ice nucleate present, rendering higher crop yields.

Economic importance

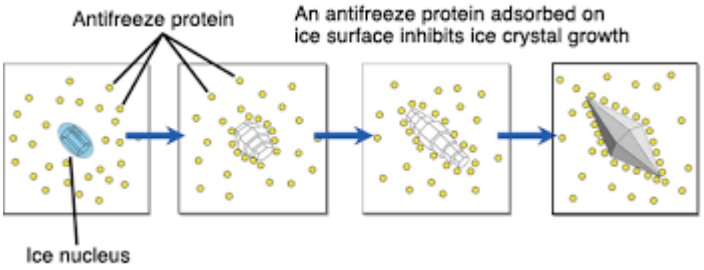
P. syringae commonly inhabits plant surfaces, its ice nucleating nature incites frost development, freezing the buds of the plant and destroying the occurring crop. The introduction of an ice-minus strain of *P. syringae* to the surface of plants would incur competition between the strains. Should the ice-minus strain win out, the ice nucleate provided by *P. syringae* would no longer be present, lowering the level of frost development on plant surfaces at normal water freezing temperature – 0 °C (32 °F).

Even if the ice-minus strain does not win out, the amount of ice nucleate present from ice-plus *P. syringae* would be reduced due to competition. Decreased levels of frost generation at normal water freezing temperature would translate into a lowered quantity of crops lost due to frost damage, rendering higher crop yields overall.

Antifreeze proteins:

Antifreeze proteins (AFPs) or ice structuring **proteins** (ISPs) refer to a class of polypeptides produced by certain animals, plants, fungi and bacteria that permit their survival in subzero environments. AFPs bind to small ice crystals to inhibit the growth and recrystallization of ice that would otherwise be fatal.

Mechanism:



Commercial and medical applications

Numerous fields would be able to benefit from the protection of tissue damage by freezing. Businesses are currently investigating the use of these proteins in:

- Increasing freeze tolerance of crop plants and extending the harvest season in cooler climates
- Improving farm fish production in cooler climates
- Lengthening shelf life of frozen foods
- Improving cryosurgery
- Enhancing preservation of tissues for transplant or transfusion in medicine
- Therapy for hypothermia
- Human Cryopreservation (Cryonics)

Microbial herbicides:

- **Microbial** weed control represents an innovative means to manage troublesome weeds and utilize the naturally occurring biological **herbicides** produced by soil microorganisms. These compounds kill or hinder the growth of weeds so that beneficial plant species can gain a competitive advantage.
- A **bioherbicide** is a biologically based control agent for weed. In irrigated **agriculture**, weed control through chemical **herbicides**, creates spray drift hazards and adversely affects the environment. Besides, pesticide residues (**herbicides**) in food commodities, directly or indirectly affect human health.
- Bioherbicides are made up of microorganisms (e.g. bacteria, viruses, fungi)

Examples of Bioherbicide:

DeVine:

A liquid suspension of spores of the fungus *Phytophthora palmivora* that causes root rot in the weed plants , used against Strangle vine *Morrenia odorata*

Collego

Wettable powder containing the fungal spores of *Colletotrichum gleosporoides* and *C. aeschynomone* causing stem and leaf blight , used against Joint vetch weed *Aeschonyme spp.* in Rice

Bipolaris

It is a suspension of fungal spores of *Bipolaris sorghicola* , used against Johnson grass weed *Sorghum halapense*

Biolophos

Fermented product of *Streptomyces hygroscopicus* , used as general herbicide

Product F

Product from *Fusarium oxysporium* , used against Orobranche in sunflower crop

ABG5003

Cercospora rodmanii used in control of water hyacinth *Eichhornia crassips*

Biopesticide:

Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals. For example, canola oil and baking soda have pesticidal applications and are considered **biopesticides**.

Classes of biopesticides

1. Biochemical pesticides:

- Naturally occurring substances that control pests by non-toxic mechanisms

- Includes substances that interfere with mating, such as insect sex pheromones, as well as various scented plant extracts that attract insect pests to traps

2. Microbial biopesticides:

- Consist of a microorganism (e.g., a bacterium, fungus, virus or protozoan) as the active ingredient.

- Active ingredient is relatively specific for its target pest

- Eg: some Bt ingredients control moth larvae found on plants, other Bt ingredients are specific for larvae of flies and mosquitoes.

3. Plant – incorporated protectants (pips):

- Pesticidal substances that plants produce from genetic material that has been added to the plant.

- Eg: scientists can take the gene for the Bt pesticidal protein and introduce the gene into the plant's own genetic material.

- Then the plant, instead of the Bt bacterium, manufactures the substance that destroys the pest.

Advantages of biopesticides:

- Less toxic than conventional pesticides.
- Effect only the target pest and closely related organisms
- whereas conventional pesticides are broad spectrum pesticides.
- Effective in very small quantities and often decompose quickly, resulting in lower exposures and largely avoiding pollution problems caused by conventional pesticides.
- When used as a component of Integrated Pest Management programs, biopesticides can greatly reduce the use of conventional pesticides while crop yields remain high.

BACTERIAL BIOPESTICIDES:

- Mainly 4 categories:

1.Crystalliferous spore formers (Bacillus thuringiensis)

2.Obligate pathogens (Bacillus papiliae)

3.Potential pathogens (Serratia marcesens)

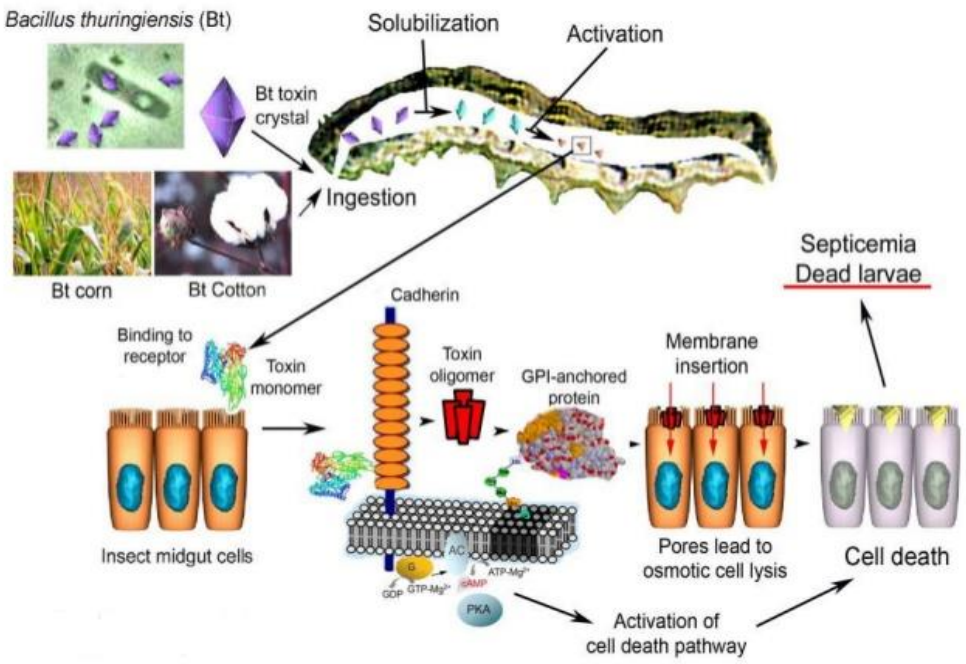
4.Facultative pathogens (Pseudomonas aeruginosa) Out of these four, 1 and 2 are important biopesticides.

Bacillus thuringiensis

- Gram positive, spore forming, facultative bacterium with nearly 100 subspecies and varieties divided into 70 serotypes.
- Specific, safe and effective tool for insect control.
- Insecticidal property resides in Cry family of crystalline proteins that are produced in the parasporal crystals and are encoded by the cry genes

***B.thuringiensis* CRY proteins:**

- Cry proteins are globular molecules with 3 structural domain connected by single linkers.
- This 3 domain family is characterised by protoxins of two different lengths, one being longer with C – terminal extension necessary for toxicity.
- This extension also has a characteristic role in crystal formation within the bacterium.
- Cry proteins are responsible for feeding cessation and death of the insect.



Mechanism of Cry Protein:

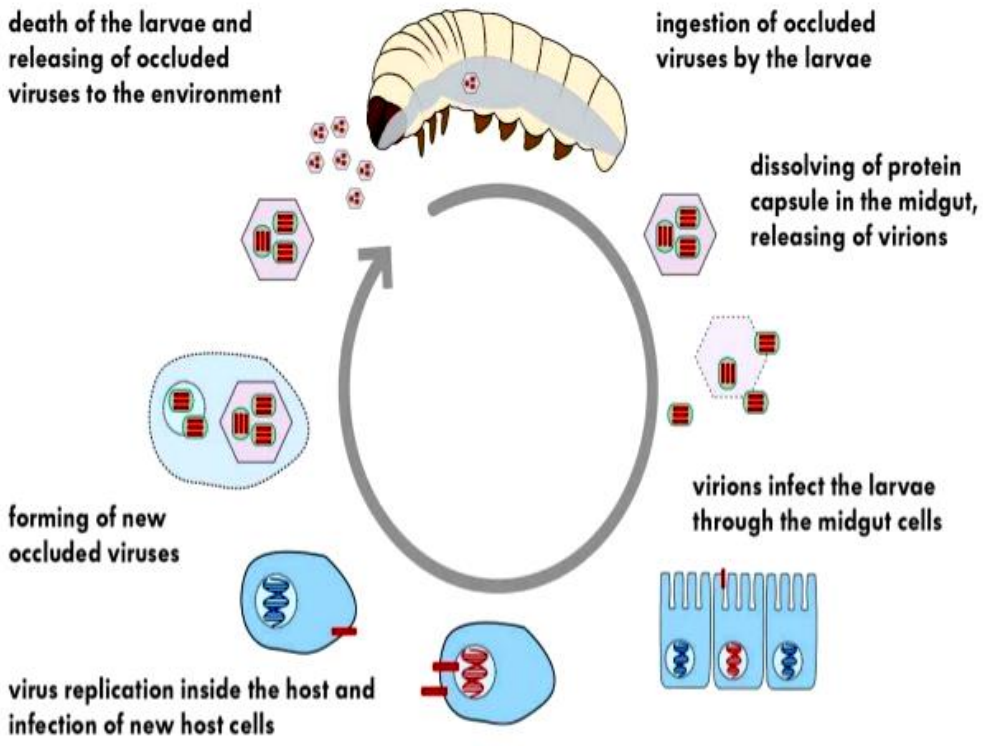
- Cry protoxins are ingested and then solubilised, releasing a protease resistant biologically active endotoxin, before it is being digested by protease of the gut to remove amino acids from its C and N terminal ends.
- The C terminal domain of the active toxin binds to the specific receptors on brush border membranes of the midgut.
- It is followed by the insertion of the hydrophobic region of the toxin into the cell membrane
- This creates a disruption in the osmotic balance because of the formation of transmembrane pores.
- Ultimately cell lysis occurs in the gut wall leading to leakage of gut contents.
- This induces starvation and lethal septicaemia of the target pest.

Pseudomonas fluorescens (Phenazine):

- This bacteria is used to control damping off caused by *Pythium sp., Rhizoctonia solani, Gaeumannomyces graminis.*
- It has ability to grow quickly in the rhizosphere

VIRAL BIOPESTICIDES:

- The viruses used for pest control are: DNA containing baculoviruses (BVs) Nucleopolyhedrosis viruses (NPVs) Granuloviruses (GVs) Acoviruses Parvoviruses Polydnaviruses Pox viruses RNA containing recoviruses Cytoplasmic polyhedrosis viruses Nodaviruses Picorna like viruses Tetraviruses
- They are narrow spectrum
- After application to plant surface, baculovirus occlusion bodies (OBs) are rapidly inactivated by solar UV radiation (280 – 320nm).
- Efficacy can be improved by the use of formulations that include stilbene derived optical brighteners, which increase susceptibility to NPV infection.
- UV inactivation can be controlled by creating systems which filter UV radiation such as plastic greenhouse structures



Mechanism of Viral Biopesticide:

- Replication of virus occurs in the nuclei or cytoplasm of the target cell
- The expression of viral proteins occurs in 3 phases:
  - Early phase ie, 0-6 hr post infection
  - Late phase ie, 6-24 hr post infection
  - Very late phase ie, upto 72 hr post infection

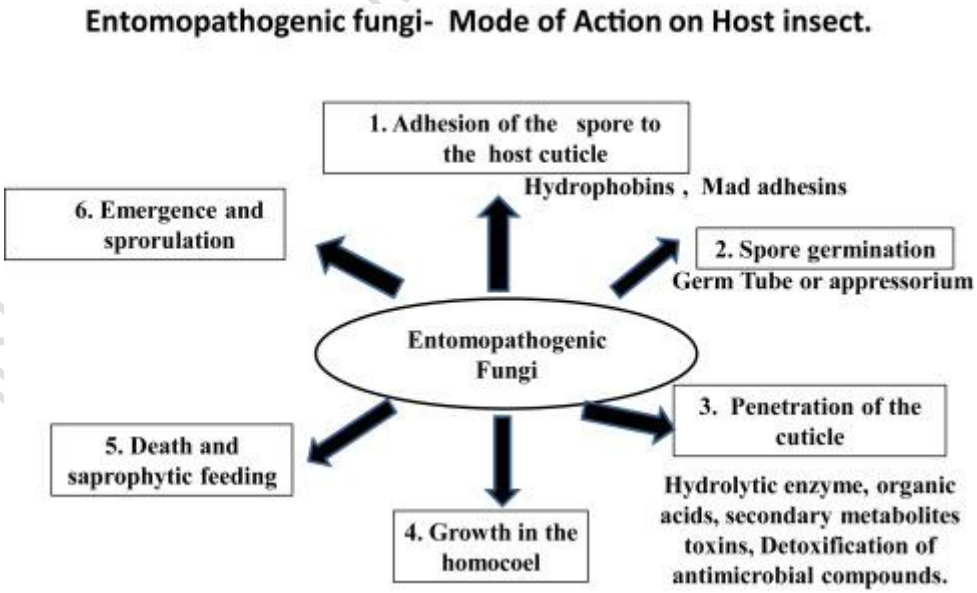
- ❖ It is at the late phase that the virions assemble as the 29kDa occlusion body protein is synthesised.
- ❖ Virions of NPVs are occluded within each occlusion body to develop polyhedra whereas the GV virion is occluded in a small occlusion body to generate granules
- ❖ Infected nuclei can produce 100s of polyhedra and 1000s of granules per cell.
- ❖ These can create enzootics, deplete the pest populations and ultimately create significant impact on the economic threshold of the pest.

Endamopathogenic fungi:

An **entomopathogenic fungus** is a **fungus** that can act as a parasite of insects and kills or seriously disables them.

Examples of Endamopathogenic fungi:

Many common and/or important entomopathogenic fungi are in the order Hypocreales of the Ascomycota: the asexual (anamorph) phases *Beauveria*, *Isaria* (was *Paecilomyces*), *Hirsutella*, *Metarhizium*, *Nomuraea* and the sexual (teleomorph) state *Cordyceps*; others (*Entomophthora*, *Zoophthora*, *Pandora*, *Entomophaga*) belong in the order Entomophthorales of the Zygomycota.



UNIT IV: Plant and algal biotechnology and bioremediation

Plant biotechnology (PBT) encompasses a multitude of scientific tools and techniques for screening and genetic manipulation of **plants** to develop beneficial or useful **plant**/**plant** products. The proficiency of these tools and techniques could be augmented by nanotechnological interventions.

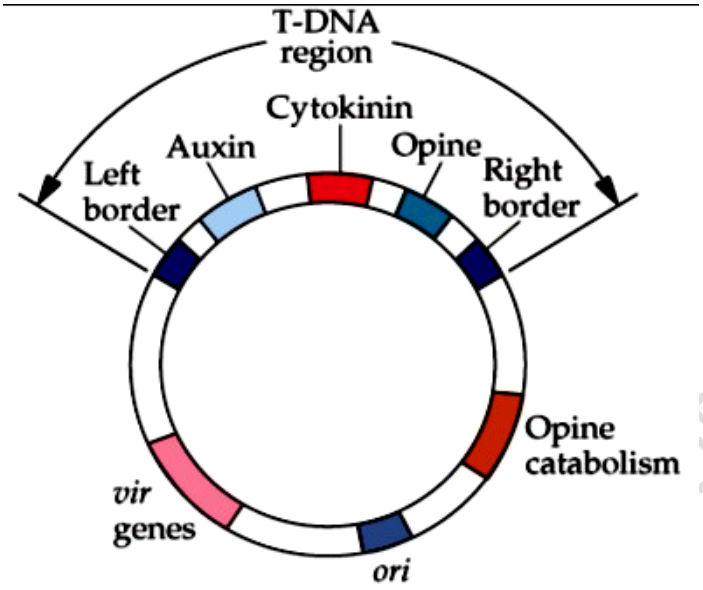
Algal biotechnology is a technology developed using **algae**. This will assist with the development of commercial-scale micro **algal** culturing techniques for the production of bioactive compounds, aquaculture feed, fine chemicals, and renewable fuels.

Bioremediation is a process used to treat contaminated media, including water, soil and subsurface material, by altering environmental conditions to stimulate growth of microorganisms and degrade the target pollutants.

Ti plasmid

- A **Ti** or **tumour inducing** plasmid is a plasmid that often, but not always, is a part of the genetic equipment that *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* use to transduce their genetic material to plants.
- The Ti plasmid is lost when *Agrobacterium* is grown above 28 °C. Such cured bacteria do not induce crown galls, i.e. they become avirulent.
- pTi and pRi share little sequence homology but are functionally rather similar.
- The Ti plasmids are classified into different types based on the type of opine produced by their genes.
- The different opines specified by pTi are octopine, nopaline, succinamopine and leucinopine.
- The plasmid has 196 genes that code for 195 proteins. There is one structural RNA.
- The plasmid is 206,479 nucleotides long, the GC content is 56% and 81% of the material is coding genes. There are no pseudogenes.
- The modification of this plasmid is very important in the creation of transgenic plants.

Structure of Ti Plasmid:



Vir (virulent) Gene:

1. Transfer the T-DNA to plant cell

2. Acetosyringone (AS) (a flavonoid) released by wounded plant cells activates vir genes.

3. virA,B,C,D,E,F,G (7 complementation groups, but some have multiple ORFs), span about 30 kb of Ti plasmid. **Function of vir genes:**

- ✓ **virA** - transports acetosyringone into bacterium, activates virG post-translationally (by phosphorylation)
- ✓ **virG** - promotes transcription of other vir genes
- ✓ **virD2-** endonuclease/integrase that cuts T-DNA at the borders but only on one strand.
- ✓ **virE2** - can form channels in membranes
- ✓ **virE1** - chaperone for virE2
- ✓ **virD2** & **virE2** also have NLSs, gets T-DNA to the nucleus of plant cell
- ✓ virB - operon of 11 proteins, gets T-DNA through bacterial membranes

Opines:

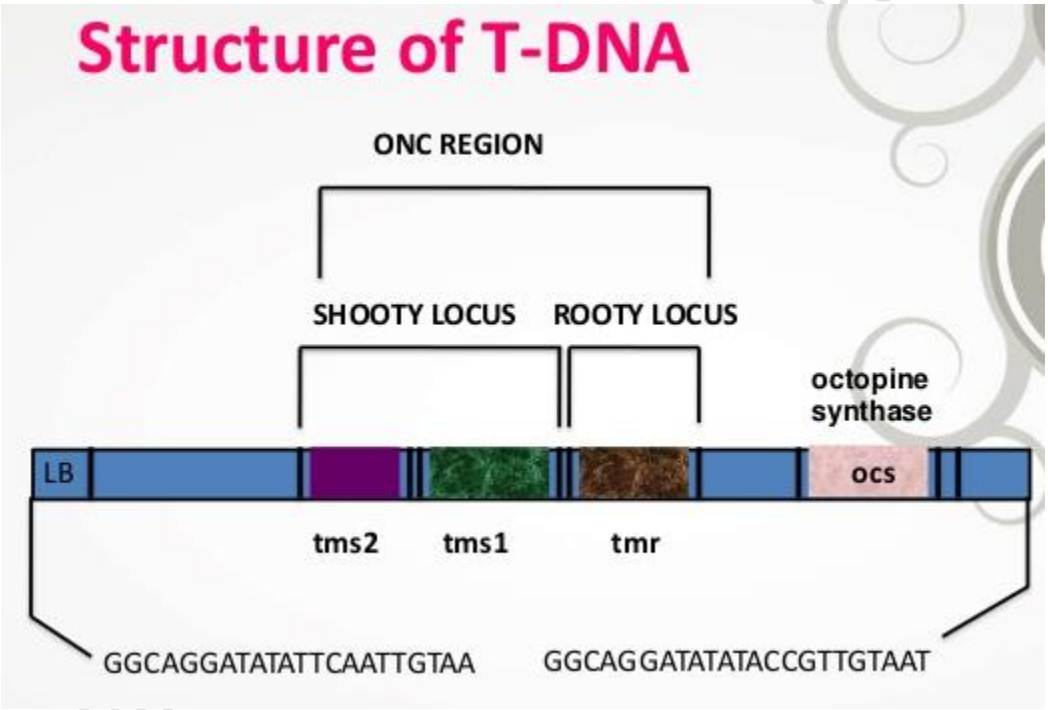
- ❖ Derivatives of amino acids synthesized by T-DNA
- ❖ Ti plasmids can be classified according to the opines produced:

1. Nopaline plasmids

2. Octopine plasmids

3. Agropine plasmids

- ❖ Nopaline plasmids : carry gene for synthesizing nopaline in the plant and for utilization (catabolism) in the bacteria.
- ❖ Octopine plasmids : carry genes to synthesize octopine in the plant and catabolism in the bacteria.
- ❖ Agropine plasmids : carry genes for agropine synthesis and catabolism.



Ti Plasmid-Derived Vector Systems:

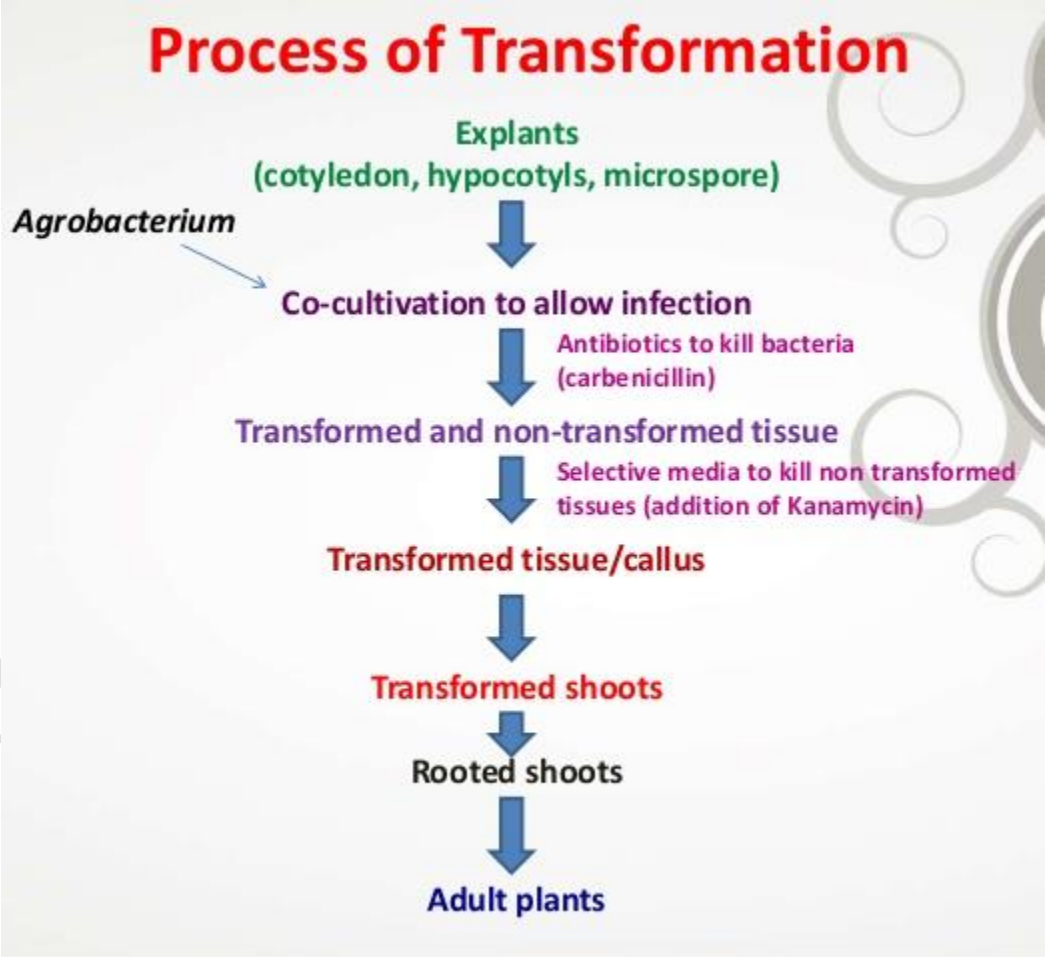
- ❖ Using Ti plasmid as a vector it is possible to insert a desiredϖ DNA sequence (gene) into the T DNA region of Ti plasmid.
- ❖ There are several limitations to use Ti plasmids directly as cloning vectors :-
  - LARGE SIZE
  - TUMOR INDUCTION PROPERTY
  - ABSENCE OF UNIQUE RESTRICTION SITES
- ❖ Agrobacterium plasmids are disarmed by deleting naturally occurring T-DNA encoded oncogenes and replacing them with foreign genes of interest.

- The right and left border sequences of T-DNA which is required for T-DNA integration.
- A multiple cloning site.
- An origin of replication
- A selectable marker gene

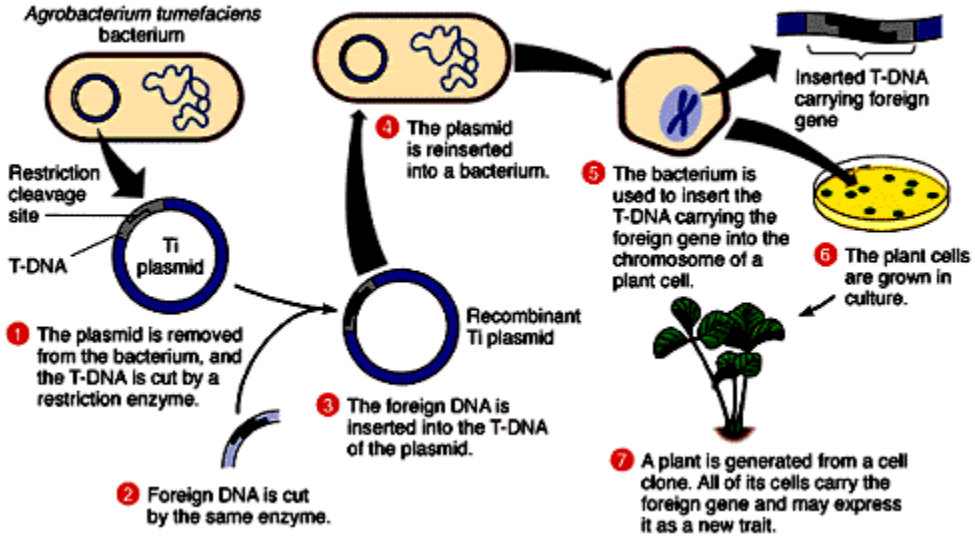
Agrobacterium mediated transformation:

The important requirements for Agrobacterium- mediated gene transfer in higher plants are as follows:-

- The plant explants must produce acetosyringone for activation of Vir genes.
- The induced Agrobacterium should have access to cells that are competent for transformation.
- Explants include cotyledon, leaf, thin tissue layer, peduncle, hypocotyls, stem, microspores

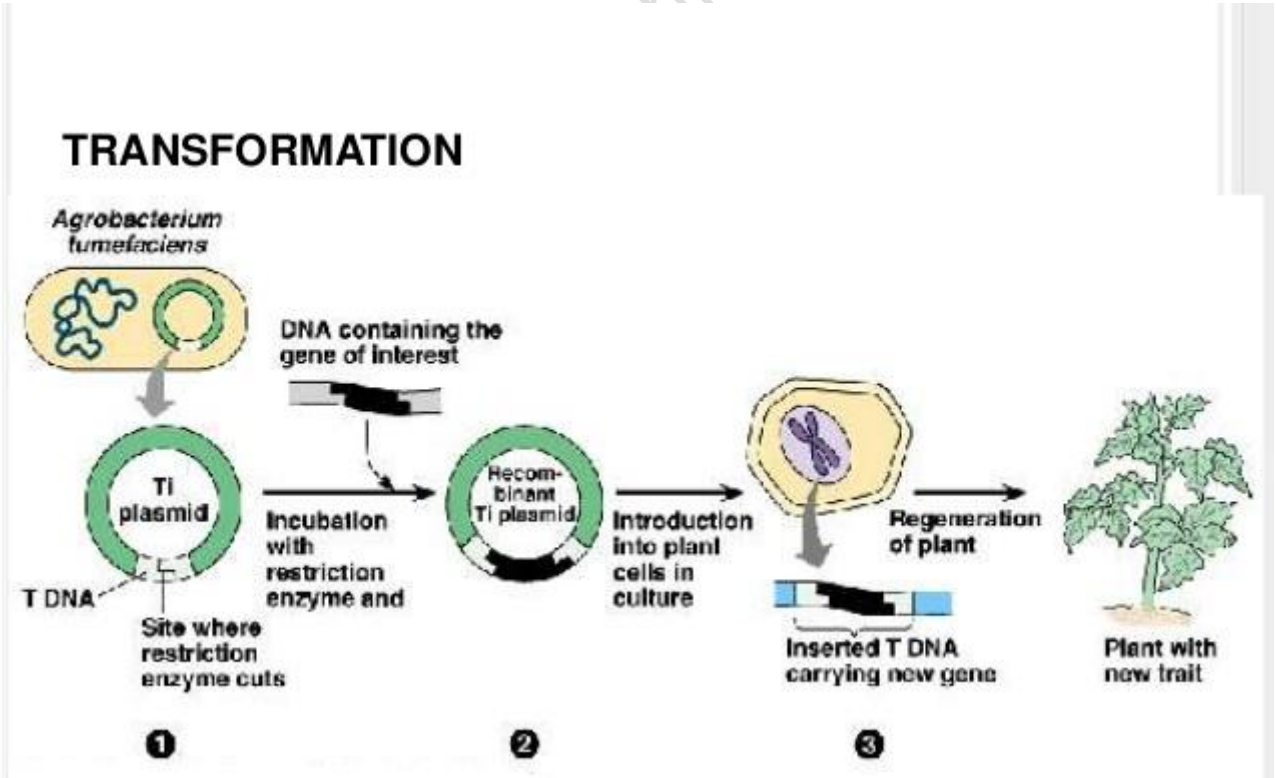


Insect, Virus and Herbicide Resistant Plant:

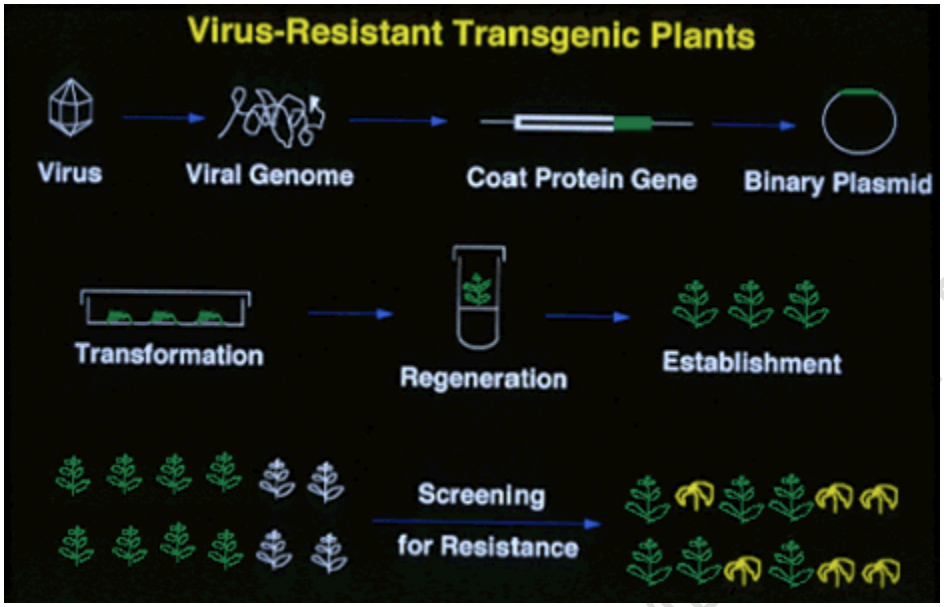


Insect resistant transgenic plants:

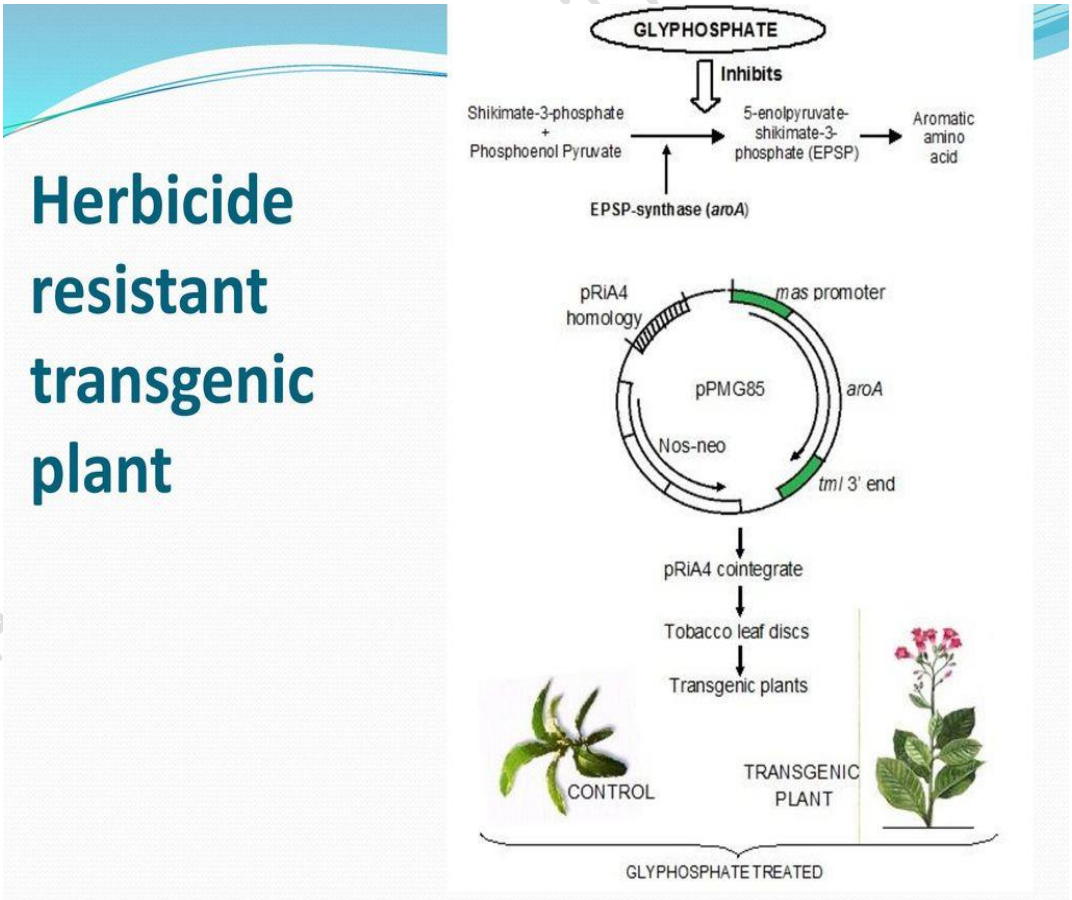
Crops that either naturally or through genetic engineering are able to resist **insect** damage. **Insect-resistant crops** generally produce compounds that are toxic to **insects** that attempt to eat the **resistant plants**.



Virus Resistant Plants:



Herbicide Resistant Plant:



Stress and senescence tolerance in plants:

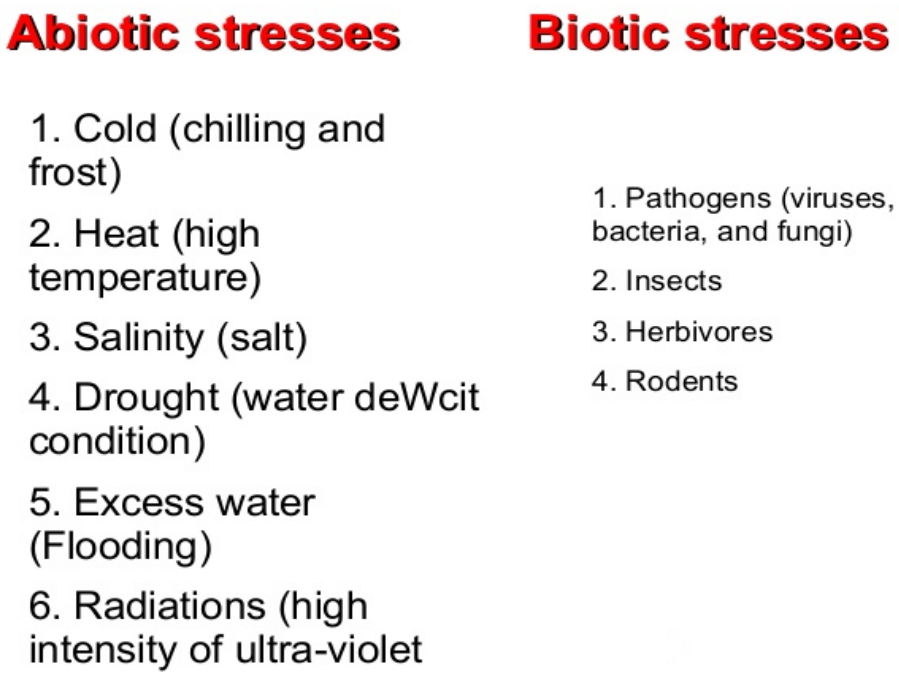
Stress-induced **senescence** and **plant tolerance** to abiotic **stress**. **Senescence** is an age-dependent process, ultimately leading to **plant** death that in annual crop **plants** overlaps with the reproductive stage of development

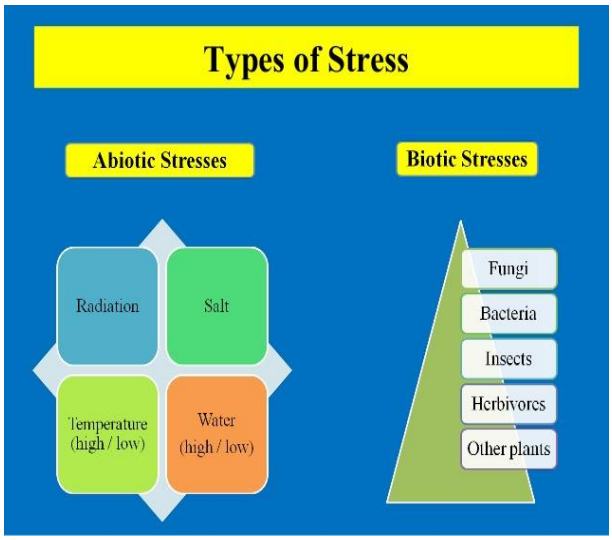
Plant senescence

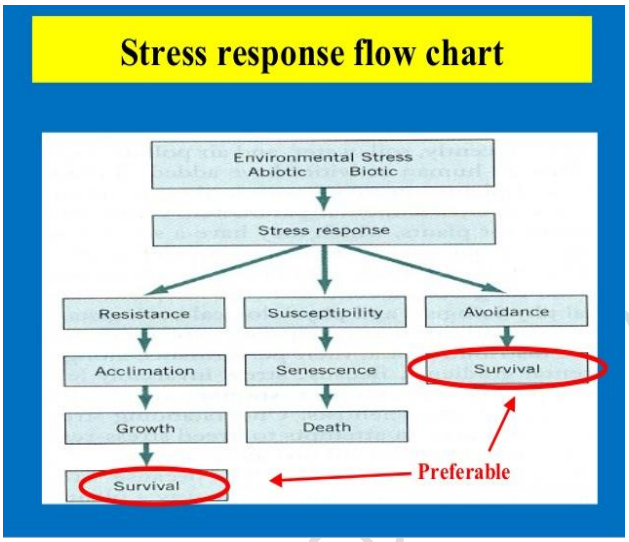
Plant senescence is the process of aging in plants. Plants have both stress-induced and age-related developmental agingChlorophyll degradation during leaf senescence reveals the carotenoids, and is the cause of autumn leaf color in deciduous trees. Leaf senescence has the important function of recycling nutrients, mostly nitrogen, to growing and storage organs of the plant. Unlike animals, plants continually form new organs and older organs undergo a highly regulated senescence program to maximize nutrient export.

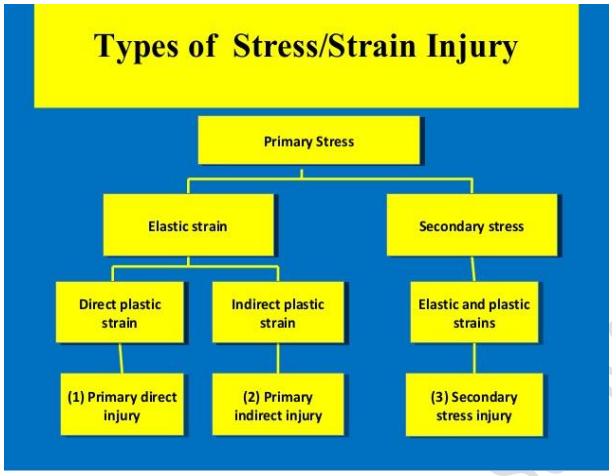
STRESS:

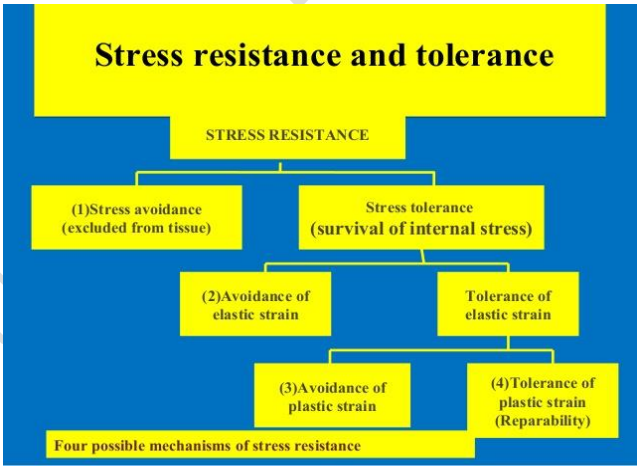
1. **Biotic stress** is stress that occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial and harmful insects, weeds, and cultivated or native plants.
2. **Abiotic stress** is defined as the negative impact of non-living factors on the living organisms in a specific environment. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the organism

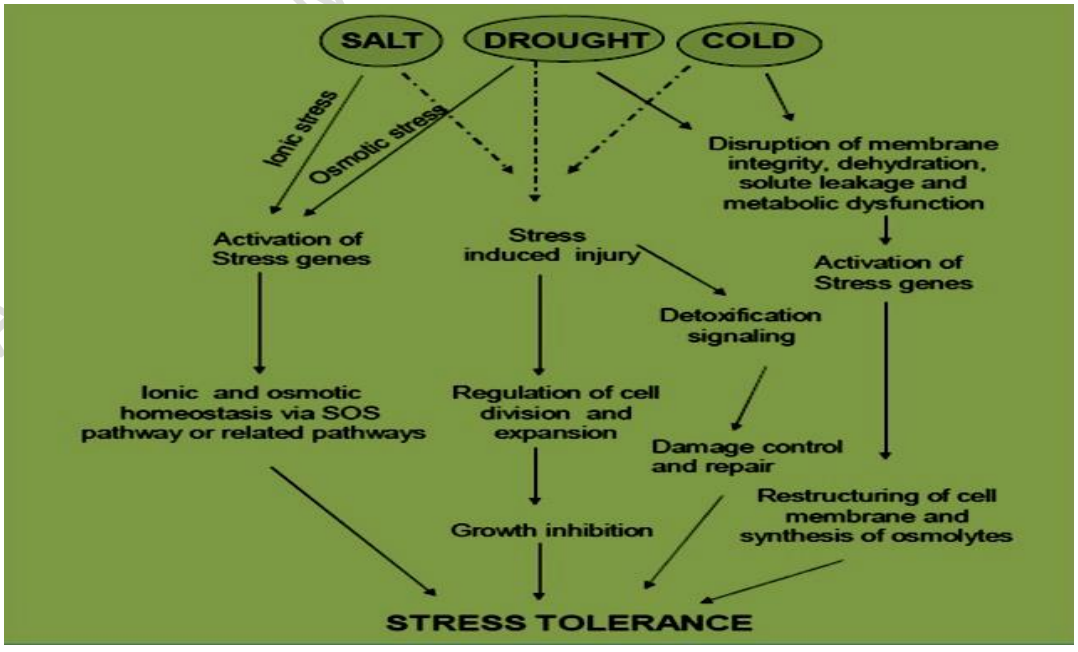



Types of Stress
Abiotic Stresses
Biotic Stresses
Radiation
Salt
Temperature (high / low)
Water (high / low)
Fungi
Bacteria
Insects
Herbivores
Other plants


Stress response flow chart
Environmental Stress
Abiotic Biotic
Stress response
Resistance
Susceptibility
Avoidance
Acclimation
Senescence
Survival
Growth
Death
Survival
Preferable


Types of Stress/Strain Injury
Primary Stress
Elastic strain
Secondary stress
Direct plastic strain
Indirect plastic strain
Elastic and plastic strains
(1) Primary direct injury
(2) Primary indirect injury
(3) Secondary stress injury


Stress resistance and tolerance
STRESS RESISTANCE
(1)Stress avoidance (excluded from tissue)
Stress tolerance (survival of internal stress)
(2)Avoidance of elastic strain
Tolerance of elastic strain
(3)Avoidance of plastic strain
(4)Tolerance of plastic strain (Reparability)
Four possible mechanisms of stress resistance


SALT
DROUGHT
COLD
Ionic stress
Osmotic stress
Disruption of membrane integrity, dehydration, solute leakage and metabolic dysfunction
Activation of Stress genes
Stress induced injury
Activation of Stress genes
Detoxification signaling
Ionic and osmotic homeostasis via SOS pathway or related pathways
Regulation of cell division and expansion
Damage control and repair
Restructuring of cell membrane and synthesis of osmolytes
Growth inhibition
STRESS TOLERANCE

Plants must be stress resistant to survive:

- Avoidance also possible by morphological adaptations
  - − Deep tap roots in alfalfa allow growth in arid conditions
  - − Desert CAM plants store H2O in fleshy photosynthetic stems
- Stress resistant plants can tolerate a particular stress
- Resurrection plants (ferns) can tolerate dessication of protoplasm to < can rehydrate dried leaves less than 7% H_2O
- Plants may become stress tolerant through

  – Adaptation: heritable modifications to increase fitness • CAM plants' morphological and physiological adaptations to low H2O environment

  – Acclimation: nonheritable physiological and biochemical gene expression

  • Cold hardening induced by gradual exposure to chilling temperature, cold-hardy plants Alfalfa taproot

Abiotic Stress:

1. **Temperature:**
   - Plants exhibit a wide range of Topt (optimum temperature) for growth
   - This is because their enzymes have evolved for optimum activity at a particular T
   - Properly acclimated plants can survive overwintering at extremely low Ts
   - Environmental conditions frequently oscillate outside ideal T range
   - Deserts and high altitudes: hot days, cold nights
   - Three types of temperature stress affect plant growth – Chilling, freezing, heat
2. **Chilling Injury:**
   - Common in plants native to warm habitats − Peas, beans, maize, Solanaceae
   - It Affects − seedling growth and reproduction − multiple metabolic pathways and physiological processes λ Cytoplasmic streaming λ Reduced respiration, photosynthesis, protein synthesis λ Patterns of protein expression
   - Initial metabolic change precipitating metabolic shifts thought to be alteration of physical state of cellular membranes
   - Temperature changes lipid and thus membrane properties
   - Chilling sensitive plants have more saturated FAs in membranes: these congeal at low temperature gel transition occurs abruptly at transition
   - Liquid crystalline - gell transition occurs abruptly at transition temperature

BIOTIC STRESS: Pathogen (e.g., fungus) invasion

- Plant reaction to invading pathogens center around the hypersensitive reaction
- The hypersensitive reaction initiates many changes in plant physiology and biochemistry

– Early activation of defense related genes to synthesize pathogenesis related (PR) proteins

• Protease inhibitors to stop cell wall lysis by specific enzymes expressed by pathogen

• Bacterial cell wall lytic enzymes (chitinase, glucanase)

– Change cell wall composition

• Express enzymes providing structual support to cell walls via synthesis of lignin, suberin, callose, glycoproteins

– Synthesize secondary metabolites to isolate and limit the pathogen spread

• These include isoflavonoids, phytoalexins

– Apoptosis at invasion site to physically cut off rest of plant.

- Pathogens may be more or less potentially infectious to a host

– Virulent on susceptible hosts

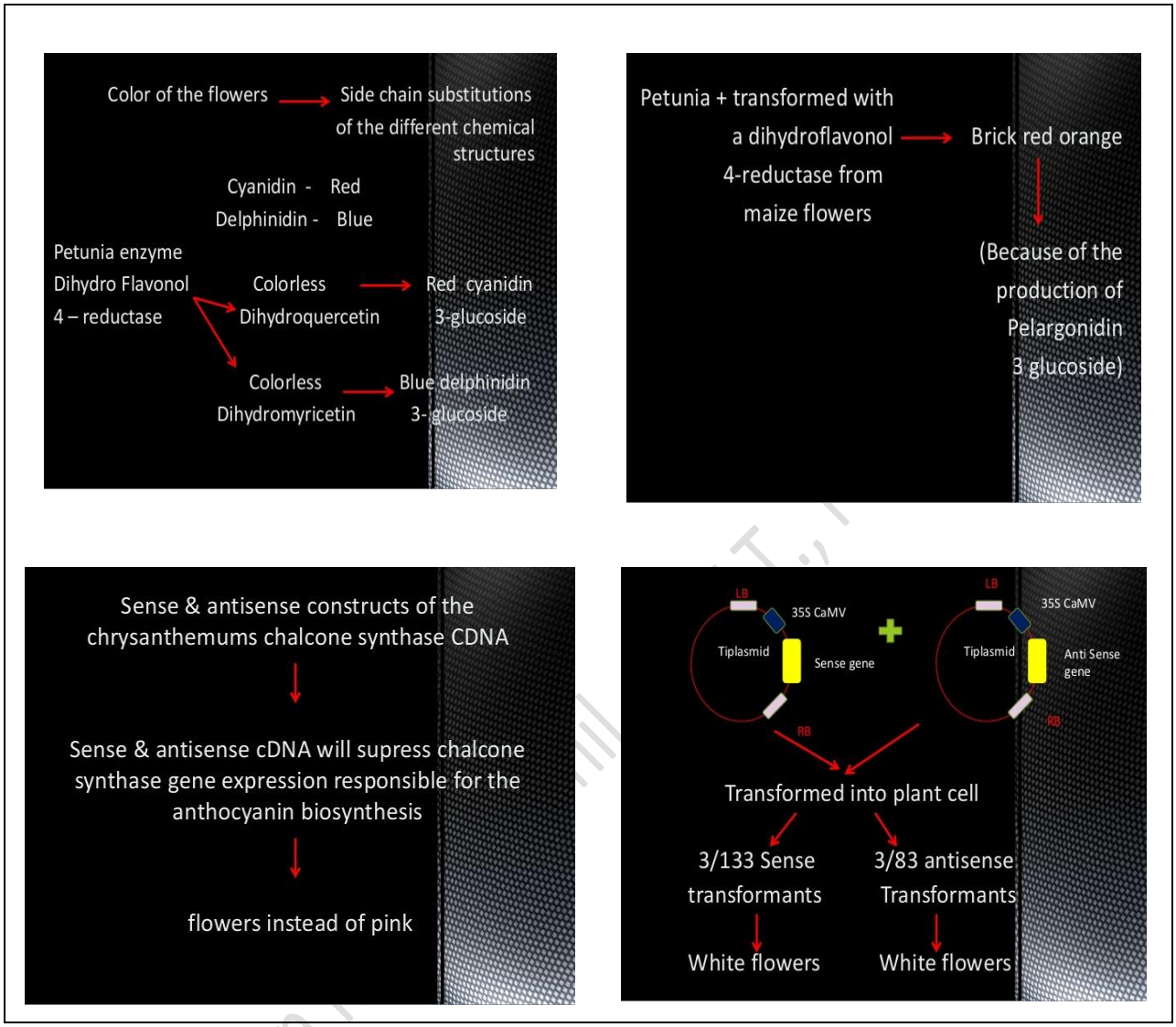
– avirulent on non-susceptible hosts

- Pathogens carry avirulence (avr) genes and hosts carry resistance (R) genes
- The normal presence of both prevents pathogens from attacking the plant
- Infection occurs when pathogen lacks avr genes or plant is homozygous recessive for resistance genes (rr)
- In these cases, the plant cannot initiate the hypersensitive reaction
- **Pathogen:** avr genes may code for proteins that produce elicitors – bits of pathogen: polysaccharides, chitin, or bits of damaged plant: cell wall polysaccharides
- **Plant:** R genes may be elicitor receptors

Modification of flower pigmentation & nutritional content

- To improve flower appearance and postharvest lifetime.
- By traditional breeding techniques thousands of new varieties that differ from one another in color, shape, and plant architecture has been created.
- But this is a slow and pains taking procedure
- Uniquely colored flowers can be developed by manipulating the genes for enzymes in the anthocyanin biosynthesis pathway.
- Anthocyanin is a flavonoids common type of flower pigmentation.

- Four plants 1. Roses 2. Carnations 3. Tulips 4. Chrysanthemums • Used to form unique colours.

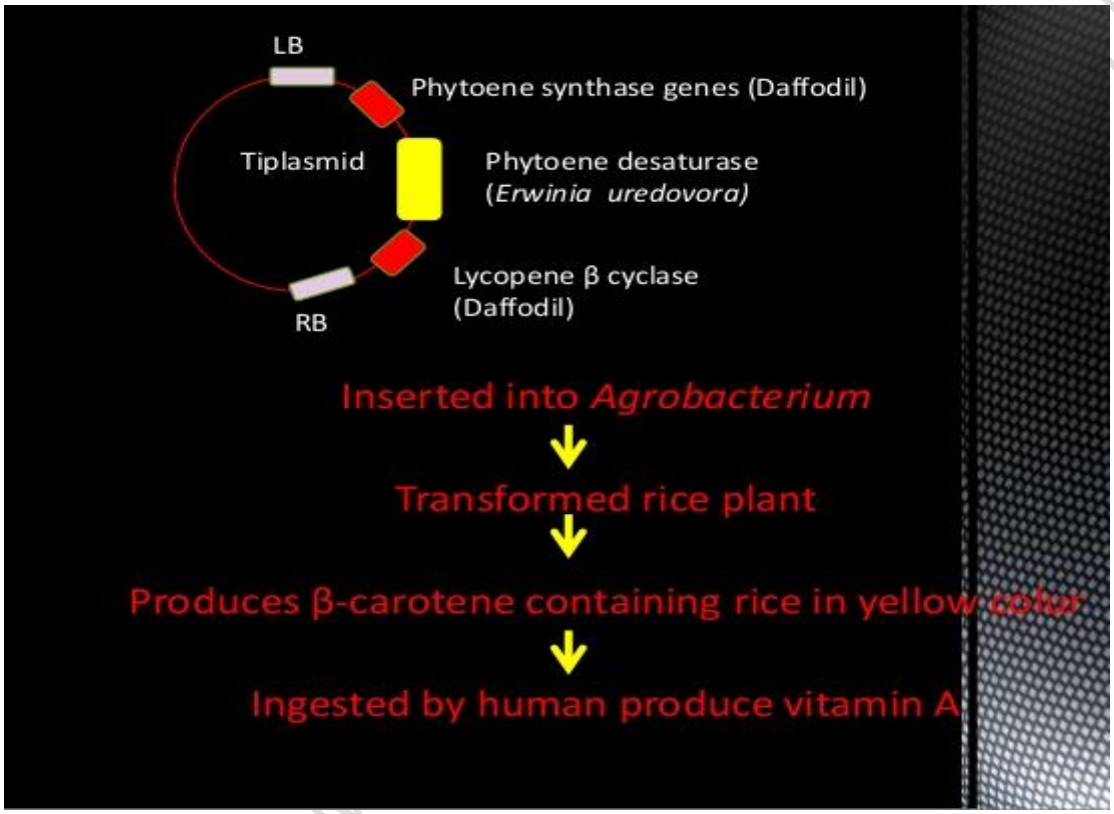


Modification of nutritional content:

- ✪ Genetic engineering has been used to improve
  - i. The nutritional quality of several different plants, including corn and pea, by modification of aminoacid.
  - **ii.** The fatty acid composition of both edible and non-edible oil-producing crops
  - **iii.** The taste of fruits and vegetables by the introduction of monellin, a sweet tasting protein.

1. **VITAMINS (VIT A):**
   - ✓ Rice is the staple food of approximately half of the world's population.
   - ✓ It is a poor source of several nutrients and vitamins like vit A

- ✓ To overcome vitamin A deficiency genetic engineering of rice to produce Vitamin A from β- Carotene
- ✓ Which is common carotenoid pigment normally found in plant photosynthetic membranes
- ✓ Agrobacterium mediated transfromation to introduce the entire βcarotene biosynthetic pathway into the rice.

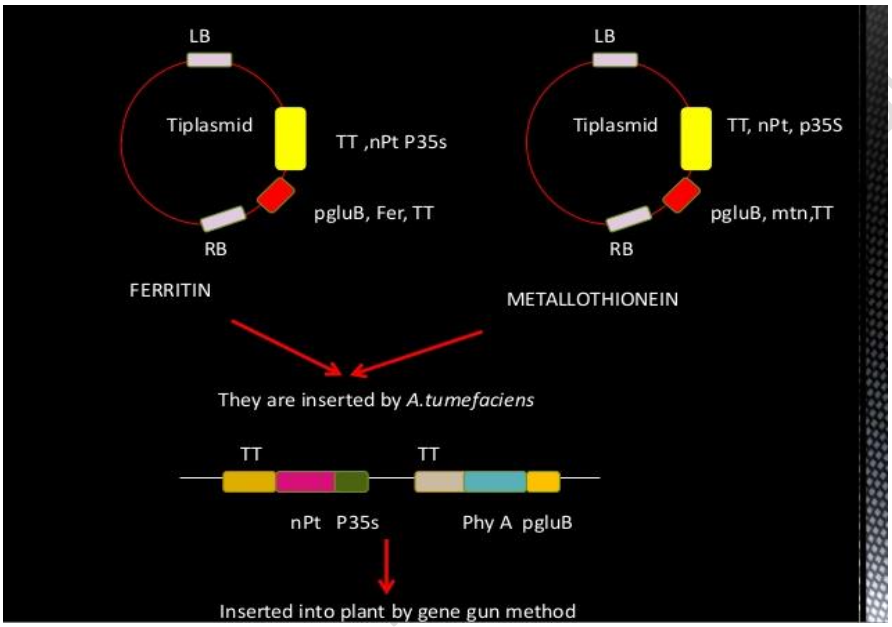


2. **IRON:**
   - ✓ WHO has estimated that ironϖ deficiency affects approximately 30% of world's population.
   - ✓ A number of crops are rich in iron isϖ often prevented by the phytic acid present in plants
   - ✓ First step toward developing food crops with high enough level of iron in rice plants to express soybean protein ferritin
   - ✓ Ferritin Iron storage Found in carries upto protein animals, plants, 4500 iron bacteria atoms
   - ✓ Soybean ferritin cDNA was cloned into a binary vector under the transcriptional control of the rice seed storage protein glutein promoter

- ✓ Introduced into plants by electroporation
- ✓ Ferritin is expressed only in seeds not in any other tissues, iron content is increased approximately 2.5times

✪ Rice plants were transformed with three different genes.

- **To increase iron** - Ferritin encoding Content cDNA from green beans.(P.vulgaris)
- **To improve** - cDNAs encoding phytase Bioavailability & metallothinein (A.fumigatus)
- **Expression of these genes controlled by** – endosperm specific promoter



Plant as Bioreactor:

✪ Plant bioreactors refer to the use of transgenic plants and cell cultures of plants to make unlimited quantities of commercially important substances like recombinant proteins including antibodies and vaccines using biotechnology oriented techniques

✪ Using genetic engineering, cereal plants, fruits plants, legumes and vegetable plants have the capacity to become low cost bioreactors to make molecules that in the normal scheme of things would not have been available from plants.

✪ Human growth hormone was the first drug that was produced using plant bioreactors, in this case from the transgenic tobacco

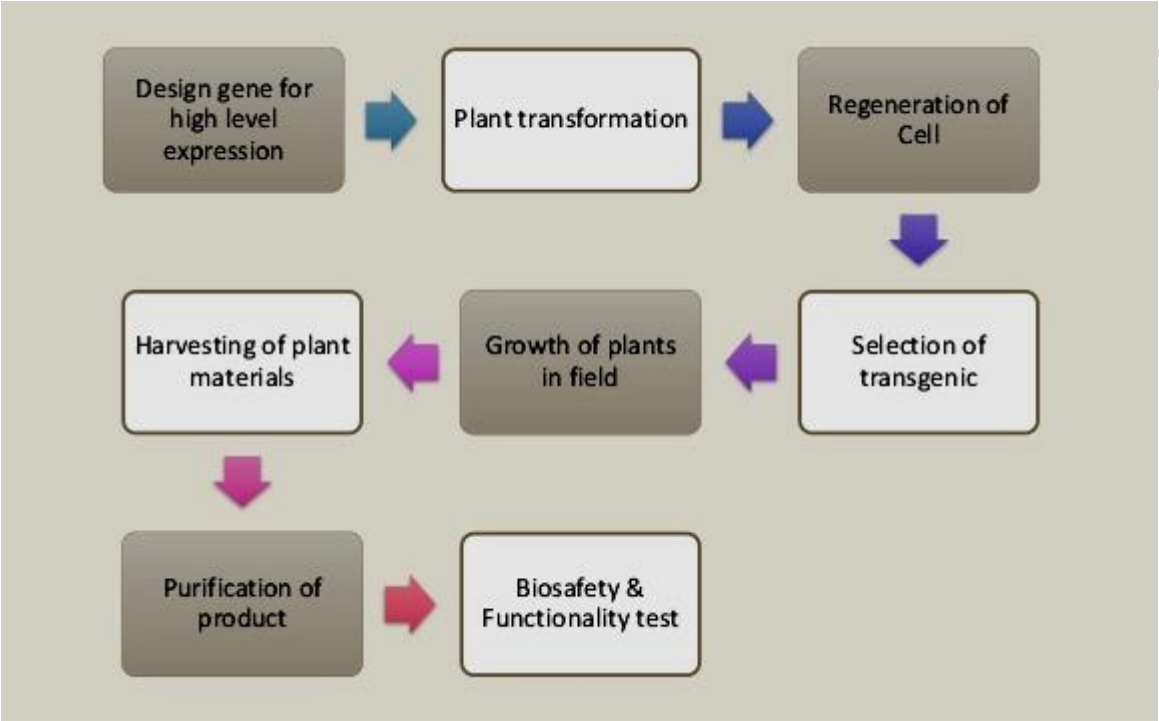
✪ Most of the research has been directed towards using plant bioreactors

- Therapeutic proteins
- Edible vaccines
- Antibodies for immunotherapy

✪ There are two basic processes that are used to produce recombinant proteins in plants

1. generating the transgenic plants by stable integration of transgene into plant genome
2. transient expression of the transgene using plant viruses as vectors

✪ The other techniques used for direct gene transfer are electroporation, polythene glycol mediated gene uptake and particle bombardment.



TYPES OF PLANT REACTORS:

✪ There are different plant bioreactors classified based on where the protein is produced

1. SEED-BASED PLANT BIOREACTORS
2. PLANT SUSPENSION CULTURES
3. HAIRY ROOT SYSTEM BIOREACTOR
4. CHLOROPLAST BIOREACTOR

1. **CHLOROPLAST BIOREACTOR** :

■ The nuclear chromosomes of chloroplasts are inserted with the foreign genes that are responsible for required product.

■ Insulin, interferons and other proteins can be prepared in chloroplast bioreactor

■ An example is the high yield in the expression of human serum albumin protein in chloroplast

2. **HAIRY ROOT SYSTEM BIOREACTOR** :

■ This has rhizosecretion caused due to infection of agro bacterium rhizogenes and is highly stable and suitable for different biopharmaceuticals

■ It offers extreme biosynthetic stability and is suitable for making biopharmaceuticals as for example scopolamine in Hyoscyamus muticus L. hairy root culture.

3. PLANT SUSPENSION CULTURES:

■ In this plant cells are grown under sterile conditions as suspension or callus cultures and given the appropriate hormonal supplements for growth and are used in expression of recombinant proteins, secondary metabolites and antibodies.

■ For example, is the expression of 80-kDa human lysosomal protein

4. **SEED BASED PLANT BIOREACTORS**

■ Seed is the most suitable bioreactor because of their large protein accumulation during its development

■ But specificity of expression and subcellular storage environment are the factors that will decide which seeds are used for producing desired products

■ There are two types of seed based plant bioreactors **1) Seed protein storage vacuole bioreactors 2) Seed oil body bioreactors**

1. Seed protein storage vacuole bioreactors

- The protein storage vacuoles in seeds contain some dominant sub compartments like matrix, globoid and crystalloids which are best for storing recombinant protein.

Matrix is suitable for soluble storage proteins, globoids for hydrolytic enzymes and crystalloids for some intrinsic protein sequences.

2. Seed oil body bioreactors

- This bioreactor can store a large amount of macromolecules.
- It has oleosin proteins which are ideal carriers of heterologous proteins encircling the seed oil body.
- This also provides recognition signal for lipase binding during oil mobilization in seedlings

- An example is the successful expression of the human lysosomal enzyme alpha-L-iduronidase in Arabidopsis thaliana seeds
- The advantage of these systems is that, proteins do not degrade at ambient temperature and are stable for long term storage

Microalgae:

Microalgae have been used as **food** by humans for thousands of years. **Microalgae** can convert solar energy to chemical energy by fixing CO_2, and its efficiency is ten times greater than terrestrial plants.

1. **Single Cell Protein:**
   - SCP are dried cells of micro organisms which can be used as dietary protein supplement.
   - They are used as animal feed & can be used for human feed as protein supplement.
   - Also called 'Novel Food' & 'Minifood'.
2. **Raw materials**

   • Production of SCP requires micro-organisms that serve as the protein source and the substrate that is biomass on which they grow

   • There is a variety of both the sources that can be used for the production of SCP.

   • The biomass used can be plant biomass or organic biomass.

   • The micro-organisms used belong to the group of Algae, Fungi and Bacteria.
3. **A list of the micro-organisms used for SCP production**:
   - ✓ **Fungi**• *Aspergillus fumigatus*• *Aspergillus niger*• *Rhizopus cyclopium*
   - ✓ **Yeast**• *Saccharomyces cerevisae*• *Candida tropicalis*• *Candida utilis*
   - ✓ **Algae**• *Spirulina sps.*• *Chlorella pyrenoidosa*• *Chondrus crispus*
   - ✓ **Bacteria**• *Pseudomonas fluroescens*• *Lactobacillus*• *Bacillus megaterium*
4. **Biomass:**

   • Biomass also plays a very important role in the production of SCP.

   • Selection of biomass depends on the micro-organisms used for the production.

   • For eg. Algae are cultivated on sewage whereas Yeast are cultured on agro-industrial wastes.

   ✓ **Algal Biomass:**

   • Algae grows auto- tropically.

   • Requires low intensity of light.

   • Temperature – 35 - 40 C & pH – 8. 5 -10.5

   • Cultivated in large trenches of sewage oxidation ponds.

   ✓ **Bacterial & Fungal biomass:**

   • Bacteria & fungi can be grown easily on a wide range of substrates

   • They require a minimum temperature of 15º-34ºc & a pH of 5- 7.

   ✓ **Yeast biomass**:

   • Cultivated on agro- industrial wastes such as molasses, starchy materials, fruit pulp, wood pulp, etc.

• Requires a temperature of 30 -34 c & pH of 3.5- 4.5.

• Also requires addition of inorganic acids & sulphur supplements in the form of salts.

5. SCP production:

• Selection of suitable strain

• Fermentation

• Harvesting

• Post harvest treatment

• SCP processing for food

✈ **Selection of strain:**

• It a very critical step as the quality of protein depends totally on the microbe that is used for the production.

• Thus careful selection of the strain should be done.

• Care should be taken that the selected strain should not produce any toxic or undesirable effects in the consumer.

✈ **Fermentation:**

• It can be carried out in the fermentor which is equipped with aerator, thermostat, pH, etc. or in the trenches or ponds.

• Microbes are cultured in fed- batch culture.

• Engineers have developed deep lift fermentor & air lift fermentor .

✈ **Harvesting:**

• When the colonies of microbes are fully developed, they are then harvested.

• The bulk of cells are removed from the fermentor by decantation.

✈ **Post harvest treatment:**

• After harvesting, the cells are subjected to a variety of processes.

• Post harvesting treatments includes steps like separation by centrifugation, washing, drying, etc.

6. **Processing for food:**

It includes

1. Liberation of cell proteins by destruction of indigestible cell wall.

A. Mechanical methods: • Crushing, crumbling, grinding, pressure homogenization, etc.

B. Chemical methods: • Enzymes & salts are used to digest or disrupt the cell wall.

• Salts like NaCl, sodium dodecyl sulfate, etc. whereas nuclease enzymes are used.

C. Physical methods: • Freeze- thaw, osmotic shock, heating & drying.

2. Reduction of nucleic acid content:

• Chemical & enzymatic treatments are preferred.

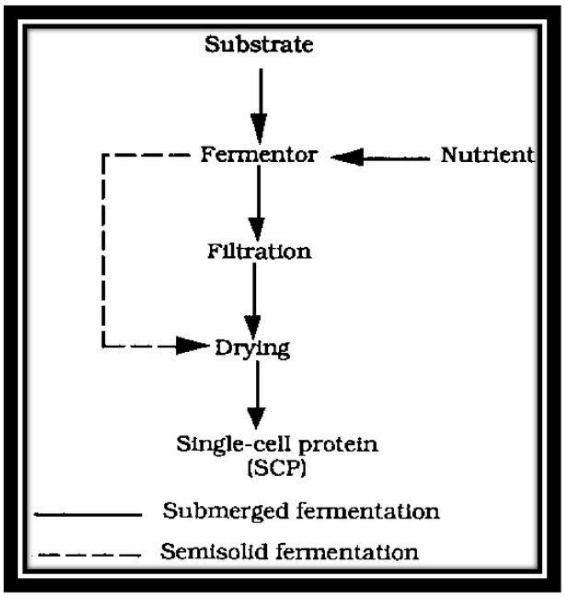
• Chemicals which are used includes acidified alcohol, salts, acids & alkalies.

• Use of such chemicals leads to formation of lygino-alanine which causes hypersensitivity skin reactions.

• Enzymes which are used include ribonuclease & nuclease enzymes

• These enzymes can be used exogenously or can be induced endogenously.

Basic Steps of SCP production:



7. Applications:

1. **As protein supplemented food**
   - Also source of vitamins, amino acids, minerals, crude fibers, etc.
   - Supplemented food for undernourished children.
2. **As health food**
   - Controls obesity
   - Provides instant energy
   - Example- Spirulina- part of diet of US Olympic team.

3. **In therapeutic and natural medicines**
    - Reduce body weight, cholesterol, stress.
    - Lowers blood sugar level in diabetic(due to presence of B - linolenic acid)
    - Prevents accumulation of cholesterol in body.
    - Healthy eyes and skin (beta carotene)
    - Beta carotene (anti cancer substance- UN National Cancer Research Institute)
    - Increase lactation.
4. **In cosmetics**
    - Important role in maintaining healthy hair (vitamin A and B).
    - Many herbal beauty products.
    - Biolipstics and herbal face cream (Phycocyanin).
    - Capable of replacing coal tar dye based cosmetics.
5. **Poultry and cattle feed**
    - Excellent, convenient source of protein and other nutrients.
    - Used to feed cattle, fishes etc.

Food coloring:

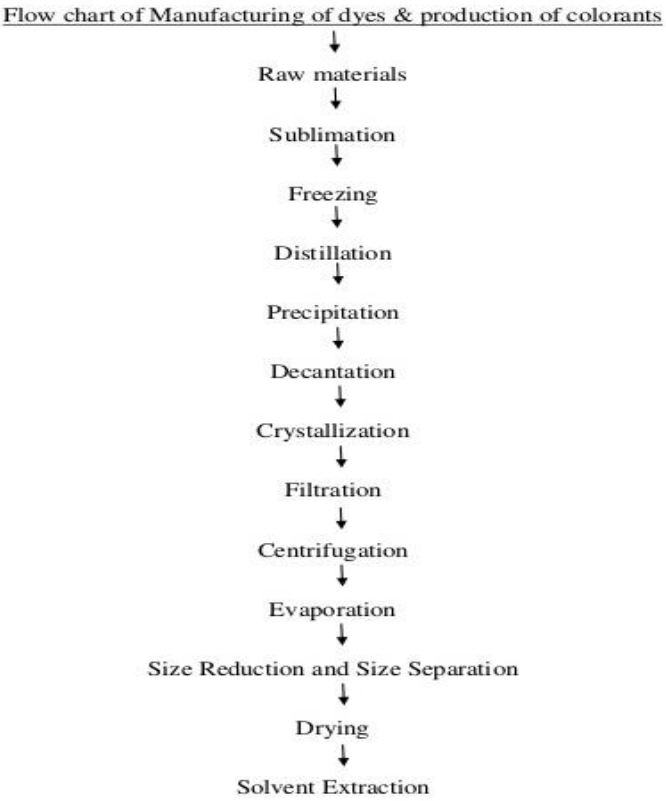
Food coloring, or **color additive**, is any dye, pigment or substance that imparts color when it is added to food or drink. They come in many forms consisting of liquids, powders, gels, and pastes. Food coloring is used both in commercial food production and in domestic cooking. Food colorants are also used in a variety of non-food applications including cosmetics, pharmaceuticals, home craft projects, and medical devices.

Purpose of food coloring

People associate certain colors with certain flavors, and the color of food can influence the perceived flavor in anything from candy to wine.Sometimes the aim is to simulate a color that is perceived by the consumer as natural, such as adding red coloring to glacé cherries (which would otherwise be beige), but sometimes it is for effect, like the green ketchup that Heinz launched in 1999. Color additives are used in foods for many reasons including:

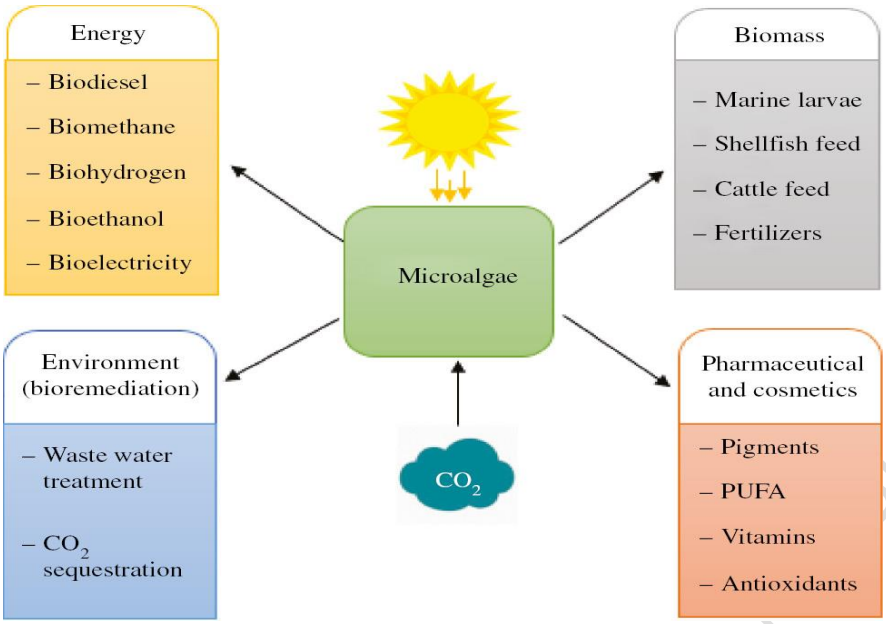
- To make food more attractive, appealing, appetizing, and informative
- Offset color loss due to exposure to light, air, temperature extremes, moisture and storage conditions
- Correct natural variations in color

- Enhance colors that occur naturally
- Provide color to colorless and "fun" foods
- Allow consumers to identify products on sight, like candy flavors or medicine dosages



Fuel Production:

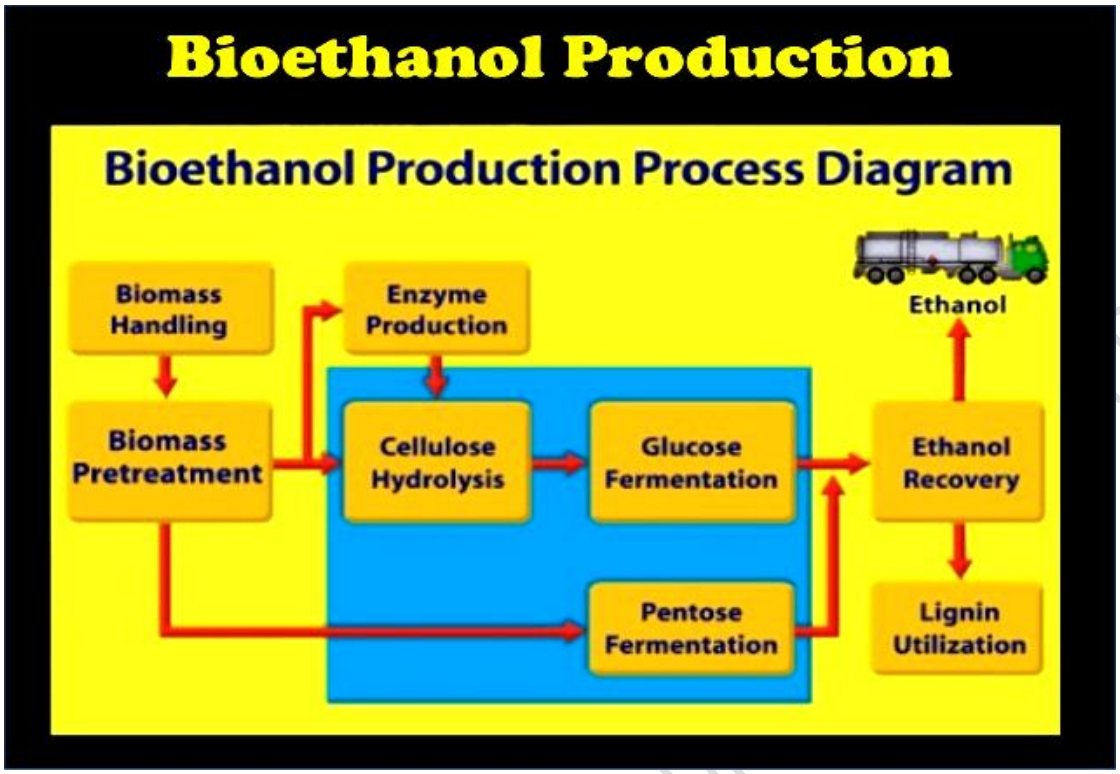
Microalgae has been reported to **produce** biogas as source of **fuel**, although the yield of biogas formation is quite low because of the sensitivity of **algal** cells to bacterial degradation and low carbon and nitrogen (C:N) ratio, which leads to the formation of inhibitor (ammonia).



Biofuels are liquid fuels that have been developed from other materials such as plants or animal waste matter by microbial action

Bioethanol:

- Bioethanol is an alcohol made by fermentation, mostly from carbohydrates produces in sugar or starch crops such as corn or sugarcane.
- Cellulosic biomass, derived from non-food sources such as trees and grasses, is also being developed as a feedstock for ethanol production.
- Used to substitute petrol fuel for the road transport vehicles
- One of the widely used alternative automotive fuels in the world (Brazil & USA are the largest ethanol producers)
- Much more environment friendly and have low toxicity level

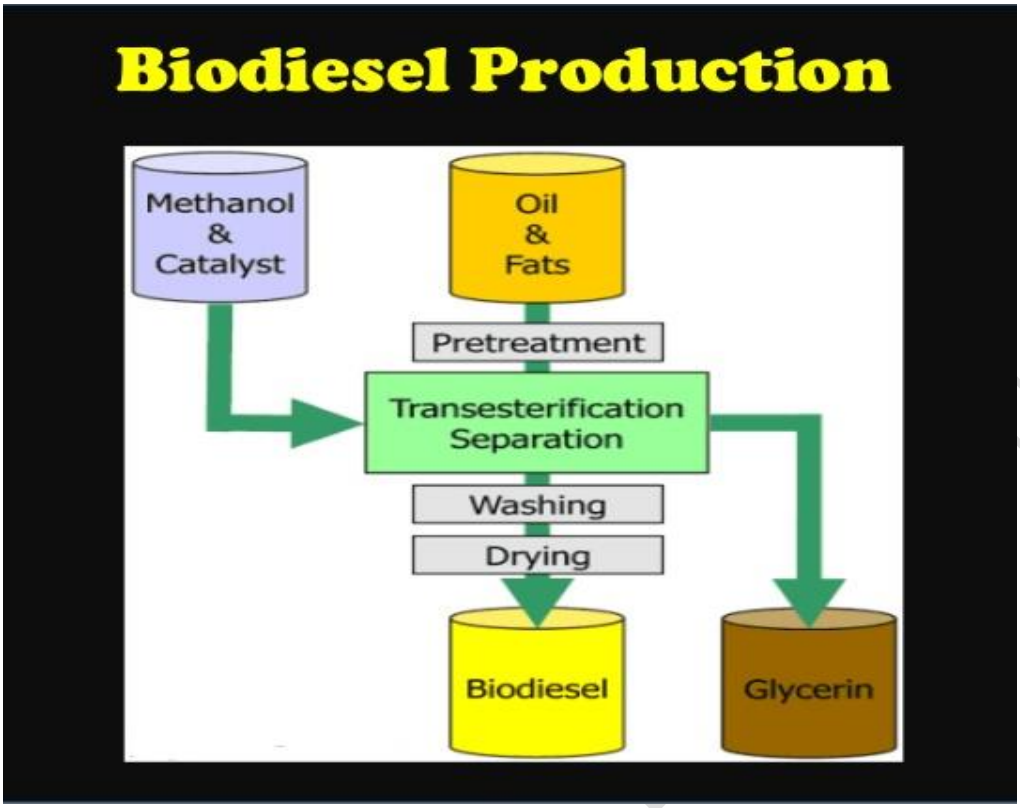


Applications of Bioethanol:

- ✈ Transport fuel to replace gasoline Fuel for power generation by thermal combustion
- ✈ Fuel for fuel cells by thermochemical reaction
- ✈ Fuels in cogeneration systems
- ✈ Feedstock in the chemical industry
- ✈ Blending ethanol with small portion of gasoline is more cost-effective

Biodiesel:

- ✈ Biodiesel is a variety of ester-based oxygenated fuels derived from natural, renewable biological sources such as vegetable oils.
- ✈ Biodiesel operates in compression ignition engines like petroleum diesel thereby requiring no essential engine modifications.
- ✈ Unlike fossil diesel, pure biodiesel is biodegradable, non- toxic and essentially free of sulphur and aromatics.



Uses of Biodiesel:

- Biodiesel is environmentfriendly.
- It can help reduce dependency on foreign oil.
- It helps to lubricate the engine itself, decreasing engine wear.
- It can be used in almost any diesel with little or no engine modification.
- It is safer than conventional diesel.
- Less global warming.

Uses of biofuels:

1. **Cars and Trucks:** Diesel cars and trucks can run on biodiesel.
2. **Aircraft:** Recent testing has shown the viability of biofuel use in the aviation industry, and use of biofuels to power aircraft is expected to increase substantially in the next decade.
3. **Off-Road Equipment**: A large percentage of off-road equipment -- such as vehicles used in agriculture, mining, forestry, construction, and power and heat production -use diesel fuel, making this equipment suitable for biodiesel use
4. **Small Engines:** Small engines, like those found in lawn mowers and chainsaws, can use ethanol blends up to 10 percent without problems

Advantages of biofuels:

1. **Cost:** Biofuels have the potential to be significantly less expensive than gasoline and other fossil fuels.

2. **Source material:** Whereas oil is a limited resource that comes from specific materials, biofuels can be manufactured from a wide range of materials including crop waste, manure, and other byproducts. This makes it an efficient step in recycling.
3. **Renewability:** It takes a very long time for fossil fuels to be produced, but biofuels are much more easily renewable as new crops are grown and waste material is collected.
4. **Security:** Biofuels can be produced locally, which decreases the nation's dependence upon foreign energy
5. **Economic stimulation:** Because biofuels are produced locally, biofuel manufacturing plants can employ hundreds or thousands of workers, creating new jobs in rural areas.
6. **Lower carbon emissions:** When biofuels are burned, they produce significantly less carbon output and fewer toxins, making them a safer alternative to preserve atmospheric quality and lower air pollution.

Disadvantages of biofuels:

1. **Production carbon emissions:** Several studies have been conducted to analyze the carbon footprint of biofuels, and while they may be cleaner to burn, there are strong indications that the process to produce the fuel including the machinery necessary to cultivate the crops and the plants to produce the fuel - has hefty carbon emissions.
2. **High cost:** To refine biofuels to more efficient energy outputs, and to build the necessary manufacturing plants to increase biofuel quantities, a high initial investment is often required.
3. **Food prices:** As demand for food crops such as corn grows for biofuel production, it could also raise prices for necessary staple food crops.
4. **Food shortages:** There is concern that using valuable cropland to grow fuel crops could have an impact on the cost of food and could possibly lead to food shortages.
5. **Water use:** Massive quantities of water are required for proper irrigation of biofuel crops as well as to manufacture the fuel, which could strain local and regional water resources.

UNIT V: Animal Biotechnology and IPR:

Animal Biotechnology: Transgenic animals are **animals** (most commonly mice) that have had a foreign gene deliberately inserted into their genome. Such **animals** are most commonly created by the microinjection of DNA into the pronuclei of a fertilised egg which is subsequently implanted into the oviduct of a pseudopregnant surrogate mother.

Methods of creation of transgenic animals

The three principal methods used for the creation of transgenic animals are **DNA microinjection, embryonic stem cell-mediated gene transfer** and **retrovirus-mediated gene transfer**.

Transgenic Animals:

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it

- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.

- Transgenic sheep and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesize human proteins in the "white" of their eggs.

An example:

Normal mice cannot be infected with polio virus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus. So normal mice cannot serve as an inexpensive, easily-manipulated model for studying the disease. However, transgenic mice expressing the human gene for the polio virus receptor

- can be infected by polio virus and even
- develop paralysis and other pathological changes characteristic of the disease in humans.

Two methods of producing transgenic mice are widely used:

- Transforming **embryonic stem cells** (**ES** cells) growing in tissue culture with the desired DNA;
- Injecting the desired gene into the **pronucleus** of a fertilized mouse egg.

Method 1: The Embryonic Stem Cell Method

Embryonic stem cells (**ES** cells) are harvested from the **inner cell mass** (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, **including its gametes**.

1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing

- the gene you desire (e.g., the insulin gene);
- **vector** DNA to enable the molecules to be inserted into host DNA molecules;
- **promoter and enhancer sequences** to enable the gene to be expressed by host cells.

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells.

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

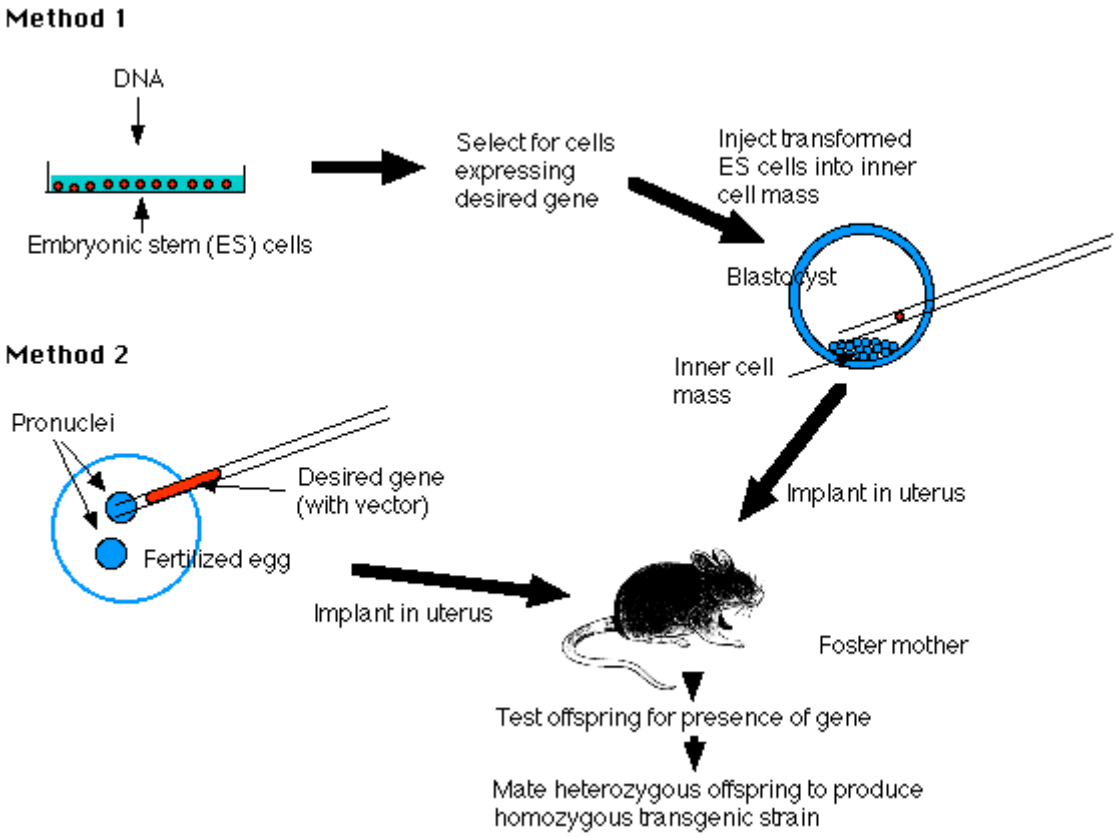
- Prepare a **pseudopregnant** mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they **implant** successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

- Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10–20% will have it, and they will be heterozygous for the gene.

7. Establish a transgenic strain

- Mate two heterozygous mice and screen their offspring for the 1 in 4 that will be **homozygous** for the transgene.
- Mating these will found the transgenic strain.



Method 2:The Pronucleus Method

1. Prepare your DNA as in Method 1

2. Transform fertilized eggs

- Harvest freshly fertilized eggs before the sperm head has become a pronucleus.
- Inject the male pronucleus with your DNA.
- When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo.

3. Implant the embryos in a pseudopregnant foster mother and proceed as in Method 1.

Method of creating Transgenic Cattle:

Step 1. Designing the gene construct

The first step is to design a gene construct. The gene construct is a unit of DNA that includes:

- an antibiotic resistance gene – to select cells that have taken up the gene construct
- a tissue-specific promoter sequence – to signal the start of expression of the protein in cells of the appropriate tissue, for example, in mammary cells in lactating cows
- the desired gene – for example, bovine casein or human myelin basic protein
- a stop sequence – to define the end of the information for making the protein.

Step 2. Sourcing the transgene

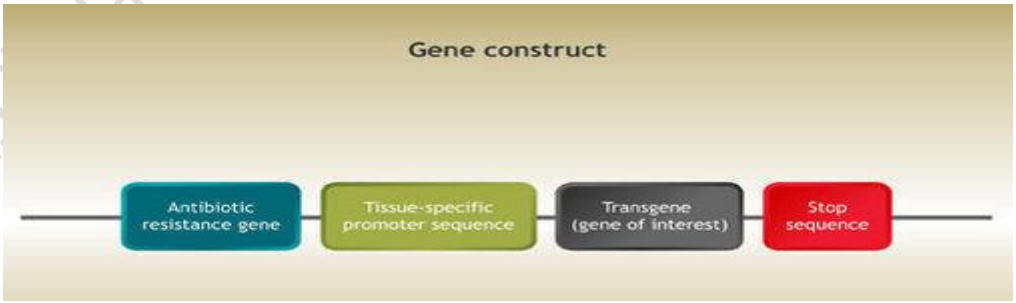
In the past, the gene would have been extracted from the source organism's DNA. Now, however, if the desired gene sequence is known, it can simply be synthesised in a lab. There are companies that make genes to order within a couple of weeks.

Step 3. Making the gene construct

The gene is usually supplied in a vector. A vector is a small piece of DNA, often a plasmid, into which a foreign piece of DNA can be inserted. When the gene of interest is in a vector, it can be sent from one lab to another, it can be stored, it can be manipulated or it can be used to transform bacteria to produce more copies of the gene of interest.

Vectors have multiple restriction enzymes sites (also called multiple cloning sites) so the gene can be inserted into the vector and then cut out from the vector using restriction enzymes. This article has more information on restriction enzymes.

After the gene is cut from the vector, it is then pasted into the multiple cloning site of the gene construct using a method known as ligation. This article has more information on DNA ligation.



Step 4. Transfecting bovine cells

The gene construct is incorporated into the genome of a bovine (cow) cell using a technique called transfection. During transfection, holes are made in the cell membrane that allow the DNA to enter. The

holes can be made by applying an electrical pulse or by adding chemicals to the cells. Once inside the cell, the gene construct may enter the nucleus and incorporate into the cell's genome.

Step 5. Selecting for transgene positive cells

After transfection, an antibiotic is added to select the bovine cells that have incorporated the gene construct. Transgenic bovine cells will survive treatment with an antibiotic, because they contain an antibiotic resistance gene making them resistant to the antibiotic. Cells without the gene construct will have no resistance to the antibiotic and will die. In addition to antibiotic selection, polymerase chain reaction (PCR) is used to check that the bovine cell contains the transgene.

Step 6. Making a transgenic embryo using nuclear transfer

Nuclear transfer is used to create a whole animal from a single transgenic bovine cell.

The transgenic bovine cell is fused with a bovine oocyte that has had its chromosomes removed (called an enucleated oocyte). An electrical pulse is applied to help fuse the cells. Once fused with the oocyte, the transgenic cell's chromosomes are reprogrammed to direct development into an embryo. After 7 days, the transgenic embryo will have about 150 cells and can be transferred into a recipient cow for further development to term.

Step 7. Confirming the cow is transgenic

If the embryo develops to full term, after 9 months, the cow will give birth to a calf. To confirm that the calf is transgenic, scientists can check using:

- PCR to determine the presence or absence of the transgene
- quantitative PCR (q-PCR) to determine the number of copies of the transgene
- fluorescent in situ hybridisation (FISH) to visualise where the transgene is in the chromosome and whether the transgene has integrated into more than one chromosome.

When the calf is lactating (either after being induced to lactate or after having its own progeny), its milk is checked to determine if the transgenic protein is being expressed.

Step 1. Designing the gene construct

The first step is to design a gene construct. The gene construct is a unit of DNA that includes:

- an antibiotic resistance gene – to select cells that have taken up the gene construct
- a tissue-specific promoter sequence – to signal the start of expression of the protein in cells of the appropriate tissue, for example, in mammary cells in lactating cows
- the desired gene – for example, bovine casein or human myelin basic protein
- a stop sequence – to define the end of the information for making the protein.

Step 2. Sourcing the transgene

In the past, the gene would have been extracted from the source organism's DNA. Now, however, if the desired gene sequence is known, it can simply be synthesised in a lab. There are companies that make genes to order within a couple of weeks.

Step 3. Making the gene construct

The gene is usually supplied in a vector. A vector is a small piece of DNA, often a plasmid, into which a foreign piece of DNA can be inserted. When the gene of interest is in a vector, it can be sent from one lab to another, it can be stored, it can be manipulated or it can be used to transform bacteria to produce more copies of the gene of interest.

Vectors have multiple restriction enzymes sites (also called multiple cloning sites) so the gene can be inserted into the vector and then cut out from the vector using restriction enzymes. This article has more information on restriction enzymes.

After the gene is cut from the vector, it is then pasted into the multiple cloning site of the gene construct using a method known as ligation. This article has more information on DNA ligation.

Step 4. Transfecting bovine cells

The gene construct is incorporated into the genome of a bovine (cow) cell using a technique called transfection. During transfection, holes are made in the cell membrane that allow the DNA to enter. The holes can be made by applying an electrical pulse or by adding chemicals to the cells. Once inside the cell, the gene construct may enter the nucleus and incorporate into the cell's genome.

Step 5. Selecting for transgene positive cells

After transfection, an antibiotic is added to select the bovine cells that have incorporated the gene construct. Transgenic bovine cells will survive treatment with an antibiotic, because they contain an

antibiotic resistance gene making them resistant to the antibiotic. Cells without the gene construct will have no resistance to the antibiotic and will die. In addition to antibiotic selection, polymerase chain reaction (PCR) is used to check that the bovine cell contains the transgene.

Step 6. Making a transgenic embryo using nuclear transfer

Nuclear transfer is used to create a whole animal from a single transgenic bovine cell.

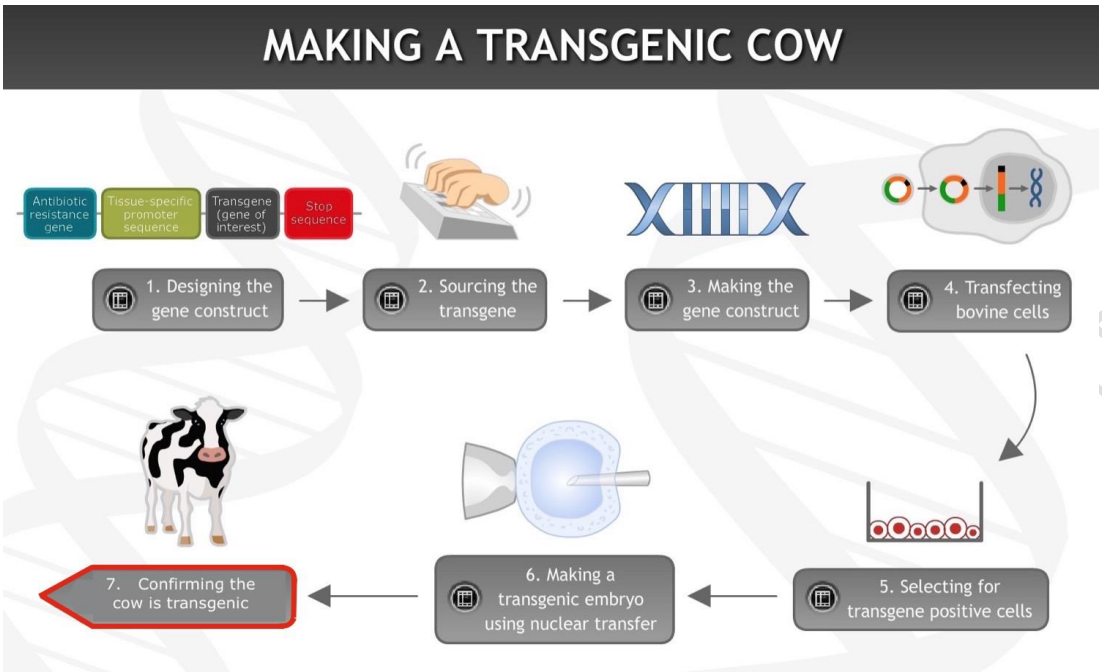
The transgenic bovine cell is fused with a bovine oocyte that has had its chromosomes removed (called an enucleated oocyte). An electrical pulse is applied to help fuse the cells. Once fused with the oocyte, the transgenic cell's chromosomes are reprogrammed to direct development into an embryo. After 7 days, the transgenic embryo will have about 150 cells and can be transferred into a recipient cow for further development to term.

Step 7. Confirming the cow is transgenic

If the embryo develops to full term, after 9 months, the cow will give birth to a calf. To confirm that the calf is transgenic, scientists can check using:

- PCR to determine the presence or absence of the transgene
- quantitative PCR (q-PCR) to determine the number of copies of the transgene
- fluorescent in situ hybridisation (FISH) to visualise where the transgene is in the chromosome and whether the transgene has integrated into more than one chromosome.

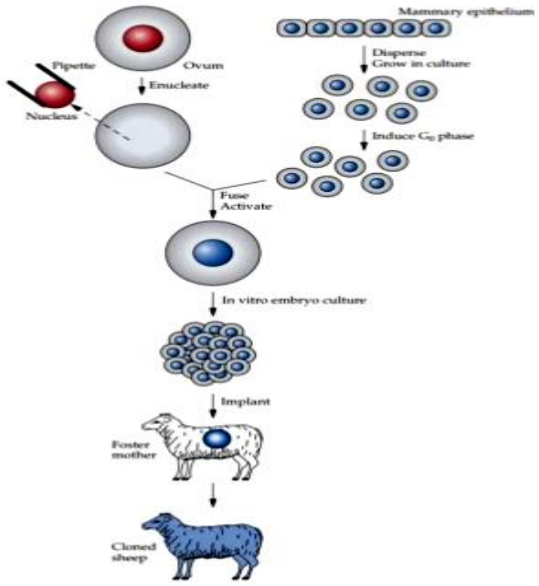
When the calf is lactating (either after being induced to lactate or after having its own progeny), its milk is checked to determine if the transgenic protein is being expressed.



Transgenic Sheep:

Dolly Sheep

Dolly the sheep was the first mammal to be cloned from an adult cell. In this, the udder cells from a 6-year-old Finn Dorset white sheep were injected into an unfertilized egg from a Scottish Blackface ewe, which had its nucleus removed. The cell was made to fuse by electrical pulses. After the fusion of the nucleus of the cell with the egg, the resultant embryo was cultured for six to seven days. It was then implanted into another Scottish Blackface ewe which gave birth to the transgenic sheep, Dolly.



Applications of Transgenic Animals

1. **Normal Physiology and Development**

In transgenic animals, a foreign gene is introduced due to which the growth factor is altered. Hence, these animals facilitate the study of gene regulation and their effect on the everyday functions of the body.

2. **Study of Diseases**

Transgenic animals are specially designed to study the role of genes in the development of certain diseases. Moreover, in order to devise a cure for these diseases, the transgenic animals are used as model organisms. These transgenic models are used in research for the development of medicines. For example, we have transgenic models for diseases such as Alzheimer's and cancer.

3. **Biological Products**

A number of biological products such as medicines and nutritional supplements are obtained from transgenic animals. Research for the manufacture of medicines to treat diseases such as phenylketonuria (PKU) and hereditary emphysema is going on. The first transgenic cow, Rosie, in 1997, produced milk which was rich in human protein (2.4 grams per litre). This milk contains the human gene alpha-lactalbumin and could be given to babies as an alternative to natural cow milk.

4. **Vaccine Safety**

Transgenic animals are used as model organisms for testing the safety of vaccines before they are injected into humans. This was conventionally done on monkeys.

Human Gene Therapy:

Gene therapy is a technique in which a functioning **gene** is inserted into a **human** cell to correct a **genetic** error or to introduce a new function to the cell. Many methods, including retroviral vectors and non-viral vectors, have been developed for both ex vivo and in vivo **gene** transfer into cells.

Types of gene therapy.:

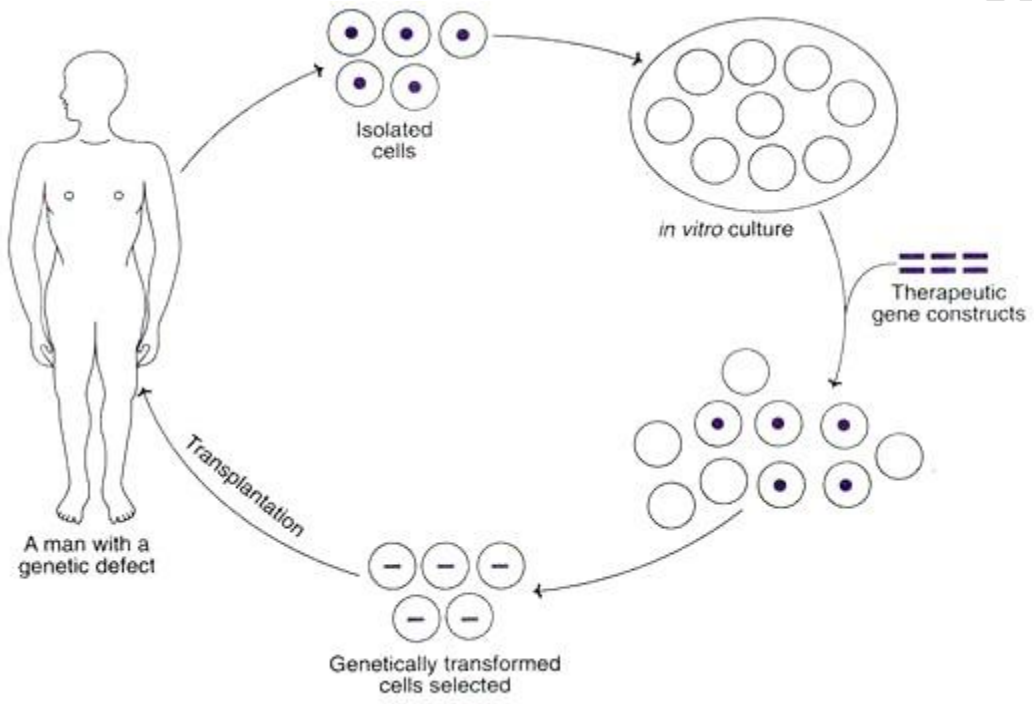
The two types are:

- (1) **Ex Vivo Gene Therapy –** This involves the transfer of genes in cultured cells (e.g., bone marrow cells) which are then reintroduced into the patient.

- (2) **In Vivo Gene Therapy** - The direct delivery of genes into the cells of a particular tissue is referred to as in vivo gene therapy.

1. Ex Vivo Gene Therapy:

The ex vivo gene therapy can be applied to only selected tissues (e.g., bone marrow) whose cells can be cultured in the laboratory. The technique of ex vivo gene therapy involves the following steps



1. Isolate cells with genetic defect from a patient.

2. Grow the cells in culture.

3. Introduce the therapeutic gene to correct gene defect.

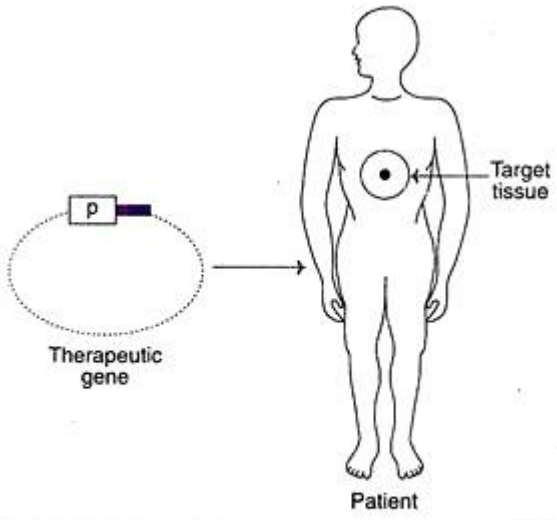
4. Select the genetically corrected cells (stable trans-formants) and grow.

5. Transplant the modified cells to the patient.

2. In Vivo Gene Therapy:

The direct delivery of the therapeutic gene (DNA) into the target cells of a particular tissue of a patient constitutes in vivo gene therapy . Many tissues are the potential candidates for this

approach. These include liver, muscle, skin, spleen, lung, brain and blood cells. Gene delivery can be carried out by viral or non- viral vector systems. The success of in vivo gene therapy mostly depends on the following parameters



Diagrammatic representation of in vivo *gene therapy. (p-Promoter gene specific for therapeutic gene)*

Applications:

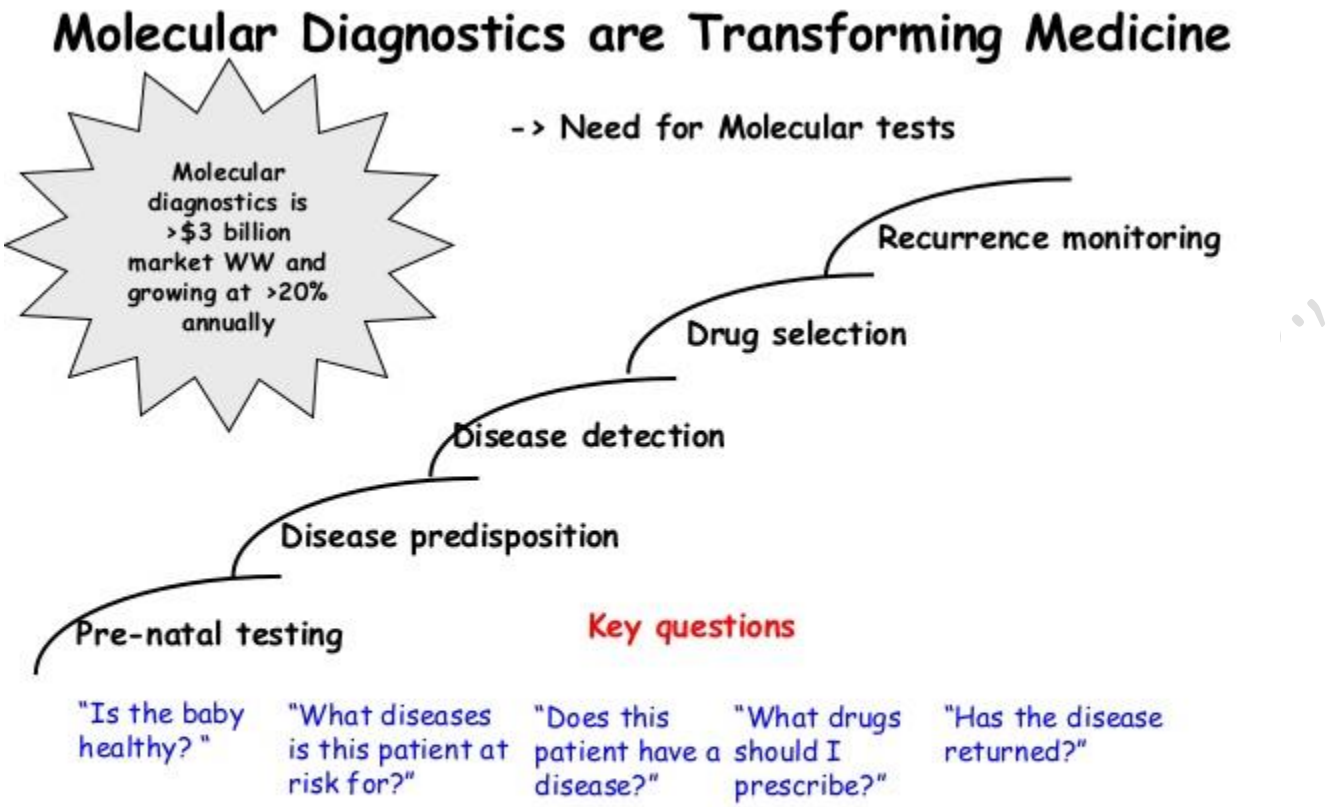
Gene therapy can deliver to target cells **genes** that code for the missing biological factor. Cancer, infectious diseases, cardiac disease, neurological disorders and some inherited conditions are among the areas into which **gene therapy** research is being carried out.

Molecular Diagnosis:

The use of molecular biology techniques to expand scientific knowledge of the natural history of diseases, identify people who are at risk for acquiring specific diseases, and diagnose human diseases at the molecular level.

Molecular Diagnostics Significance:

To face the near future, the medical practitioner not only understand molecular biology, but must also embrace the use of this rapidly expanding body of information in his medical practice, whether practicing family medicine pediatrics, oncology, obstetrics and gynecology, pathology, or any other medical specialty.



Characteristics of a Detection System:

• A good detection system should have 3 qualities:

- Sensitivity
- Specificity
- Simplicity

• **Sensitivity** means that the test must be able to detect very small amounts of target even in the presence of other molecules.

• **Specificity**: the test yields a positive result for the target molecule only.

• **Simplicity:** the test must be able to run efficiently and inexpensively on a routine basis.

Molecular Diagnostics Immunological Diagnostics Methods

1. Radioimmunoassay

2. Enzyme-Linked ImmunoSorbent Assay (ELISA)

3. Western Blotting

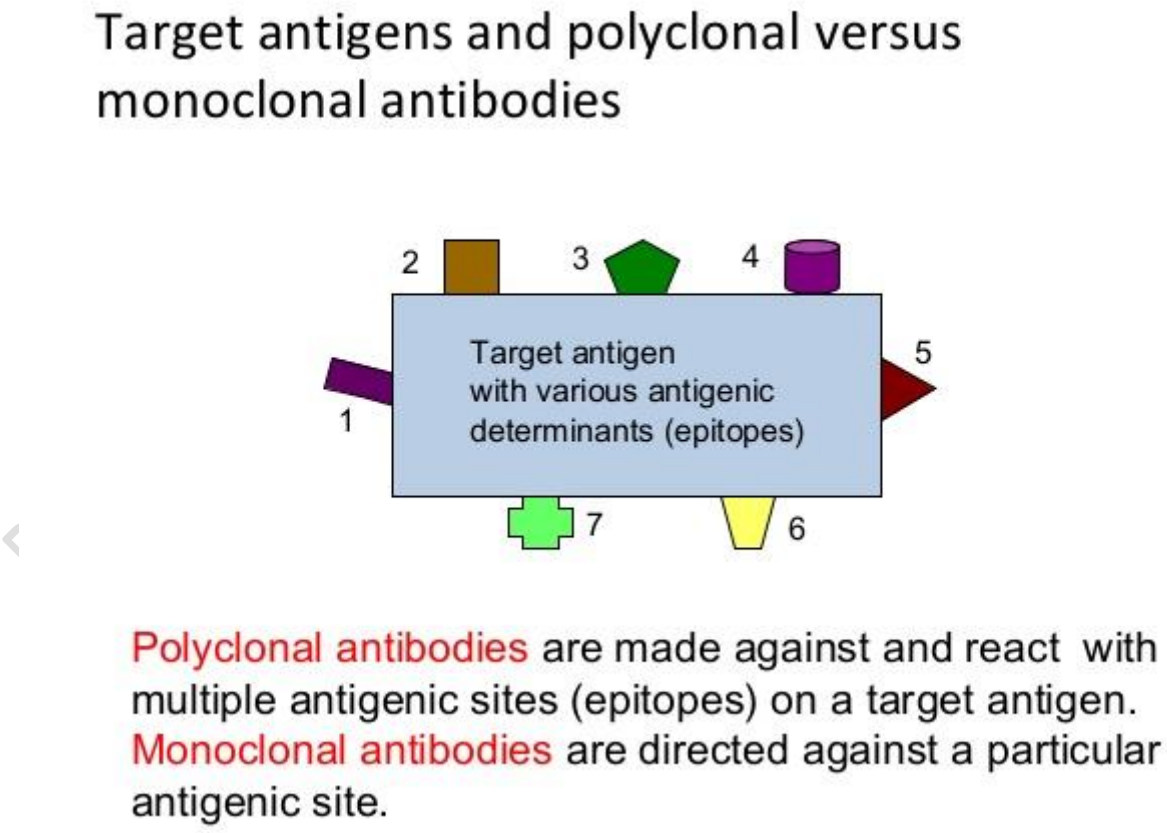
4. Immunoprecipitation

5. Immunofluorescence

6. Flow Cytometry and Fluorescence

7. Alternatives to Antigen-Antibody Reactions

8. Immunoelectron Microscopy



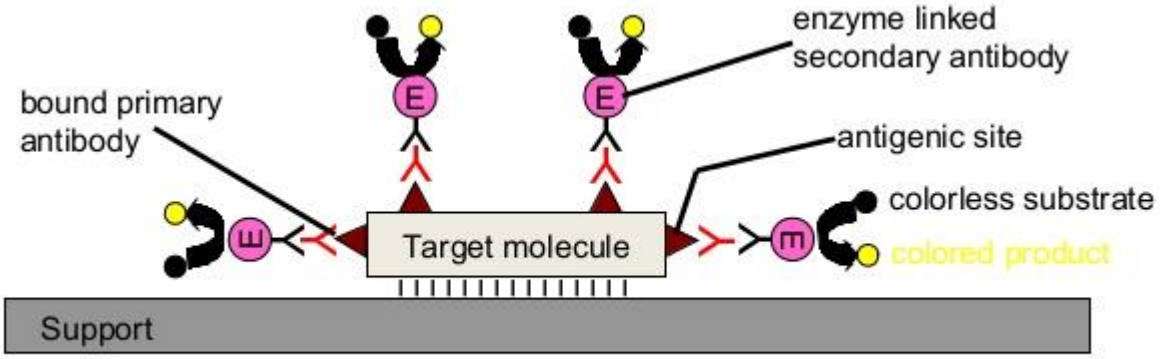
Enzyme-Linked Immunosorbent Assay (ELISA): Immunological detection

A. Bind sample to the support (commonly plastic or a membrane)

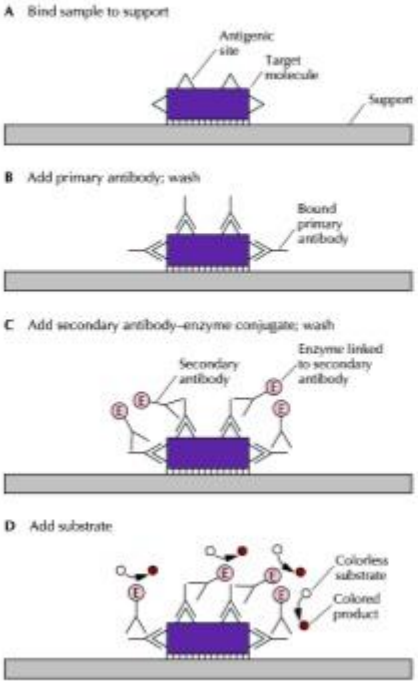
B. Add primary antibody; wash

C. Add secondary antibody-enzyme conjugate; wash

D. Add substrate

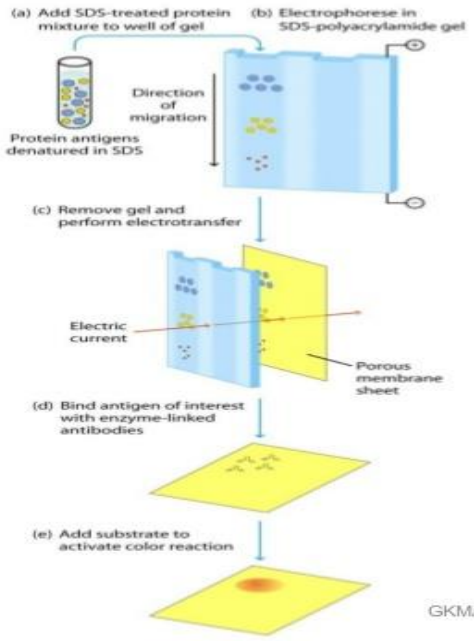


Immunological Diagnostics Methods - ELISA



- Addition of a **specific antibody** (primary antibody) which will bind to the test molecule if it is present.
- **Washing** to remove unbound molecules.
- Addition of **secondary antibody** which will bind to the primary antibody.
- The secondary antibody usually has attached to it an **enzyme** e.g. **alkaline phosphatase**.
- **Wash** to remove unbound antibody.
- Addition of a **colourless substrate** which will react with the secondary antibody to give a **colour reaction** which indicates a positive result.

-> can be used for quasi High-throughput!!!



Western blot

SDS-Page: separates the components according to their molecular weight.

Blot: the proteins in the gel are transferred to the sheet of nitrocellulose or nylon by the passage of an electric current.

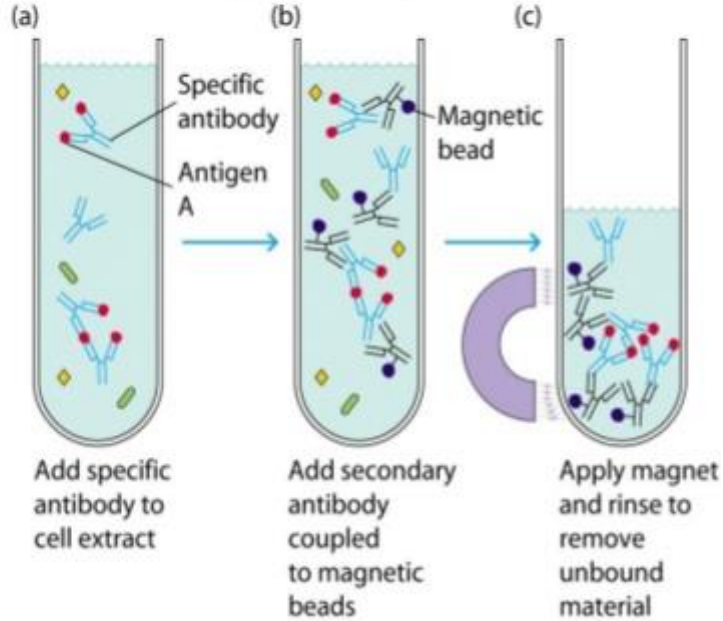
Immunoreaction: probed with Ab & then radiolabeled or enzyme-linked 2nd Ab.

Detection: a position is visualized by means of an ELISA reaction.

GKM/M.MEDPAEDS/LECT 01/2015

Immunoprecipitation

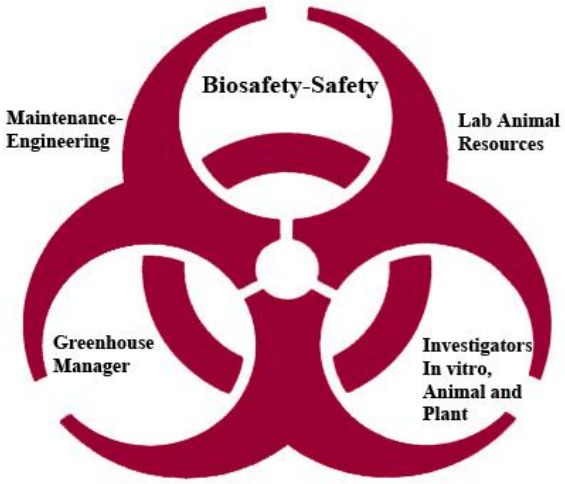
Immuno-precipitates can be collected using magnetic beads coupled to a secondary antibody.



BIOSAFETY:

Biological safety or **biosafety** is the application of knowledge, techniques and equipment to prevent personal, laboratory and environmental exposure to potentially infectious agents or biohazards. Biosafety defines the containment conditions under which infectious agents can be safely manipulated.

BIOSAFETY SYMBOL:



BIOSAFETY IS RELATED TO SEVERAL FIELDS:

- In **ecology** (referring to imported life forms from beyond **ecoregion** borders),
- In **agriculture** (reducing the risk of alien viral or transgenic genes, or prions such as BSE/"MadCow", reducing the risk of food bacterial contamination)
- In **medicine** (referring to organs or tissues from biological origin, or genetic therapy products, virus; levels of **lab containment** protocols measured as 1, 2, 3, 4 in rising order of danger),
- In **chemistry** (i.e., nitrates in water, PCB levels affecting fertility) and
- In **exobiology** (i.e., NASA's policy for containing alien microbes that may exist on space samples - sometimes called **"biosafety level 5"**).

BIOSAFETY ISSUES:

- ❖ Blood borne pathogens (BBP)
- ❖ Laboratory Safety
- ❖ Biosafety Issues Infectious substance
- ❖ Biological waste disposal

- Recombinant DNA (rDNA) and diagnostic specimen shipping
- Bioterrorism and Select agents
- Respiratory Protection
- Occupational safety and health in the use of research animals
- Biohazards used in animal models

BIOHAZARDOUS MATERIALS:

- Viruses
- Bacteria
- Chlamydiae/Rickettsiae
- Fungi
- Prions
- Recombinant DNA
- Transgenic Plants, Animals and Insects
- Human and Primate Cells, Tissues, and Body Fluids
- Brain Tissue from Demented Patients
- Viral Vectors
- Replication deficient viruses

PRINCIPLES OF BIOSAFETY:

- Practice and Procedures
  - Standard Practices
  - Special Practices & Considerations
- Safety Equipment Facility
- Design and Construction
- Increasing levels of protection
- Biosafety Levels 1-4 (BSL)
  - Increasing levels of employee and environmental protection
  - Guidelines for working safely in research & medical laboratory facilities
- Animal Biosafety Levels 1- 4 (ABSL)
  - Laboratory animal facilities
  - Animal models that support research
  - Guidelines for working safely in animal research facilities

BIOSAFETY CONCEPTS:

(1) Standard Microbiological Practices

- Most important concept / Strict adherence
- Aware of potential hazard
- Trained & proficient in techniques
- Supervisors responsible for:
  - Appropriate Laboratory facilities
  - Personnel & Training
- Special practices & precaution
- Occupational Health Programs

(2) Safety Equipment

- Primary Containment Barrier
- Minimize exposure to hazard
- Prevent contact / Contain aerosols
- Engineering controls/ equipment
- Personal Protective Equipment (PPE)
- Gloves, gowns, Respirator, Face shield, Booties
- Biological Safety Cabinets
- Covered or ventilated animal cage systems

(3) **Facility Design and Construction**

- Secondary Barrier/ Engineering controls
- Contributes to worker protection
- Protects outside the laboratory
- Environment & Neighborhood

- Ex. Building & Lab design, Ventilation, Autoclaves, Cage wash facilities, etc.

ETHICS:

The field of ethics (or moral philosophy) involves systematizing, defending, and recommending concepts of right and wrong behavior

BIOETHICS:

Bioethics is the study of the ethical issues emerging from advances in biology and medicine. It is also moral discernment as it relates to medical policy and practice. Bioethicists are concerned with the

ethical questions that arise in the relationships among life sciences**,** biotechnology**,** medicine**,** politics**,** law, and philosophy**.** It includes the study of values **(**"the ethics of the ordinary"**)** relating to primary care and other branches of medicine.

- ❖ The term **"bioethics"** was introduced in the **70's** by **Van Rensselaer Potter** for a study aiming at ensuring the **preservation of the biosphere**
- ❖ It was later used to refer a study of the ethical issues arising from health care, biological and medical sciences

Some historical examples:

- ✪ Abortion
- ✪ Contraception
- ✪ Kidney dialysis machine (Who had the priority?)
- ✪ Organ transplant, artificial ventilator, and brain death
- ✪ *In vitro* fertilization (IVF)
- ✪ Cloning and stem cell research
- ✪ Genetic engineering

LEGALITY: Definition:

Legality can be defined as an act, agreement, or contract that is consistent with the law or state of being lawful or unlawful in a given jurisdiction.

PRINCIPLE OF LEGALITY:

- ❖ Legality is the legal ideal that requires all law to be clear, ascertainable and non-retrospective.
- ❖ It requires **decision makers to resolve disputes by applying legal rules** that have been declared beforehand, and not to alter the legal situation retrospectively by discretionary departures from established law.
- ❖ In **criminal law** it can be seen in the general prohibition on the imposition of criminal sanctions for acts or omissions that were not criminal at the time of their commission or omission.
- ❖ The principle is also thought **to be violated** when the sanctions for a **particular crime are increased with retrospective effect.**

In administrative law it can be seen in the desire for state officials to be bound by and apply the law rather than acting upon whim. As such advocates of the principle are normally against discretionary powers.

MORALITY:

- Morality speaks of a system of behavior in regards to standards of right or wrong behavior.
- The word carries the concepts of:

  (1) Moral standards, with regard to behavior;

  (2) Moral responsibility, referring to our conscience; and

  (3) A moral identity, or one who is capable of right or wrong action.

- Common synonyms include ethics, principles, virtue, and goodness. Morality has become a complicated issue in the multi-cultural world we live in today. Let's explore what morality is, how it affects our behavior, our conscience, our society, and our ultimate destiny.

Morality principles concerning the distinction between right and wrong or good and bad behaviour.

ETHICS:

- **Ethics:** "the **rules of conduct** recognized in respect to a **particular class of human actions** or a particular group, culture"

Ethics and **morals** relate to "right" and "wrong" conduct. While they are sometimes used interchangeably, they are different: **ethics** refer to rules provided by an external source, e.g., codes of conduct in workplaces or principles in religions. **Morals** refer to an individual's own principles regarding right and wrong.

COMPARISON CHART:

Ethics versus Morals comparison chart

	Ethics	Morals
What are they?	The rules of conduct recognized in respect to a particular class of human actions or a particular group or culture.	Principles or habits with respect to right or wrong conduct. While morals also prescribe dos and don'ts, morality is ultimately a personal compass of right and wrong.
Where do they come from?	Social system - External	Individual - Internal
Why we do it?	Because society says it is the right thing to do.	Because we believe in something being right or wrong.
Flexibility	Ethics are dependent on others for definition. They tend to be consistent within a certain context, but can vary between contexts.	Usually consistent, although can change if an individual's beliefs change.
The "Gray"	A person strictly following Ethical Principles may not have any Morals at all. Likewise, one could violate Ethical Principles within a given system of rules in order to maintain Moral integrity.	A Moral Person although perhaps bound by a higher covenant, may choose to follow a code of ethics as it would apply to a system. "Make it fit"
Origin	Greek word "ethos" meaning "character"	Latin word "mos" meaning "custom"
Acceptability	Ethics are governed by professional and legal guidelines within a particular time and place	Morality transcends cultural norms

BASIC PRINCIPLES IN BIOETHICS:

In bioethics they are **four basic principles** and they were proposed by **Beaucham and Childress (1979):**

- Autonomy
- Beneficence
- No malfeasance
- Justice

Bioethics we find several grounded ethical theories. Two of these are **deontological ethics** and **utilitarian ethics.**

1. **Deontological ethics**

   - Proposed by Immanuel Kant
   - It consists in that reason identifies actions like good or bad, independent of their consequences. (Importance / Effect of what has gone before)

2. **Utilitarian ethics**

   - Proposed by Jeremy Bentham and John Stuart-Mill
   - It says that actions are good or bad depend on their consequences. (Importance / Effect of what has gone before)
   - The balance between purposes that give benefits or damage is produced by utilitarian ethics.

These **principles can be grouped in two levels**:

- **Minimum levels:** obligations that generate universal duties and these involve negative transitive duties (facts that you cannot do other people). Here, there are principles of no malfeasance and justice.
- **Maximum levels:** they are related with the choice of the vital project that every person chooses to depend on their scale of values. They generate imperfect obligations: facts that I can auto impose, but I cannot call for other people (neither other people to me). Here, there are principles of autonomy and beneficence.

- **The Principle of Respect for autonomy:**

Autonomy is Latin for "self-rule" We have an obligation to respect the autonomy of other persons, which is to respect the decisions made by other people concerning their own lives. This is also called the principle of human dignity. It gives us a negative duty not to interfere with the decisions of competent adults, and a positive duty to empower others for whom we're responsible.

Corollary principles: honesty in our dealings with others & obligation to keep promises.

- **The Principle of Beneficence:**

We have an obligation to bring about good in all our actions.

Corollary principle? We must take positive steps to prevent harm. However, adopting this corollary principle frequently places us in direct conflict with respecting the autonomy of other persons.

- **The Principle of non malfeasance:**

(It is not "non-malfeasance," which is a technical legal term & it is not "no malevolence," which means that one did not intend to harm.)

We have an obligation not to harm others: "First, do no harm."

Corollary principle: Where harm cannot be avoided, we are obligated to minimize the harm we do.

Corollary principle: Don't increase the risk of harm to others.

Corollary principle: It is wrong to waste resources that could be used for good.

Combining beneficence and non malfeasance: Each action must produce more good than harm.

- **The Principle of justice**

We have an obligation to provide others with whatever they are owed or deserve. In public life, we have an obligation to treat all people equally, fairly, and impartially.

Corollary principle: Impose no unfair burdens.

Combining beneficence and justice: We are obligated to work for the benefit of those who are unfairly treated.

INTELLECTUAL PROPERTY (IPR)

In common sense intellectual property is a product of mind. It is similar to the property (**consisting of movable and immovable thing**) like a house or car where in the property or owner may use his property as his wishes nobody else can use his property without his permission as per Indian law.

Types of Intellectual Property

- Patents
- Copyright
- Trademarks
- Related Rights
- Geographical Indications
- Industrial Designs
- Unfair Competition
- Enforcement of Intellectual Property Rights
- Emerging Issues in Intellectual Property
    1. Biotechnology
    2. Traditional Knowledge

Patents

A patent is a government granted and secured legal right to prevent other forms of making, using or selling the invention covered by the patent. A patent is a personal property which can be licensed or sold by the person/organization like any other property.

Examples:

- Electric lighting- patents held by Edison and Swan
- Plastic- patents held by Baekeland
- Ballpoint pens- patents held by Biro
- Microprocessors- patents held by Intel.
- Telephones-patents held by Bell

Patents for:

- **The drug substance itself:**
  - Chemical composition of the API
- **Method of use:**
  - Use of the drug to treat a particular condition
- **The formulation:**
  - The physical form of a drug and method of administration
- **The process of making it:**
  - Manufacturing methods

Copyright

Copyright aims at providing protection to authors (writers, artists, music composers, etc) on their creations. Such creations are usually designated as 'works".

The best example of copyright is the authored and edited books, or audio and video cassettes, which cannot be reproduced without the permission of the person (author, editor or publisher), who holds the copyright. In biotechnology, the copyright may cover DNA sequence data which may be published.

Trademarks

A trademark is a sign that is used to identify certain goods and services as those produced or provided by a specific person or enterprises.

E.g. "DELL" is trademark that identifies goods (computers and computer related objects).

E.g. "CITY BANK" is a trademark that relates to services (banking and financial services).

Related Rights

Related rights provide protection to the following persons or organizations:

- **Performers** (actors, musicians, singers, dancers, or generally people who perform), in their performances
- **Producers of sound recordings** (for example, cassette recordings and compact discs) in their recordings and

- **Broadcasting organizations,** in their radio and television programs.

Sometimes, these rights are also referred to as neighboring rights.

Industrial Designs

An industrial design is the ornamental or aesthetic aspect of an article. The design may consist of three-dimensional features, such as the shape of an article, or two-dimensional features, such as patterns, lines or color.

Industrial designs are applied to a wide variety of products of industry and handicrafts such as technical and medical instruments, watches, jewelry, house ware, electrical appliances, vehicles, architectural structures, textile designs and other luxury items.

To be protected under most national laws, an industrial design must appeal to the eye. This means that an industrial design is primarily of an aesthetic nature, and does not protect any technical features of the article to which it is applied.

Unfair Competition

Unfair competition is generally understood as any act of competition that is contrary to honest practices in industrial or commercial matters.

A dishonest practice is not something that can be defined with precision.

The standard of fairness or honesty may change from country to country, as well as evolve with time. It is, therefore, difficult to attempt to encompass all existing acts of unfair competition in one definition.

Enforcement (a law) of Intellectual Property Rights

A publisher may own copyright in a book, which has been reproduced and sold without his or her consent, at a cut price.

A sound producer, who has invested large amounts of money, in terms of talent and technical skill, in producing records, sees that copies of it are sold on the market, at cheap prices, without his authorization.

Someone else's trade mark may have been used by a company on similar or identical goods of lesser quality, harming thus the reputation of the legitimate owner, and inflicting on him or her serious financial loss, let alone exposing customer's health to danger.

Somebody may be using the geographical denomination of "Roquefort" on cheese manufactured elsewhere than in the region of Roquefort in France, thus deceiving the consumers as well as taking away business from legitimate producers.

In all such cases intellectual property rights (i.e. copyright, related rights, trademarks and geographical indications) have been infringed. It is important that in such cases enforcement mechanisms be called into play to protect not only the legitimate interests of the rights of the owners, but also of the public.

Emerging Issues in Intellectual Property

Intellectual property plays an important role in an increasingly broad range of areas, ranging from the internet to health care, to nearly all aspects of science and technology, literature and the arts.

The following two topics, ***Biotechnology*** and ***Traditional Knowledge***, are now being discussed at length at the international arena.

- **Biotechnology**

Biotechnology is a field of technology of growing importance in which inventions may have a significant effect on our future, particularly in medicine, food, agriculture, energy and protection of the environment.

The science of biotechnology concerns living organisms, such as plants, animals, seeds and microorganisms, as well as biological material, such as enzymes, proteins and plasmids (which are used in "genetic engineering")

- **Traditional Knowledge**

Traditional knowledge-used here broadly to refer to tradition-based innovations and creations resulting from intellectual activity in the industrial, scientific, literary or artistic fields-had been largely over-looked in the IP community until quite recently.

It is now increasingly recognized that the economic value of traditional knowledge assets could be further enhanced by the use of IP.

IPR in India:

Patent Administration in India

The Head Office is in **Kolkata**

Four branches:

1. **Kolkata**
2. **Mumbai**
3. **Delhi**
4. **Chennai**

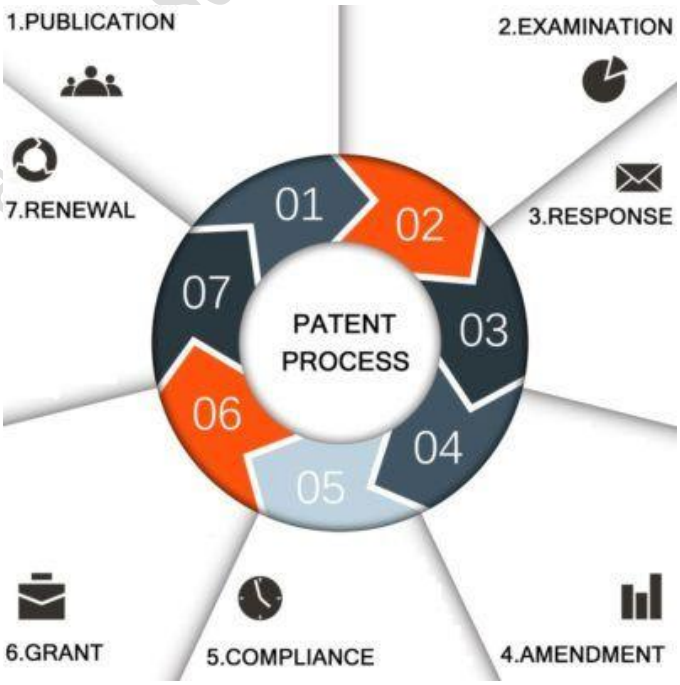
Who can file Patent Application in India

Either alone or jointly:

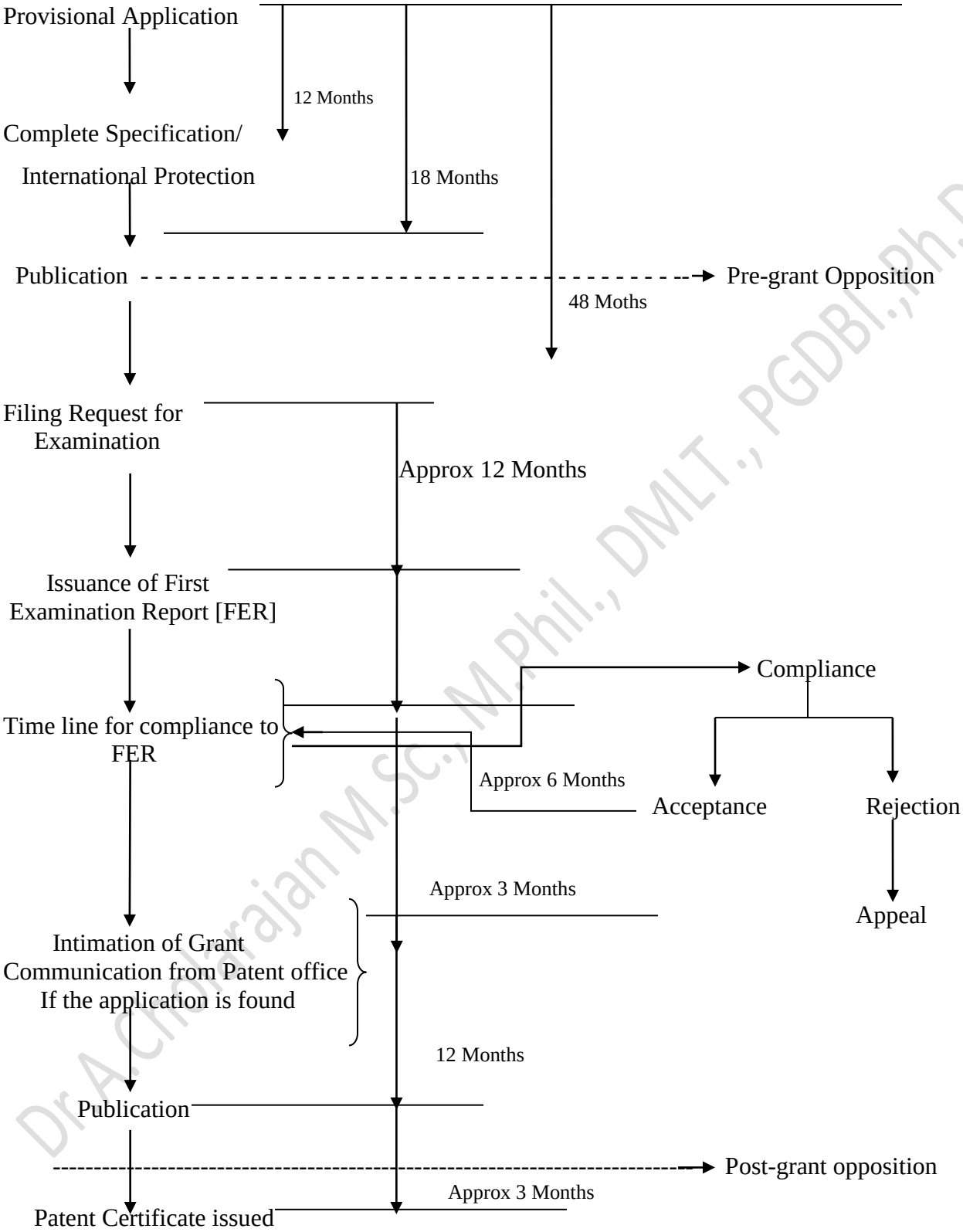
✔ By any person claiming to be true and first inventor(s)

✔ By any person being the assignee of person claiming to be true and first inventor(s)

✔ By the legal representative of any deceased person who can immediately after his death is entitled to make such application



PATENT PROCEDURE IN INDIA



Documents Required / Patent Forms***

✔Application Form (Form 1)

✔ Proof of Right to Apply (Paragraph 9 of Form 1)

✔ Provisional or complete Specification (Form 2)

✔ Statement of Foreign Filing (Form 3)

✔ Declaration as to Inventorship (Form 5)

✔Request for Early Publication (Form 9)

✔ Request for Examination (Form 18)

✔ Power of Attorney, if required (Form 26)

✔ Certified Copy of Convention Application, if required

✔ Abstract of Invention

✔ Drawing(s), if any

Indian Patent Office:

- The **Indian Patent Office** is administered by the Office of the Controller General of Patents, Designs & Trade Marks (CGPDTM).
- This is a subordinate office of the Government of India and administers the Indian law of Patents, Designs and Trade Marks.

Patent administration

The CGPDTM reports to the Department of Industrial Policy and Promotion (DIPP) under the Ministry of Commerce and Industry and has five main administrative sections:

- Patent Office
- Designs Registry
- Trademarks Registry
- Geographical indications Registry
- Rajiv Gandhi National Institute of Intellectual Property Management (NIIPM)
- Patent Information System

The patent office is headquartered at Kolkata with branches in Chennai, New Delhi and Mumbai, but the office of the CGPDTM is in Mumbai.

The Indian Patent Office has 526 Patent Examiners, 97 Assistant Controllers, 42 Deputy Controllers, 1 Joint Controller, and 1 Senior Joint Controller, all of whom operate from four branches. Although the designations of the Controllers differ, all of them (with the exception of the Controller General) have equal authority in administering the Patents Act.

International Patent Office:

- The **World Intellectual Property Organization** (**WIPO**) is one of the 17 specialized agencies of the United Nations (UN)
- WIPO was created in 1967 "to encourage creative activity, to promote the protection of intellectual property throughout the world"
- WIPO currently has 191 member states, administers 26 international treaties, and is headquartered in Geneva, Switzerland
- The current Director-General of WIPO is Francis Gurry, who took office on 1 October 2008

1. Food and Agriculture Organization (FAO)

2 International Civil Aviation Organization (ICAO)

3 International Fund for Agricultural Development (IFAD)

4 International Labour Organization (ILO)

5 International Maritime Organization (IMO)

6 International Monetary Fund (IMF)

7 International Telecommunication Union (ITU)

8 United Nations Educational, Scientific and Cultural Organization (UNESCO)

9 United Nations Anti-Terrorism Coalition (UNATCO)

10 United Nations Industrial Development Organization (UNIDO)

11 Universal Postal Union (UPU)

12 World Bank Group (WBG)

12.1 International Bank for Reconstruction and Development (IBRD)

12.2 International Finance Corporation (IFC)

12.3 International Development Association (IDA)

13 World Health Organization (WHO)

14 World Intellectual Property Organization (WIPO)

15 World Meteorological Organization (WMO)

16 World Tourism Organization (UNWTO)

17 Former specialized agencies

Present Indian Scenario

PATENTS IN THE INDIAN SCENARIO:

The laws pertaining to Patent in India is governed by the Patents Act, 1970 which has been amended twice by The Patents (Amendment) Act, 1999 and The Patents (Amendment) Act, 2002. The new Patent Act, 2002 has although been notified on June 25th 2002, however, currently only limited sections of it have been made applicable vide Gazette Notification from the Government of India, dated May 20, 2003. Although, it is being implemented in phased manner, however, it is a matter of time before the new Act shall be applicable in its entirety.

In the current scenario, the old Acts i.e. The Patent Act, 1970 and The Patent Rules, 2003 are applicable except for the sections made applicable through the Gazette Notification, as stated above.

WHAT IS PATENTABLE:

Patents are granted in respect of any invention in goods. An invention means any new and useful art, process, method or manner of manufacture, machine, apparatus or other article, or substance produced by manufacture, and includes any new and useful improvement in any of them.

No patent is granted in respect of claims for the substances themselves; however, claims for the methods or processes of manufacture are patentable. However, in compliance with its commitment under the TRIPS agreement, India has been given time to introduce product patent by the year 2005.

WHO CAN APPLY:

Both the Indian nationals and foreigners can make an application for patent in India. But, in case of foreigners applying for patent in India, it is necessary that the country of such applicant should also be providing such reciprocal rights to the Indian nationals.

Application for patents can be made by any person claiming to be the true and first inventor of the invention or by his assignee or legal representative. An application for patent can be made by any of these persons either alone or jointly with any other person. Two or more companies as assignees may also make an application jointly.

STEPS INVOLVED IN GRANT OF PATENT:

1. Filing of an application for grant of a patent accompanied by either a provisional specification or a complete specification before any public disclosure of the invention.
2. In case provisional specification accompanies the original application, then filing of the complete specification within 12 months from the date of filing of the provisional specification. The said period may be extended by a further period of 3 months by paying appropriate fee for extension.
3. Over coming objections, if any laid by the examiner after the technical examination of the application by the patent office.
4. Acceptance of the application and advertisement of such acceptance in the official gazette.
5. Overcoming opposition, if any, to the grant of a patent.
6. Grant and sealing of the patent.
7. Maintenance of patent by payment of renewal fee.
8. Enforcement/revocation.

PATENT COOPERATION TREATY:

Patent Cooperation Treaty is the sister treaty of the Paris Convention, which is administered by the World Intellectual Property (WIPO). The PCT facilitates filing of patent applications under a single umbrella and provides for simplified procedure for the search and examination of such applications.

Under the Paris convention an inventor gets a grace period of 12-months to file a patent application in other member countries after filing in the home country. This period of grace is extended to 30-months under the PCT, whereby an inventor can file an "international patent application" in each of the PCT member countries within this prescribed period. In India a grace period of 31 months is granted for such "international patent application".

FILING PROCEDURE UNDER THE PCT:

1) International Phase

2) National Phase

1. International Phase

India being one of the contracting state in the "PCT", any Indian applicant may file an international application in the standard format [Form "PCT" /RO/101] through any of the Indian Patent Offices as the Receiving Office i.e. The Patent Office, Kolkata, and its branch Offices at New Delhi, Mumbai, Chennai (RO/IN) along with the copy of Specification and Statutory Fees. Language of filing may be either in English or Hindi.

At the time of filing the said application the applicant is also required to mention the number of countries wherein eventually registration of patent is desired to be sought and also has to specify the name of the International Search Authority and the International Preliminary Examination Authority.

The following are the documents that must accompany a PCT Application filed through an Indian Patent office as the receiving office:

1. PCT Request (Form PCT/RO/101).
2. The complete specification in triplicate.
3. Power of Attorney
4. Certified priority Document.

On the receipt of the application, the patent office shall prepare a certified copy of the priority documents and transmit the same to the International Bureau and the International Search Authority with intimation to the applicant. Thereafter the international search is conducted and the copy of the search report is also forwarded to the applicant.

2. <u>National Phase</u>

Once the formalities under step one are duly complied and the applicant receives the International Search Report or once the Final International Preliminary Examination Report is complete and issued, the application enters the National Phase.

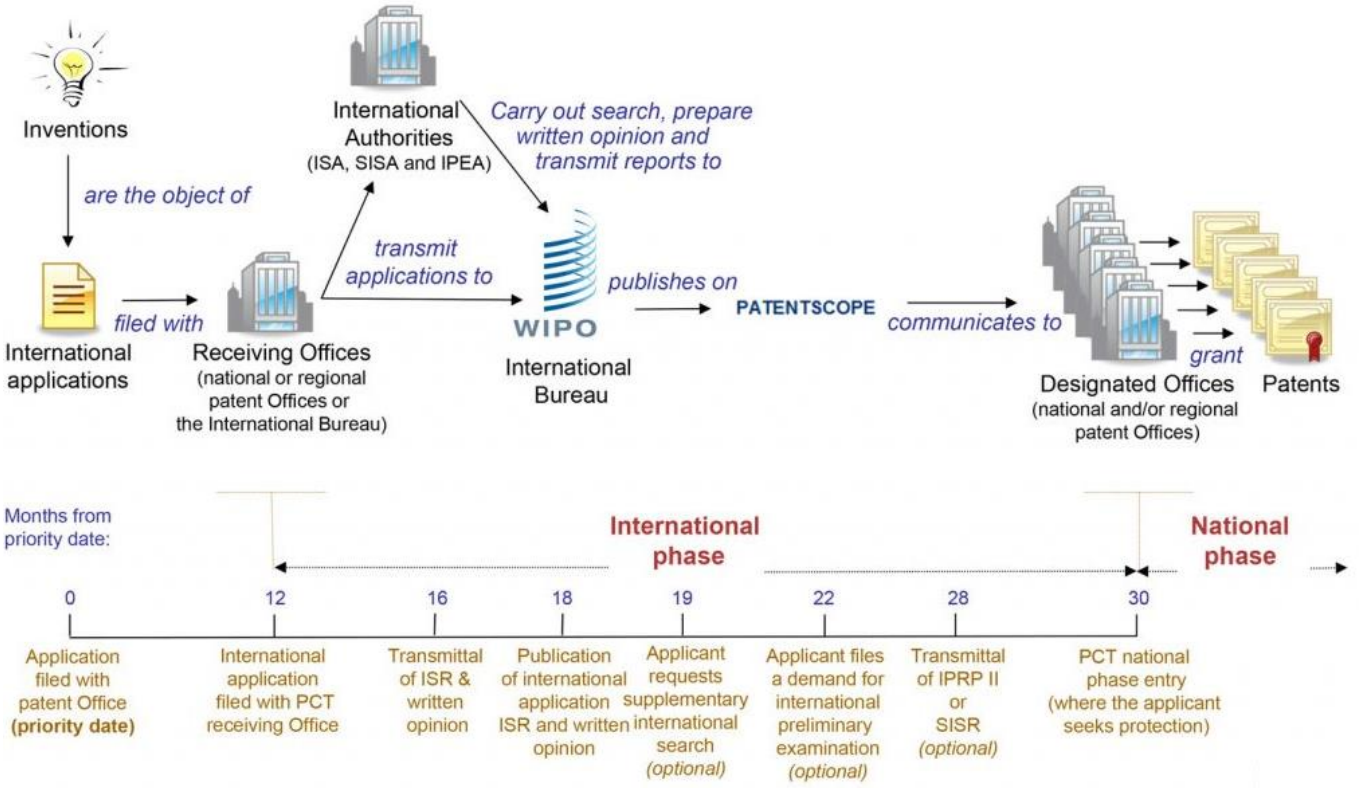
Filing of National Phase Application in India requires the request for the grant of patent to be made to the competent Receiving Office i.e. The Patent Office, Kolkata, and its branch Offices at New Delhi, Mumbai, Chennai in the prescribed form i.e. Form 1A. Language of filing may be either in English or Hindi.

Further the following information/ documents are also required to be submitted along with the necessary fees with the Patent office:

1. Request (PCT/RO/101)
2. Drawings (where applicable)
3. P.A./G.P.A. (where applicable)
4. The Specification including drawing figures as published in the "PCT" Gazette;
5. Verified English translation of international application, if not in English;
6. International Search Report;
7. International Preliminary Examination Report (if India is elected for using the IPE results);
8. Certified Copies of the Priority Documents;
9. Particulars of amendments made to the specification/claims during the "PCT" International Phase;
10. Verified English translation of amendments filed during the international phase.
11. Such other information and documents that the patent office may require to be submitted.

Process of filling a PCT-application:

- ***Filing:*** you file an international application with a national or regional patent Office or WIPO (if permitted by your State's national security provisions), complying with the formality requirements, in one language (preferably English), and you pay **one set of fees**.
- ***International Search:*** an "International Searching Authority" (ISA) identifies the published patent documents and technical literature ("prior art"). So it's a check whether your invention is patentable or not.
- ***International Publication:*** after the expiration of 18 months from the earliest filing date, the content of your international application is disclosed to the world.
- ***Supplementary International Search (optional):*** a second authority identifies published documents which may not have been found by the first ISA.
- ***International Preliminary Examination (optional):*** another way to get clarification whether your invention is patentable. A third ISAs carries out an additional patentability analysis, usually on an amended version of the application.
- ***National Phase:*** after the end of the PCT procedure, usually at 30 months from the earliest filing date of your initial application, from which you claim priority, you start to pursue the grant of your patents directly before the national (or regional) patent Offices of the countries in which you want to obtain them.



Advantages of PCT:

- Use of the PCT saves effort—time, work, money—for any person or firm seeking protection for an invention in a number of countries
- Use of the PCT also helps the applicant to make decisions about the prosecution of the application before the various national Patent Offices in the PCT National Phase of processing
- The saving arises primarily from the fact that, under the PCT, the applicant files one application—the PCT international application—in one place, in one language and pays one initial set of fees, and that this PCT international application has the effect of a national or regional application, which, without the PCT, he would have to file separately for each country or region
- The help to the applicant in the PCT National Phase prosecution of the application follows from the "advice" he obtains from the PCT international search report, a report which is established for each PCT international application, according to high, internationally regulated standards, by one of the Patent Offices that are highly experienced in examining patent applications and that have been specially appointed to carry out international searches.

Patentability

Inventions which are new, involve an inventive step and are susceptible of industrial application are **patentable** even if they concern a product consisting of or containing biological material *. Biological material which is isolated from its natural environment or produced by means of a technical process may also be the subject of an invention.

The following are **not patentable**:

- plant and animal varieties;
- Essentially biological processes for the production of plants or animals, such as crossing or selection. This exclusion from patentability does not, however, affect the patentability of inventions which concern a microbiological process *;
- The human body and the simple discovery of one of its elements, including the sequence or partial sequence of a gene.

However, an element isolated from the human body or produced by means of a technical process, including the sequence or partial sequence of a gene, may constitute a patentable invention.

The following inventions include those that are unpatentable where their exploitation would be **contrary to public policy or morality**:

- processes for cloning human beings;
- processes for modifying the germ-line genetic identity of human beings;
- uses of human embryos for industrial or commercial purposes;
- Processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes.

Patenting process:

An invention is patentable only if it is:

- New and previously undisclosed
- Distinguished by an inventive step not obvious to someone expert in that technology

- Capable of industrial application - that is, it is physically possible to make the invention

Some issues to consider before deciding to patent:

- Do you really need a patent? Would some combination of other forms of IPR protect your idea adequately? And be honest with yourself - are you perhaps motivated more by vanity (the prospect of a patent in your name) than by commercial necessity?
- Have you studied the total cost of patenting (which should include annual renewal fees in every country in which you have protection)? Is your invention likely to earn enough income to justify the cost? Normally, you should not apply for a patent until you have thoroughly researched the commercial and financial potential of your idea.
- Is the time right to apply for a patent? Application starts a sequence of events which cannot be delayed. Do you apply for a patent early on, or wait until the invention is market-ready and more capable of quickly recouping its IPR costs? Later may be better than sooner, but circumstances will vary so you should always seek the advice of a patent attorney.
- Does your invention have a short product life cycle? The patenting process typically takes 3-4 years. If your invention is aimed at a highly competitive market in which products are rapidly replaced or improved, your patent may be worth little by the time it is granted.
- Who will pay to enforce your patent? National IP offices do not enforce patents or monitor them for infringement. These are the responsibilities of the patent owner or a licensee. Until funds are potentially available to enforce your patent - from royalties or sales income - it may offer limited practical protection.
- How strongly might your patent resist legal challenge? You will definitely need a patent attorney's advice on the strength of your claims. This is important because the validity of patent claims is often challenged, usually by competitors who want to copy a successful product. If they succeed, you may be left with a valueless patent and an order to pay the victor's legal costs.

Applying for a patent

Applying for a patent is a legal process governed by strict timescales and usually immovable deadlines. It is not something to rush into! To maximize your chances of a worthwhile patent you should:

- Study the application procedure in detail.
- Aim to apply not in haste, but **strategically** - at a time and for a reason that most benefits your exploitation plans.
- Use a patent attorney! **Do not** do it all yourself - the risk of making mistakes is too great.

Here is only a very brief guide to the application process for a European Patent according to the European Patent Convention (EPC).

✈ Applying for a patent at a national IP office is roughly similar to stages 1-6 below, but an application must be made in the local language.

Making an international application through the Patent Co-operation Treaty (PCT) involves a single procedure for stages 1-4, but 30 months after filing the application goes through stages 5 and 6 in every national or regional IP office where you wish to take up protection.

Choosing your route for a patent application (EPC, PCT, national and regional, or combinations thereof will depend on:

- Your invention.
- Your business plan.
- Your available funds.
- Your intended market.
- The likeliest sources of infringing products.

A patent attorney will be able to advise you on the route that is best **for you and your invention**.

Stage 1: Beginning the process:-

Your patent attorney must provide documentation consisting of:

- A request for a patent.
- Details of the applicant (you).
- A description of the invention.
- Claims.
- Drawings (if any).
- An abstract.

A fee must also be paid. In order to avoid delay, it is vital that all documentation conforms in every detail to official requirements. Your patent attorney will ensure that it does. At the EPO, applications are accepted in English, French or German.

For your patent attorney to prepare all the information about your invention, he or she will obviously need to work closely with you. **Do not assume that you know best because it is your invention**. You must trust the skill and judgement of your patent attorney, as patenting involves a complex mix of law and technology. The claims in particular need to be drafted with skill, as they are the most important aspect of a patent.

Stage 2: Filing date and initial examination:-

If your documentation appears correct, your application is given a **filing date** - also known as your **priority date**. After filing there is a **formalities examination** to ensure that your documentation is correct and complete.

At any time in the next 12 months you can file for patent protection in other countries and have those later filings treated as if they had been filed on your priority date. In practice, this gives you a year to decide how many countries you wish to include in your patent protection.

Stage 3: Search:-

A **search report** is sent to you, listing and including copies of all prior art documents found by an experienced examiner and regarded as relevant to your invention. The search is based mainly on your claims for novelty, but your description and any drawings will also be taken into account. The report will often include an initial opinion on the patentability of your invention.

Stage 4: Publication:-

Your application is **published** 18 months after the filing date. Your invention will appear in databases accessible to other people around the world. It will act as **prior art** against any future patent applications from other inventors or companies for similar inventions.

You then have six further months to make two decisions:

- Do you want to continue with your application? You indicate ‘yes' by requesting a more thorough (‘substantive') examination.

- Which countries do you want to include (‘designate') in your patent protection? Designation fees must be paid.

After your patent is granted, you may claim damages for infringements originating as far back as the publication date of your application. However, to enjoy this right in some countries it may be necessary to file a translation of your claims with their national IP office and for them to publish the translated claims.

Stage 5: Substantive examination:-

If you request **substantive examination**, the EPO has to decide whether your invention **and** your application meet the requirements of the European Patent Convention. For maximum objectivity there are usually three EPO examiners, one of whom maintains contact with your patent attorney. This stage will often involve dialogue between the examiners and your patent attorney, which may result in the re-drafting of key parts of your application. Your patent attorney will defend your application, and this is one more reason why it is essential to have professional representation.

Stage 6: Decision to grant a patent:-

If the examiners decide to grant a patent, and all fees have been paid and any claims translations filed, the decision is reported in the European Patent Bulletin. The **decision to grant** takes effect on the date of publication.

Stage 7: Validation:-

What you have now got is a ‘bundle' of individual national patents. After the EPO decision to grant is published, your patent has to be **validated** in each designated state within a specific time limit. If this is not done, your patent may not be enforceable in that state. In some states, validation may include having to file (and pay for) a translation of the whole patent, or just a translation of the granted claims.

Stage 8: Opposition:-

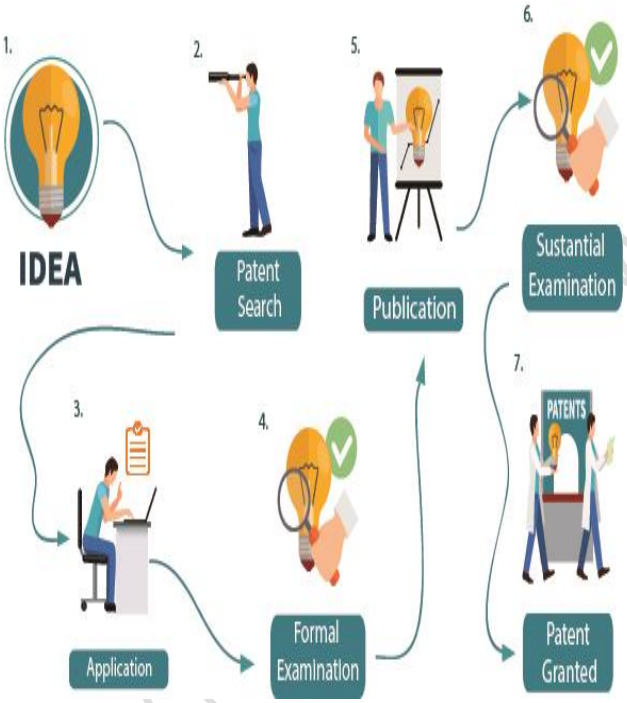
A granted patent may be **opposed** by third parties - usually the applicant's competitors - if they believe it should not have been granted. After the grant is reported in the European Patent Bulletin they have nine months in which to file notice of opposition. The most common charge is that the invention is

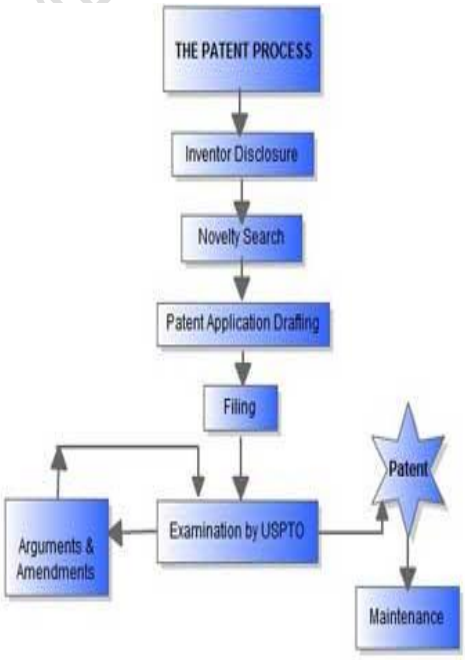
not novel or lacks an inventive step. The case will be examined by an EPO team, again of three examiners.

Opposition is the last chance to attack a European patent **as a single entity in a single forum**. Later, the patent can only be challenged in national courts and a ruling in one country has no effect on the patents for the same invention in other countries. This gives competitors a strong incentive to challenge an invention during the opposition period, as challenging patents in separate national courts can be much more expensive.

Stage 9: Appeal:-

All EPO decisions are open to appeal. Responsibility for decisions on appeals is taken by independent boards of appeal.





Step by step guide for how to get patent in India

Step 1: Write down the invention (idea or concept) with as much details as possible

Collect all the information about your invention such as:

- Area of invention
- Description of the invention what it does

- How does it work
- Advantages of the invention

Ideally, if you have worked on the invention during research and development phase you should have something call lab record duly signed with date by you and respective authority.

Step 2: include drawings, diagrams or sketches explaining working of invention

The drawings and diagrams should be designed so as to explain the working of the invention in better way with visual illustrations. They play an important role in patent application.

Step 3: check whether the invention is patentable subject matter

All inventions may not be patentable, as per Indian patent act there are certain inventions that are not patentable explained in detail in(inventions not patentable)

Step 4a: Patentability search

The next step would be finding out whether your invention meets all patentability criteria as per Indian patent act? That is,

- Novelty
- Non-obviousness
- Industrial application
- Enabling

The detailed explanation for patentability criteria is given here (what are patentability criteria's). The patentability opinion is provided by the patent professionals up on conducting extensive search and forming patentability report.

Step 4b: Decide whether to go ahead with patent

The patentability report and opinion helps you decide whether to go ahead with the patent or not, chances are what you thought as novel might already been patented or know to public in some form of information. Hence this reports saves lots of time, efforts and cost of the inventor by helping him decide whether to go ahead with the patent filing process or not.

Step 5: Draft (write) patent application

In case you are at very early stage in the research and development for your invention, then you can go for **provisional application**. It gives following benefits:

- Secures filing date
- 12 months of time to file complete specification
- Low cost

After filing provisional application, you secure the filing date which is very crucial in patent world. You get 12 months of time to come up with the complete specification, up on expiry of 12 months your patent application will be abandoned.

When you complete the required documents and your research work is at level where you can have prototype and experimental results to prove your inventive step you can file complete specification with patent application.

Filing the provisional specification is the optional step, if you are at the stage where you have complete information about your invention then you can directly go for complete specification.

Step 6: Publication of the application

Up on filing the complete specification along with application for patent, the application is published after 18 months of first filing.

An early publication request can be made along with prescribed fees if you do not wish to wait till the expiry of 18 months from the date of filing for publishing your patent application.

Generally the patent application is published within a month form request form early publication.

Step 7: Request for examination

The patent application is examined only after receiving request for examination that is RFE. Up on receiving this request the controller gives your patent application to a patent examiner who examinees the patent application with different patentability criteria like:

- Patentable subject matter

- Novelty
- Non-obviousness
- Inventive step
- Industrial application
- Enabling

The examiner creates a first examination report of the patent application upon reviewing it for above terms. This is called patent prosecution. Everything happening to patent application before grant of patent is generally called as patent prosecution.

The first examination report submitted to controller by examiner generally contains prior arts (existing documents before the date of filing) which are similar to the claimed invention, and same is reported to patent applicant.

Step 8: respond to objections

Majority of patent applicants will receive some type of objections based on examination report. The best thing to do it analyse the examination report with patent professional (patent agent) and creating a response to the objections raised in the examination report.

This is a chance for an inventor to communicate his novelty over prior arts found in the examination report. The inventor and patent agent create and send a response to the examination that tries to prove to controller that his invention is indeed patentable and satisfies all patentability criteria's.

Step 9: clearing all objections

This communication between controller and patent applicant is to ensure that all objections raised in the patent application are resolved. (if not the patent will not be granted) and the inventor has his fair chance to prove his point and establish novelty and inventive step over existing prior arts.

Up on finding the patent application in order of grant, it is grant to the patent applicant as early as possible.

Step 10: Grant of patent

The application would be placed in order for grant once it is found to be meeting all patentability requirements. The grant of patent is notified in the patent journal which is published time to time.

Present Indian Scenario

PATENTS IN THE INDIAN SCENARIO:

The laws pertaining to Patent in India is governed by the Patents Act, 1970 which has been amended twice by The Patents (Amendment) Act, 1999 and The Patents (Amendment) Act, 2002. The new Patent Act, 2002 has although been notified on June 25th 2002, however, currently only limited sections of it have been made applicable vide Gazette Notification from the Government of India, dated May 20, 2003. Although, it is being implemented in phased manner, however, it is a matter of time before the new Act shall be applicable in its entirety.

In the current scenario, the old Acts i.e. The Patent Act, 1970 and The Patent Rules, 2003 are applicable except for the sections made applicable through the Gazette Notification, as stated above.

WHAT IS PATENTABLE:

Patents are granted in respect of any invention in goods. An invention means any new and useful art, process, method or manner of manufacture, machine, apparatus or other article, or substance produced by manufacture, and includes any new and useful improvement in any of them.

No patent is granted in respect of claims for the substances themselves; however, claims for the methods or processes of manufacture are patentable. However, in compliance with its commitment under the TRIPS agreement, India has been given time to introduce product patent by the year 2005.

WHO CAN APPLY:

Both the Indian nationals and foreigners can make an application for patent in India. But, in case of foreigners applying for patent in India, it is necessary that the country of such applicant should also be providing such reciprocal rights to the Indian nationals.

Application for patents can be made by any person claiming to be the true and first inventor of the invention or by his assignee or legal representative. An application for patent can be made by any of these persons either alone or jointly with any other person. Two or more companies as assignees may also make an application jointly.

STEPS INVOLVED IN GRANT OF PATENT:

1. Filing of an application for grant of a patent accompanied by either a provisional specification or a complete specification before any public disclosure of the invention.
2. In case provisional specification accompanies the original application, then filing of the complete specification within 12 months from the date of filing of the provisional specification. The said period may be extended by a further period of 3 months by paying appropriate fee for extension.
3. Overcoming objections, if any laid by the examiner after the technical examination of the application by the patent office.
4. Acceptance of the application and advertisement of such acceptance in the official gazette.
5. Overcoming opposition, if any, to the grant of a patent.
6. Grant and sealing of the patent.
7. Maintenance of patent by payment of renewal fee.
8. Enforcement/revocation.