5th SEM B.Sc. ZOOLOGY

CALICUT UNIVERSITY

BIOTECHNOLOGY, MICROBIOLOGY & IMMUNOLOGY

2019 ADMISSION

Prepared by

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FIFTH SEMESTER B.Sc. ZOOLOGY PROGRAMME
ZOOLOGY CORE COURSE- VI [Theory]
BIOTECHNOLOGY, MICROBIOLOGY AND IMMUNOLOGY
Code: ZOL5B07T
[72 hours] [4 hours per week] [4 Credits]

Section A: BIOTECHNOLOGY (24 hrs)

MODULE 1: Genetic Engineering and Animal cell culture (12 Hrs)

Genetic Engineering (10 hrs)

Concept and scope of biotechnology – Mention branches of biotechnology. Introduction to the concept of Recombinant DNA Technology: Cloning vectors (Plasmid, pBR322, Phages, Cosmids, Virus vectors, YAC vector and Bac vector).

Enzymes: Type II Restriction endonucleases, polynucleotide kinase, exonuclease, terminal transferase, reverse transcriptase and DNA ligase.

Construction of Recombinant DNA: Preparation of vector and donor DNA, Joining of vector DNA with the donor DNA, Introduction of recombinant DNA into the host cell and selection of transformants (brief account).

Animal Cell Culture (2 hrs)

Cell culture media (Natural and Defined), Preparation and Sterilization, Primary cell culture, Cell Lines, Pluripotent Stem Cells, Cryopreservation of cultures. Somatic cell fusion and HAT selection of hybrid clones – production of monoclonal antibodies.

MODULE 2: Transgenic Organisms (5 hrs)

Transfection Methods: (Chemical treatment, Electroporation, Lipofection, Microinjection, Retroviral vector method, Embryonic stem cell method and Shot Gun Method). Transgenic Animals: (Fish, Pig, Sheep, Rabbit, Mice, Goat and Insects), Knock Out Mice. Human Cloning and Ethical Issues of transgenic Animals.

MODULE 3: Applications of Biotechnology (7hrs)

Molecular diagnosis of genetic diseases (Cystic Fibrosis, Huntington's Disease and Sickle Cell Anemia). Vaccines and Therapeutic agents, Recombinant DNA in Medicines (Recombinant Insulin and Human Growth Hormone).

Human gene therapy (gene therapy for severe combined immune deficiency).

Enzymes in detergents and leather industries, Heterologous protein production, Biofiltration, Bioremediation, Bioleaching, Molecular pharming and Bioreactors.

Molecular markers (brief account) RFLP, RAPD, VNTR, SNPs and their uses.

Section B: MICROBIOLOGY (24 hrs)

MODULE 4: Introduction and Methods in Microbiology (8 hrs)

Introduction (1 hr)

Microbial Diversity: Archaebacteria, Eubacteria, Prochlorophyta, Algae, Fungi, Protozoa, Viruses, Viroids, Prions, Mycoplasma and Rickettsias

Methods in Microbiology (7 hrs)

Sterilization: Physical and Chemical methods - Dry and Moist Heat, Pasteurization, Radiation, Ultrasonication. Disinfection, Sanitization, Antiseptics, Sterilants and Fumigation. Preparation of culture media: Selective, Enrichment and Differential media. Plating techniques and Isolation of pure colonies. Staining: Simple staining, Negative staining and Gram staining. Culture preservation techniques: Refrigeration, Deep freezing, Freezing under liquid Nitrogen and Lyophilisation.

MODULE 5: Basic Concepts in Bacteriology and Virology (8 hrs)

Bacteria: Structure of a typical Bacterium, Different types of bacterial culture (Batch, Synchronous, Arithmetic), Bacterial growth: Growth phases, Methods of growth determination.

Basic Concepts of Virology: General characteristics and classification of viruses. Bacteriophages: Diversity, lytic and lysogenic Phages (Lambda and P1 Phage),

Applications of bacteriophages. Oncogenic Viruses. Prevention and control of Viral diseases: Antiviral compounds, Interferons and viral vaccines.

MODULE 6: Industrial and Medical Microbiology (8 hrs)

Industrial Microbiology (4 hrs)

Bioengineering of microorganisms for industrial purposes: Microbial production of industrial products (micro-organisms involved, media, fermentation conditions, downstream processing and uses) - citric acid, ethanol, wine, penicillin, glutamic acid, riboflavin, enzymes (amylase, cellulase, protease, lipase, glucose isomerase, glucose oxidase). Bioinsecticides (Bt) and Steroid biotransformation.

Medical Microbiology (4 hrs)

Normal microflora of the human body: skin, throat, gastrointestinal tract and urogenital tract.

Diseases caused by: (with reference to causative agent, symptoms and mode of transmission).

a) Bacteria: anthrax, tuberculosis, typhoid, whooping cough, pneumonia, cholera, gonorrhea, and syphilis.

b) Viruses: polio, chicken pox, herpes, hepatitis, rabies, dengue, AIDS and chikungunya.

c) Protozoa: malaria, kala-azar and toxoplasmosis.

d) Fungi: dermatomycoses and opportunistic mycoses

Bacterial drug resistance.

Section C: IMMUNOLOGY (24 hrs)

MODULE 7: Cells and organs of immune system (6 hrs)

Introduction (1 hr)

Immunity: Natural and acquired, active and passive, immunization, vaccines, mechanisms of innate immunity - barriers, inflammation, phagocytosis.

Cells of the immune system (3 hrs)

B- cells, T – cells, NK cells, monocytes, macrophages, neutrophils, basophils, eosinophils, mast cells, and dendritic cells (APCs).

Organs of the immune system (2 hrs)

Lymphoid organs: Primary (thymus, bone marrow) and secondary (lymph nodes, spleen).

MODULE 8: Antigens, antibodies, immunity and MHC (9 hrs)

Antigens (3 hrs)

Types, factors for immunogenecity, exogenous antigens, endogenous antigens, adjuvant, haptens, epitopes, antigen-antibody reaction - precipitation reaction, agglutination reaction, agglutination inhibition reaction.

Immunoglobulins (2 hrs)

Structure, classification and biological functions. Mention immunoglobulin gene families – κ and λ light chain families and the heavy chain family.

Immunity (2 hrs)

Types of Immunity: humoral and cell mediated immunity, primary and secondary response, generation of cytotoxic T- cells (CTLs), NK cell mediated cytotoxicity, ADCC and cytokines (brief).

Major Histocompatability Complex (2 hrs)

MHC, HLA, Class I MHC, Class II MHC molecules and structure. Mention Class III MHC.

MODULE 9: Autoimmune and Immunodeficiency diseases, Tumor and transplantation immunology (9 hrs)

Autoimmune diseases (2 hrs)

Auto immune diseases: Systemic (SLE, multiple sclerosis and rheumatoid arthritis). Organ specific-(Hashimoto's thyroiditis, Grave's disease, Myasthenia gravis)

Immunodeficiency disease **(3 hrs)**

Primary (Bruton's Disease, Di-George syndrome and SCID) Secondary (AIDS) – Clinical course of HIV – acute infection, seroconversion, window period, chronic latent phase – lymph adenopathy and crisis phase. Mention antiretroviral therapy (ART)

Tumor immunology **(2 hrs)**

Malignant transformation of cells, tumor antigens, immune response to tumor antigens.

Transplantation Immunology **(2 hrs)**

Transplantation Antigens, Various organ transplantation (liver, kidney, heart, skin), Xenotransplantation

MODULE 1

GENETIC ENGINEERING AND ANIMAL CELL CULTURE

GENETIC ENGINEERING

Concept and scope of biotechnology

- Term derived from the fusion of biology & technology.
- Coined by Karl Ereky.
- Deals with the exploitation of biological agents or their components for generating useful products.

Definition

i. The application of biological organisms, systems or processes constitute biotechnology (British biotechnologist).

ii. Biotechnology is the integrated use of biochemistry, microbiology and engineering sciences in order to achieve technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof (European Federation of Biotechnology).

iii. Biotechnology is the controlled use of biological agents such as microorganisms or cellular components for beneficial use (US National Science Foundation).

iv. Biotechnology is a technology using biological phenomena for copying & manufacturing various kinds of useful substances (Japanese biotechnologists).

Scope of Biotechnology

- Has made advances in two major areas namely molecular biology and microbiology.
- Production of industrially important bio chemicals including enzymes and for basic studies in molecular biology.
- Biotechnology now plays a very important role in employment, production and productivity, trade, economics and economy, human health, and the quality of human life throughout the world.
- For the protection of human health, production of monoclonal antibodies, DNA, and RNA probes, artificial vaccines for inoculation, rare and valuable drugs such as human interferon, insulin,..
- The technology for gene therapy for the treatment of genetic diseases, identification of parents / criminals using DNA or autoantibody finger printing are very accurate and reliable from even blood or semen stains, hair roots, etc. are notable achievements in the field of medical biotechnology.
- Microorganisms are being employed for several decades tor the large-scale production of a variety of bio chemicals ranging from alcohol to antibiotics and in the processing of foods and feeds (industrial biotechnology).
- Techniques for remodeling existing proteins/enzymes to enhance their efficiency and/or alter their specificity have been developed (protein/enzyme engineering).

- Several biological agents such as viruses, fungi, bacteria, etc. are being exploited for the control of plant diseases and insect pests (bio control).
- Bacteria are being utilized for detoxification of industrial effluents (wastes), in combating oil spills, for treatment of sewage, and for biogas production (environmental biotechnology).
- Microbes are also being employed for the extraction of metals from such low-grade ores, the conventional extraction from which would be uneconomical (microbial mining).
- In vitro fertilization and embryo transfer techniques have permitted childless couples suffering from one or the other kind of sterility to have their own babies (test tube babies).
- Hormone-induced super ovulation and/or embryo splitting coupled with embryo transfer can be used for the rapid multiplication of farm animals particularly cattle.
- Genetic engineering is being employed to develop transgenic animals resistant to certain diseases, capable of faster growth rates and more efficient feed conversion, and with the capacity to produce certain valuable bio chemicals and to excrete them in milk, urine, or blood from which they can be isolated and purified (molecular farming).
- In agriculture rapid and economic clonal multiplication of fruit and forest trees, production of virus-free stocks of clonal crops, creation of novel genetic variations through somaclonal variations, and transfer of novel and highly valuable genes for disease and insect resistance through genetic engineering have opened up exciting possibilities in crop production, protection, and improvement (agricultural biotechnology).

Branches of biotechnology

1. Medical biotechnology

- Red biotechnology
- Protection of human health
- Production of recombinant vaccines
- Monoclonal antibodies
- Valuable drugs
- Technology for gene therapy
- DNA fingerprinting

2. Industrial biotechnology

- Production of variety of biochemicals from alcohols to antibiotics.
- Production of enzymes – protease, amylase, lipase,..
- Protein engineering
- Microbial mining

3. Environmental biotechnology

- Detoxification of industrial effluents by bacteria
- Bacteria are used in combating oil spills.

- Treatment of sewage
- Biogas production

4. Animal biotechnology

- Invitro fertilization
- Embryo transfer
- Production of transgenic animals
- Production of valuable proteins in milk, urine or blood – molecular farming

5. Plant biotechnology

- Production of transgenic plants
- Producing plants resistant to the attack of insects, viruses, herbicides,..
- Germ plasm conservation
- Meristem culture

6. Blue biotechnology

- Marine & aquatic applications of biotechnology
- Use rarely

Introduction to the concept of Recombinant DNA Technology

- Recombinant DNA or rDNA is made by combining DNA from two or more sources.
- The process often involves combining the DNA of different organisms.
- The process depends on the ability to cut, and rejoin, DNA molecules at points identified by specific sequences of nucleotide bases called restriction sites.
- DNA fragments are cut out of their normal position in the chromosome using restriction enzymes (restriction endonucleases) and then inserted into other chromosomes or DNA molecules using enzymes called ligases.
- A fragment of DNA containing a single gene or several genes can be inserted into a vector (carrier molecule) that can be propagated within another cell.
- The product thus obtained is called recombinant DNA (rDNA).
- Recombinant DNA is one in which nucleotide sequences from two different sources are combined invitro into the same DNA molecule.
- Using this technique, a single copy of a gene or DNA segment that includes one or more genes can be isolated and cloned into an indefinite number of copies, all identical. This technique is called gene cloning.
- This became possible because bacteria, phages, and plasmids reproduce in their usual manner even after insertion of foreign DNA so that the inserted DNA will also replicate faithfully with the parent DNA.

Cloning vectors

- Molecule that has the ability to replicate in an appropriate host cell & into which the DNA fragment to be cloned called DNA insert is integrated for cloning.
- Used for the transport of foreign DNA into the host cell.
- Cloning vehicles
- Should be able to replicate autonomously
- Should be easy to isolate & purify
- Should be easily introduced into the host cell
- Should have suitable marker genes
- Should have the ability to integrate
- Should contain unique target sites
- Should contain suitable control elements

Plasmid

- Self replicating double stranded circular DNA
- Extra chromosomal entity in bacteria
- Capable of conjugation – F plasmid
- Encode resistance to antibiotics – R plasmid
- Carry genes for the utilization of unusual metabolites
- Size – less than 1 kb to 500 kb

pBR322

- First artificial cloning vector
- P – plasmid , B – Boliver & R – Rodrigues
- Contains origin of replication – ori
- Contains 4361 bp & two antibiotic resistance genes – ampicillin (amp^r) & tetracycline (tet^r)
- 20 unique recognition sites for restriction endonucleases (Bam H1, *Eco* RV, *Sph* I, *Sal* I, *Xma* III, *Nru* I, *Hind* III, *Cla* I, *Pst* I, *Sca* I, *Pvu* I).

Phages

- Bacteriophages are the viruses that infect bacterial cells
- Two bacteriophages – phage λ & M13 – used as cloning vectors
- λ genome contains origin of replication, genes for head & tail proteins and the enzymes for DNA replication, lysis, lysogeny, cohesive ends of 12 bases.
- Two kinds of vectors
    1. λ replacement vectors – two restriction sites
    2. λ insertion vectors – single restriction site

- Efficient than plasmids for cloning of large DNA fragments
- Easier to screen large number of phage plaques

Cosmids

- Hybrid vector
- Plasmid + λ phage
- Has origin of replication
- Unique restriction sites
- Selectable markers
- Length – 5 kb

Virus vectors

- Cauliflower mosaic virus, Tobacco mosaic virus, Simian virus 40, Bovine Papilloma virus
- Animal viruses can introduce the genes directly by infecting the host cells.
- Contain powerful promoters which are needed for gene expression.
- Capacity to replicate their genomes into large numbers

YAC vector

- Yeast Artificial Chromosome
- Circular forms in bacteria
- Linear forms in yeast
- Consist of centromere, telomere, origin of replication, ARS, SUP4

BAC vector

- Bacterial artificial chromosome
- Used to clone DNA sequences in bacterial cells
- Each BAC is a DNA clone containing roughly 100 – 300 thousand base pairs of cloned DNA.
- Useful for mapping & sequencing mammalian genomes

Enzymes

Type II Restriction endonucleases

- Restriction endonuclease - term was coined by Lederberg and Messelson in 1964
- Enzymes that destroy (restrict) any foreign DNA entering the host cell
- They serve as molecular scissors for cutting down the DNA into discrete reproduced fragments.
- They cut DNA at defined sites and are one of the most important groups of enzymes for the manipulation of DNA.

- These are found in bacterial cells, where they function as part of a protective mechanism called restriction - modification system.
- In this system, the restriction enzyme hydrolyses any exogenous (foreign) DNA that appears in the cell. e.g., viral DNA.
- The restriction endonucleases are a group of enzymes that recognize specific nucleotide sequences in DNA often 4 or 6 base pairs long and cut both strands of DNA within the recognition sites.
- Sometimes restriction enzymes cleave both DNA strands at precisely opposite points on the two strands yielding blunt-ended fragments.
- In some cases the two DNA strands are not cut directly opposite each other, instead, the cuts are staggered forming cohesive ends (sticky ends).
- The sequences recognized by restriction enzymes are often palindromes.
- Restriction – modification enzymes are divided into two – class I & class II
- Two classes – Class I – type II enzymes
  Class II – type I & III enzymes
- First type II enzyme isolated was *Eco* R1 in 1970.
- More than 3000 type II restriction enzymes have been characterized.
- Widely used for mapping & reconstructing DNA.

Polynucleotide kinase

- PNK
- Catalyses the reversible phosphorylation of nucleoside monophosphates, nucleic acids,..
- PNK from bacteriophage T4 is widely used in molecular biology

Exonuclease

- Degrade nucleic acids
- Breaks phosphodiester bond
- Cut off from 5’ or 3’ ends of DNA molecule
- Eg:- Bal 31, exonuclease III

Terminal transferase

- Terminal deoxynucleotidyl transferase (TdT) is a unique DNA polymerase.
- Catalyzes the polymerization of deoxyribonucleotides in the absence of a template.
- It performs DNA synthesis using only single-stranded DNA as the nucleic acid substrate.
- TdT catalyzes the template - independent addition of deoxyribonucleotides to the 3’ hydroxyl terminus of single or double-stranded DNA.
- It can also catalyze the addition of ribonucleotides and a range of unnatural nucleotides into DNA strands.

- TdT requires a single-stranded initiator that is at least three nucleotides long with a free 5' phosphate end and a free 3' hydroxyl end for extension.

Reverse transcriptase

- Used to synthesize the copy of DNA or cDNA by using mRNA as a template
- Eg:- RNA dependent DNA polymerase

DNA ligase

- Joining enzymes
- Repair broken phosphodiester bonds
- Seal discontinuities in the sugar phosphate chain
- Molecular glue
- Eg:- T4 DNA ligase

Construction of Recombinant DNA

Procedures

1. Generation of DNA fragments
2. Cutting of DNA using restriction endonucleases
3. Selection of cloning vectors
4. Fragment is inserted to the cloning vector
5. DNA ligase links cloning vector & DNA
6. Transfer of recombinant vectors into the bacterial cell by transformation or infection.
7. Identifying host cell containing recombinant DNA.

Preparation of vector and donor DNA

Generation of DNA fragments

Three methods

First

1. Fragmentation of DNA by restriction enzymes.
2. Fragments are separated by gel electrophoresis
3. Fragment is identified from the separated bands by using molecular probes.

Second

- Make a copy of gene from its mRNA, using reverse transcriptase.
- DNA formed is called complementary DNA

Third

- Artificial synthesis of gene
- Synthesized by chemical method

Joining of vector DNA with the donor DNA

Place the gene in a vector

Three methods

1. ***Cohesive end ligation***

- Common method
- Restriction enzyme produces staggered cuts & produces sticky ends
- Another fragment having complement sequence anneal with it producing a rDNA.

2. ***Homopolymer tailing***

- DNA can be cut at the desired position both in vector & clone, without staggered cleavage.
- dATP poly dA is added at 3' cut ends of vector with the help of terminal transferase.
- dTTP poly dT is added at 3' cut ends of DNA sequence to be cloned.
- Clone & vector are joined by annealing the poly dA with poly dT tail
- Ligation by DNA ligase.

3. ***Blunt end ligation by T4 DNA ligase***

- Restriction enzyme is used to cut a duplex
- Broken ends are joined with another DNA molecule
- T4 DNA ligase is used for joining
- Disadvantage – any DNA molecule may join.

Introduction of recombinant DNA into the host cell and selection of transformants

- The plasmid or phage vector must be introduced into a bacterial cell which will allow the vector to multiply.
- The process of adding new DNA to a bacterial cell is called transformation.
- The recombinant vector is added to a flask containing a culture of *E.coli*.
- Calcium chloride is added to the flask followed by a brief heat shock.
- This has the effect of making holes appear briefly in the cell surface membranes of *E.coli* making them permeable to DNA and allowing the plasmids to enter.
- The insert contains a selectable marker that allows for the identification of recombinant molecules.

Detection of transformed cells

- **Direct selection method** -This occurs when the desired gene specifies resistance to an antibiotic.
- **Blue – white selection method** - It is a powerful method for screening recombinants.
- In this method a reporter gene lac Z is inserted in the vector (encodes β-galactosidase).
- β- galactosidase breaks a synthetic substrate, X-gal into an insoluble blue coloured product.
- If a foreign gene is inserted into lac Z, this gene will be inactivated therefore no blue colour will develop.
- The host cells containing recombinant will form white coloured substrate on the medium containing X-gal.
- The host cells containing non recombinants will turn blue in colour.
- **Colony hybridization** - applied to DNA or RNA released from blotted microbial colonies.
- The microbial colonies are transferred (blotted) to a membrane.
- The cells are lysed in place to release the nucleic acids.
- The RNA or DNA (after denaturation) is fixed to the filter and hybridized with a labelled probe.
- Blocking reagent may be added prior to the probe to prevent unspecific binding.
- Excess probe is washed away and the membrane is visualized by UV or autoradiography.
- **Plaque hybridization -** This technique is used for the screening of bacteriophages from plaque-forming units.
- In this method lawn of *E.coli* cells are prepared on an agar medium which is allowed to get infected by recombinant phage particles.
- They infect *E.coli*, multiply inside cells and lyse them forming plaques.
- As in colony hybridization, a replica plate is prepared from a master plate containing plaques using a nitrocellulose filter and follows the steps exactly like in colony hybridization.
- Recombinant phage particles isolated from the plaques are used for further study.

ANIMAL CELL CULTURE

Cell culture media (Natural and Defined)

- The nutrient media used for the culture of animal cells and tissues must be able to support their survival as well as growth
- They must provide nutritional and hormonal factors.
- It is of two types
  (1) Natural media
  (2) Defined media.
- ***Natural media*** consists solely of naturally occurring biological fluids
- plasma clot, biological fluids (amniotic fluid, ascetic fluid, pleural fluid, serum, aqueous humour from the eye, insect hemolymph.
- The serum is the most widely used and is obtained from adult human blood, horse blood, or placentał cord blood, or calf blood.
- ***Defined or Artificial medium*** is a medium in which all the chemicals used are known.
- It is of different types
  (1) Media with serum - Eagles minimum essential medium, with 5 to 20 % serum. Serum provides plasma proteins, peptides, lipids, minerals, carbohydrates, and some enzymes.
  (2) Media without serum - Dulbecco's enriched modification of minimum essential medium (DME)
- Serum free media is used to avoid the influence of serum constituents in the culture like toxic effect or degradation of sensitive proteins by serum proteases.
- Chemically defined media contain contamination free ultra pure inorganic and organic constituents and may contain pure protein additives, like insulin, epidermal growth factor, etc.
- Protein-free media do not contain any protein, they only contain non protein constituents necessary for the culture of the cells.

Preparation and Sterilization

- The site of the opening is sterilized with 70% alcohol and the tissue is removed aseptically and placed in a balanced salt solution (4) disaggregation of the explants.
- Cell cultures are generally started from disaggregated explants.
- Disaggregation is by mechanical method or enzymatic method using trypsin and collagenase or using EDTA. (Ethylene diamine tetraacetic acid).
- Preparation and sterilization of the substrate plastic wares are supplied sterilized, treatments are not necessary and ready for use.
- Glass wares must be carefully washed with nontoxic detergent following, an overnight soak, thoroughly rinsed in tap water, and finally in distilled water.
- Glasswares are kept in containers or wrapped in aluminum foil and sterilized in dry heat (160°C for one hour) while screw caps are autoclaved separately.

- The quality of glassware washing and sterilization should be checked periodically.
- The various media and reagents used in cell cultures must be carefully sterilized either by autoclaving or by filtration.
- Heat stable constituents are autoclaved at 121°C for 20 minutes.
- Heat labile constituents like proteins, growth factors, serum, proteins etc must be sterilized by filtration through a 0.2 mm porosity membrane filter.
- Each filtrate should be tested for sterility to avoid failure due to contamination.
- Autoclaving is preferred to filtration since it is cheaper, needs less labor, and is uniformly effective.
- Most of the media now available commercially are usually pre-sterilized and ready for use.

Primary cell culture

- Cell culture is the complex process by which cells are grown under controlled conditions on a suitable nutrient medium.
- Freshly isolated cell cultures are called ***primary cultures***.
- A culture-derived directly from a tissue
- A primary cell culture may be obtained either by, allowing cells to migrate out from the tissue which is adhering to substrate, or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells.
- When sub culturing is done from the primary culure it is called ***secondary culture.***

Cell Lines

- A ***cell line*** is a permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space.
- Die after several subcultures called finite cell lines
- Continue to grow indefinitely are called continuous cell lines.
- Finite cell lines grow through a limited number of cell generations and have a limited life,
- The characteristics of finite cell lines are anchorage-dependent, contact inhibition and density limitation.
- ***Continuous cell lines*** are obtained either from transformed cell lines in vitro o cancerous cells.
- They divide rapidly.
- Their generation time is 12 to 14 hours.
- They have no contact inhibition and no anchorage dependence and have no or reduced density limitation.
- They show enhanced growth and proliferation due to the rapid growth rate and different cell shapes and organization of microfilaments.

Pluripotent Stem Cells

- Pluripotent stem cells are cells that have the capacity to self renew by dividing and to develop into the three primary germ cell layers of the early embryo
- Therefore into all cells of the adult body, but not extra-embryonic tissues such as the placenta.
- Two properties: self-renewal and potency.
- Self renewal is the capacity of the stem cells to divide indefinitely, producing unaltered cell daughters maintaining the same properties as the progenitor cell.
- There are two types of PSCs, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).
- ESCs are derived from the inner cell mass (ICM) of preimplantation embryos and can be indefinitely maintained and expanded in the pluripotent state in vitro.
- Pluripotent stem cells can also be obtained by inducing dedifferentiation of adult somatic cells through a recently developed in vitro technology - reprogramming
- iPSC are derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state that enables the development of an unlimited source of any type of human cell needed for therapeutic purposes.

Cryopreservation of cultures

- Cryopreservation is a method whereby cells are frozen, maintaining their viability until they are defrosted months or years later.
- Cells are cryopreserved to minimize genetic change and avoid loss through contamination.
- The freezing process involves slowly reducing the temperature of prepared celIs to -30 to -60°C followed by a transfer to temperatures less than -130°C.
- Two common cryoprotective agents are dimethyl sulfoxide (DMSO) and glycerol.
- Glycerol is used primarily for cryoprotection of red blood cells
- DMSO is used for the protection of most other cells and tissues.
- A Sugar called trehalose, which occurs in organisms capable or surviving extreme dehydration, is used for freeze drying methods of cryopreservation
- Trehalose stabilizes cell membranes, and it is particularly useful for the preservation of sperm, stem cells, and blood cells.
- An Important application of cryopreservation is in the freezing and storage of hematopoietic stem cells, which are found in the bone marrow and peripheral blood.
- Hematopoietic stem cells and mesenchymal stem cells are capable of differentiating into skeletal and cardiac muscle tissues, nerve tissue, and bone.
- Cryopreservation is also used to freeze and store human embryos and sperm.
- Cells can live for more than a decade if properly frozen.
- Parathyroid glands, veins, cardiac valves, and aortic tissue, can be successfully cryopreserved.

- Freezing is also used to store and maintain the long-term viability of early human embryos, Ova (eggs), and sperm.

Somatic cell fusion and HAT selection of hybrid clones

- Somatic cell fusion or hybrid cells can be produced by fusing different types of somatic cells from two different types of tissues or species in a cell culture media.
- In plants, it is commonly called protoplast fusion.
- Cell fusion is enhanced 100 to 1000 times by the addition of inactivated Sendai (parainfluenza) virus or polyethylene glycol (PEG).
- These agents adhere to the plasma membranes of cells and alter their properties that facilitate their fusion.
- Fusion of two cells produces a heterokaryon, i.e., a single hybrid cell with two nuclei, one from each of the cells entering fusion.
- Subsequently, the two nuclei also fuse to yield a hybrid cell with a single nucleus.
- The *steps* in a somatic cell hybridization

1. Appropriate human and mouse cells are selected and mixed in the presence of inactivated Sendai virus or PEG to promote cell fusion.
2. After some time, the cells (a mixture of man, mouse, and hybrid cells) are plated on a selective medium, e.g., HAT medium
3. This medium is supplemented with Hypoxanthine, Aminopterin, and Thymidine, hence the name HAT medium - which allows the multiplication of hybrid cells.

Production of monoclonal antibodies

- A hybridoma is a hybrid cell obtained by fusing a B-lymphocyte with usually a tumor cell.
- The hybrid cells thus produced possess the ability to produce antibodies due to the B-lymphocyte genome and the capacity for indefinite growth in vitro due to the tumor (myeloma) cell involved in fusion.
- Specific hybridomas are either cultured in vitro or passaged through the mouse peritoneal cavity to obtain monoclonal antibodies - called hybridoma technology
- A hybridoma cell culture produces large quantities of the plasma cells antibodies, called monoclonal antibodies.
- Monoclonal antibodies are homogenous antibodies produced by a single clone of genetically identical B cells
- A monoclonal antibody (mAb or moAb) preparation is specific to a single antigenic determinant (epitope) of a single antigen.
- Two approaches have been followed for the ***production of monoclonal antibodies***.
  a) In vitro production through cell culture technique
  b) In vivo production using ascites tumors in mice or rat
- The first method is economically viable and also it can be scaled up for example one kg of antibody (which can be produced through cultured cells in a single fermenter) would

require 20,000 mice if made by ascites tumors, and the risk of contamination is greatly reduced.

- Steps involved in the production of monoclonal antibodies

1. Immunize a rabbit through repeated injection of a specific antigen
2. Produce tumours in a mouse or rabbit
3. Antibodies & myeloma cells are cultured separately
4. Induce fusion of B lymphocytes to myeloma cells using PEG to produce hybridoma
5. The hybrid cells are grown in selective HAT medium
6. Select the desired hybridoma for cloning & antibody production
7. Culture selected hybridoma cells for the production of mAbs in large quantity.

MODULE 2

TRANSGENIC ORGANISMS

Transfection Methods

- Transfection – process of deliberately introducing naked or purified nucleic acids into eukaryotic cells
- Transformation – non viral DNA transfer in bacteria & non animal eukaryotic cells
- Transduction – virus mediated gene transfer in eukaryotic cells
- Transfection results in unexpected morphologies & abnormalities in target cells
- Transfection methods includes Calcium phosphate precipitation, Dextran mediated, Lepofection, Electroporation, Retroviral infection, Micro injection, Short gun method,…

Chemical treatment

Calcium phosphate precipitation

- DNA preparation for transfection is first dissolved in phosphate buffer, $CaCl_2$ & added to the DNA solution.
- Insoluble calcium phosphate which coprecipitates with the DNA is formed.
- Calcium phosphate DNA precipitate is added to the cell to be transfected
- Cells take the precipitate by phagocytosis
- 1 – 2% of cells are transfected by this method.

Dextran mediated

- DEAE – Dextran mediated
- Diethyl aminoethyl dextran – water soluble & polycationic
- It is added to the transfection solution containing DNA.
- DEAE – Dextran mediates DNA uptake by cells through endocytosis.
- Suited for transient transfection
- Transient transfection – introduced gene is gradually lost from the daughter cells transfected.

Electroporation

- Cells are mixed with DNA & exposed to pulses of high electrical voltage for a milliseconds.
- Induces pores in the cell membrane, DNA enter the cells.
- Treatment of cells with colcemide before electroporation increases the frequency of transfection.
- Linearized DNA is far efficient than circular supercoiled DNA.

Lipofection

- Delivery of DNA into cells using liposomes.
- ***Liposomes*** – small vesicles prepared from a suitable lipid & the required DNA is contained within the liposome
- Technique is simple, highly reproducible & efficient
- They fuse with plasma membrane & thereby deliver the DNA into the cells which bring about transfection.
- Used to deliver DNA into live animals by direct infection or intravenous injection.

Microinjection

- Most reliable & commonly used technique.
- DNA solution is injected directly into the nucleus of a cell or into the male pronucleus of a fertilized egg.
- Microinjection assembly consist of a low power stereoscopic dissecting microscope & two micromanipulators.
- One for a glass micropipette to hold the ovum by partial suction
- Other for a glass injection needle to introduce the DNA into the male pronucleus

Retroviral vector method

- ***Retroviruses*** – vectors for integrating the transgene into the genome of a recipient cell
- Not in regular use today
- Limitations – transfer only small pieces of DNA
  Risk of retroviral contamination

Embryonic stem cell method

- Cells from the blastocyst stage of a developing mouse embryo can proliferate in cell culture and still retain the capability of differentiating into all other cell types.
- Functional transgene can be integrated at a specific site within a gene in the genome of ES cells.
- The genetically engineered cells can be selected,grown, and used to generate transgenic animals.
- The cloned ES cells are injected into the blastocoel of blastocyst stage embryos.
- The blastocysts are obtained from donor females in a similar manner as that for microinjection.
- Alternatively, the zona pellucida of eight cells to morula stage embryo is removed and the morulae are co-cultured with the ES cells.
- The ES cells are preferentially incorporated into the inner cell mass of the developing embryo.
- It is also possible to transplant the ES cell nucleus into an enucleated fertilized ovum.

- The embryos co-cultured or injected with transfected ES cells are transferred to the surrogate mother where they complete their developmet
- Progeny derived from such embryos contain tissues derived from the ES cells i.e., they are chimeric.

Shot Gun Method

- Microprojectile bombardment
- Device – gene gun
- Enable penetration of cell wall
- Used for genetic transformation of many organisms to introduce a diverse range of desirable traits.
- It shoots foreign DNA into plant cells or tissues at a very high speed.
- High velocity microprojectiles are used to incorporate the introduced transgenes.
- The DNA is coated on the surface of gold or tungsten
- The DNA coated gold particles are coated on the lower surface of a flying disc – microprojectiles
- This is accelerated with air pressure & shot into a tissue.

Transgenic Animals

- One whose genome has been altered in a heritable manner by introduction of a foreign DNA sequence into genome of its zygote or embryo

Fish

- A fish carrying one or more foreign genes
- Introduced genes include human or rat gene for growth hormone
- Anti –freeze protein gene of polar flounders was transferred to Atlantic salmon – promoting resistance to low temperature.
- Microinjection method was used to generate transgenic fish
- Eg:- zebra fish

Pig

- The efficiency of the production of transgenic pigs is still very low compared to that of the production of transgenic mice.
- The animal grew a little faster but did not become large.
- They show an increase of 10-15% in the daily weight and 16-18% in feed efficiency which is lower to those in mice.
- There was a marked reduction in the subcutaneous fat in some of these pigs suggesting the possibility of producing leaner meat with lower fat content.

- The enviro pig is an interesting example of transgenesis as a way of overcoming the detrimental environmental impact of high phosphate pig manure.
- In this case, a phytase transgene that is expressed in the salivary gland efficiently removes the phosphate residues from phytate, (the predominant storage form of phosphorus in plant-based animal feeds, unable to ruminants pigs and poultry are unable to digest and utilize phytate) the major source of phosphate in pig feed.
- As a consequence, the phosphorous in phytate becomes metabolically accessible, which enhances growth and so significantly reduces the amount of phosphorus that is excreted.

Sheep

- Produced to achieve better growth & meet production as well as to serve as bioreactors
- Transgenic animals showed improvement in body weight gain, feed efficiency, lean meat/fat ratio, fat composition,..
- Increased wool production & improved wool quality are important objectives of transgenic sheep

Rabbit

- Aims at the production of recoverable quantities of pharmaceutically or biologically important proteins.
- IL 2, growth hormone, tissue plasminogen activators, alpha 1 antitrypsin,… are separately transferred to rabbits.
- Proteins were harvested from the milk.

Mice

- Most preferred mammal for studies on gene transfer.
- Due to short oestrous cycle & gestation period, short generation time, production of several offsprings per pregnancy, convenient in vitro fertilization, successful culture of embryo, availability of diverse array of genetic stocks,..

Goat

- John McPherson & Karl Ebert produced transgenic goats
- They expressed a heterologous protein in their milk – LatPA
- This protein is used for dissolving blood clots.
- Some human genes have been introduced in goats & their expression is achieved in mammary glands

Insects

- It is an insect that has been genetically modified, either through mutagenesis, or more precise processes of transgenesis, or cisgenesis.
- Motivations for using GM insects include biological research purposes and genetic pest management.

- The sterile insect technique (SIT) is a control strategy where male insects are sterilized, usually by irradiation, then released to mate with wild females.
- lf enough males are released, the females will mate with mostly sterile males and lay non-viable eggs.
- This causes the population of insects to Crash (the abundance of insects is extremely diminished).

Knock Out Mice

- **Knock out mice** – genetically engineered mouse in which researchers have inactivated or knocked out an existing gene by replacing it with an artificial piece of DNA.
- Causes change in mouse's phenotype.
- Used for studying the roles of genes which have been sequenced.
- Knock out mouse are produced by a technique called gene targeting
- It is carried out in mouse embryonic stem cells

Human Cloning

- Human cloning is the creation of a genetically identical copy of a human.
- The term is generally used to refer to artificial human cloning, which is the reproduction of human cells and tissue.
- Two commonly discussed types of theoretical human cloning are ***therapeutic cloning and reproductive cloning***.
- ***Therapeutic cloning*** would involve cloning cells from a human for use in medicine and transplants, it is an active area of research but is not in medical practice anywhere in the world.
- Two common methods of therapeutic cloning that are being researched are *somatic-cell nuclear transfer and (more recently) pluripotent stem cell induction.*
- In this, the growth of the embryo is stopped at the blastocyst stage.
- ***Reproductive cloning*** would involve making an entire cloned human instead of just specific cells or tissues.
- Cloned embryo is transferred into the womb of a surrogate mother

Ethical Issues of transgenic Animals

- Possibility of generating designer babies
- Humans exploit other organisms for their benefit
- Potential harm to human health
- Potential damage to the environment
- Excessive corporate dominance
- Unnaturalness

- Overexploitation by genetic engineering may harm animals
- There was concern about the release either accidental or deliberate, of genetically modified organisms into the environment.
- "Playing god", "manipulation of life", "the most threatening scientific research ever undertaken", and "man made evolution"
- Medical experiments may involve a certain amount of animal suffering.
- An example is provided by the oncomouse which was the first animal to be patented in US.
- The oncomouse is a transgenic mouse to which an oncogene has been added. Some people argue that patenting animals is itself unethical because it reduces them to the level of objects.
- Others argue that experiments such as those with oncomice cause suffering and should therefore be banned.

MODULE 3

APPLICATIONS OF BIOTECHNOLOGY

Molecular diagnosis of genetic diseases

Cystic Fibrosis

- Cystic fibrosis (CF) is a genetic disorder that affects mostly the lungs, but also the pancreas, liver, kidneys and intestine.
- Long-term issues include difficulty breathing and coughing up mucus as a result of frequent lung infections.
- Other signs and symptoms may include sinus infections, poor growth, fatty stool, clubbing of the fingers and tees, and infertility in most males.
- CF is inherited in an autosomal recessive manner.
- It is caused by the presence of mutations in both copies of the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein.
- Those with a single working copy are carriers and otherwise mostly healthy.
- CFTR is involved in the production of sweat, digestive fluids, and mucus.
- When the CFTR is not functional, secretions that are usually thin instead become thick.
- There is no known cure for cystic fibrosis.
- Women who are pregnant or couples planning a pregnancy can have themselves tested for the CFTR gene mutations to determine the risk that their child will be born with CF.
- The most prevalent pathogenic variant discovered having a deletion of a phenylalanine at position 508 of the protein

Huntinton's disease

- A hereditary neurological disorder characterized by movement, cognitive, psychiatric symptoms.
- For Huntington's disease, a genetic test is performed on blood sample.
- Check for the expanded CAG repeat characteristic of HD
- HD is caused by the expansion of a CAG trinucleotide repeat in the first exon of the Huntington (HTT) gene.
- There are three main types of HD genetic testing
  1. Diagnostic testing to confirm or rule out disease
  2 Presymptomatic testing to determine whether an at risk individual inherited the expanded allele
  3. Prenatal testing to determine whether the fetus has inherited the expanded allele.
- For testing polymerase chain reactions (PCR) and Southern blot hybridization is carried out to accurately measure the CAG trinucleotide repeat size and interpret the test results.
- Individuals who do not have HD usually have 28 or fewer repeats.
- Individuals with HD usually have 40 or more repeats.

Sickle Cell Anaemia

- Sickle cell anemia (SCA) is a chronic blood disorder initiated by inheriting a faulty (sickle) hemoglobin with chronic hemolytic anaemia and recurrent vaso occlusion as its key pathological features.
- The molecular explanation of sickle hemoglobin is a single nucleotide polymorphism (SNP) or point mutation (GAG $\longrightarrow$GTG) in the β – hemoglobin gene (HBB).
- The homozygosity for the mutated S hemoglobin (HbS) leads to a substitution of a hydrophobic valine amino acid for the normal hydrophilic glutamic acid residue at the sixth amino acid of the β- globin chain and the resulting HbS β- globin chains are substituted for normal HbA β- globin chains.
- (HbAA) indicates the normal genotype
- (HbAS) denotes carrier
- Most of the DNA based diagnostic techniques of hemoglobin disorders are based on the polymerase chain
- Restriction fragment length polymorphisms (RFLPs) arise because mutations can create or destroy the sites recognized by specific restriction enzymes, leading to variations between individuals in the length of restriction fragments produced from identical regions of the genome.
- The target DNA containing the specific sequence mutaion responsible for a globin disorder is amplified by PCR and the product is then digested by the restriction enzyme and the resulting DNA fragment separated on gels.
- The presence or absence of the recognition site is determined from the pattern of the RFLP analysis.
- The single nucleotide change in the beta globin gene that causes sickle cell anaemia by chance abolishes a *Cvn*I restriction endonuclease site.
- This enzyme recognizes the sequence CCTNAGG and cleaves the DNA between the C and T.
- In the normal gene, the DNA sequence is CCTGAGG, whereas in the sickle cell anaemia gene the sequence is CCTGTGG.
- After two primer sequences that flank the *Cvn*I site are added, a small amount of sample DNA can be amplified by PCR. T
- The amplified DNA is digested with *Cvn*l, and the cleavage products are separated by gel electrophoresis and visualized by ethidium bromide staining of the DNA in the gel.
- If the *Cvn*l site is present, then a specific set of DNA fragments is observed.

Vaccines & therapeutic agents

- A ***therapeutic agent*** means a drug, protein, peptide, gene, compound, or other pharmaceutically active ingredients.
- They are used for the treatment and care of a patient for both preventing and combating disease or alleviating pain or injury.
- A ***Vaccine*** is a biological preparation that provides active acquired immunity to a particular infectious disease.
- A vaccine typically contains an agent that resembles a disease causing micro organism and is often made from weakened or killed forms of the microbe, its toxins, or one or its surface proteins.
- The administration of vaccines is called vaccination.
- Vaccination is the most effective method of preventing infectious diseases
- Widespread immunity due to vaccination is largely responsible for the worldwide eradication of smallpox and the restriction of diseases such as polio, measles, and tetanus from much of the world
- Recombinant DNA technology can be used in various ways to create reliable vaccines.
- Immunologically active, non infectious agents can be produced by deleting the genes that cause virulence; with this deletion, a live vaccine would never be able to revert to the infectious form.
- Vaccines that use components of a pathogenic organism rather than the whole organism are called subunit vaccines or acellular vaccines.
- Recombinant DNA technology is highly suited for developing subunit vaccines.
  Eg:- Covishield vaccine.
- ***Toxoids*** - The toxins produced by Diptheria and tetanus bacilli are detoxicated and used in the preparation of vaccines.
- ***Recombinant protein vaccines*** - Recombinant DNA technology has made possible the expression of protective protein antigens in E.coli, yeast, mammalian cells, or baculovirus.
- This technology avoids the problems of growing and manipulating large amounts of a pathogen from which the antigen is purified
- Their immunogenicity is lower than that of cultured microorganisms derived proteins.
- So the addition of an adjuvant to achieve enhanced efficiency is essential.
- Eg:- hepatitis B vaccine is made by inserting a segment of the hepatitis B virus gene into a yeast cell.
- The modified yeast cell produces large amounts of hepatitis B surface antigen, which is purified and harvested and used to produce the vaccine.
- ***Protein vaccine*** - Proteins can be purified from in vitro cultures of a pathogenic microorganism.
- One of the most widely used subunit protein vaccines is the influenza vaccine composed of haemagglutinin (HA) and neuraminidase (NA) purified from the inactivated influenza virus.

- ***Polysaccharide based vaccines*** - Stimulation of an antibody response against the surface polysaccharide of pathogenic bacteria is a strategy for the development of vaccines against capsulated bacteria.
- Polysaccharide based vaccines have been developed for S. pneumoniae and Salmonella Typhimurium.
- ***Live vaccines*** (e. g., Measles, BCG, Oral polio vaccine -OPV) are prepared from live or wild organisms.
- These organisms lost their capacity to induce disease but retain their immunogenicity.
- ***Inactivated or killed vaccines*** are produced by growing bacteria or virus in culture media and then inactivating them with heat or chemicals usually formalin, when injected into the body they stimulate active immunity they are safe but generally less effective than live vaccines.
- Eg:- Covaxin against COVID 19
- ***Mixed or combined vaccine*** - When more than one kind of immunizing agent is included in the vaccine it is called a mixed or combined vaccine.
- e.g., MMR (Measles, Mumps, Rubella) DPT (Diphtheria pertussis tetanus)
- ***Polyvalent vaccines*** - vaccines which are prepared from two or more strains of the same species.
- e.g., polio, Influenza vaccines.
- ***Auto or autogenous vaccine*** The term auto or autogenous vaccine is applied when the organism in the vaccine is obtained from the patient.

Other therapeutic agents

- Animal cell products are mainly high molecular weight proteins they include several enzymes, hormones, animal vaccines, monoclonal antibodies, interferons
- Some important enzymes obtained from animal cells are asparaginase, collagenase, hyaluronidase, pepsin, rennin, trypsin, tyrosine hydroxylase, and urokinase.
- Four important hormones obtained from cultured cells include luteinizing hormone, follicle-stimulating hormone chorionic hormone, and erythropoietin.
- Several other proteins have also been produced using cultured cells.
- The most important of these proteins is the ***Tissue plasminogen activator (t-PA).***
- Recombinant t-PA is safe and effective for dissolving blood clots in patients with heart diseases and thrombin disorders.
- The t-PA is also used in acute myocardial infarction (AMI).
- The tPA catalyzes the proteolytic processing of inactive proenzyme plasminogen in the blood into active protein plasmin.
- Plasmin cleaves insoluble fibrin of blood clots into soluble fibrin fragment peptides.
- Hence blood clots dissolved and the blood vessel is opened.
- ***Blood factor VIII*** - Using recombinant DNA technology, factor VIII has been produced from mammalian cell culture and used for treating hemophilia A patients.

- Several hormones and related molecules such as erythropoietin, and human growth hormone can also be synthesized using cell cultures.
- A number of antigens that are used in viral vaccines are also been synthesized using cell cultures.

Recombinant DNA in medicines

Recombinant Insulin

- lnsulin is a hormone produced by β - cells of islets of Langerhans of the pancreas.
- Insulin is a peptide hormone produced by the pancreas and is a central regulator of carbohydrates and fat metabolism in the body.
- Consist of 51 amino acids
- Molecular weight – 5808 Da.
- Insulin hormone is a dimer of an A- Chain and a B-chain which are linked together by a disulfide bond.
- Recombinant human insulin is produced predominantly using *E. coli* and *Saccharomyces* cerevisiae for therapeutic use in humans.
- A chain consist of 21 amino acids & B chain consist of 30 amino acids
- *Hakura et al* (1977) chemically synthesize the DNA sequence of insulin for two chains A and B and separately inserted into two pBR322 plasmid vectors.
- The recombinant plasmid was then separately transformed into E.coli host.
- The recombinant host produces pro-insulin chains i.e., fused β - galactosidase-A chain and β - galactosidase-B-chain separately.
- These pro-insulin chains A and B were separated from β - galactosidase by treatment with cyanogen bromide.
- After detachment, A and B chains are joined invitro to reconstitute the naive insulin by sulfonating the peptide chains with sodium disulphonate and sodium sulfite.
- *Gilbert & Villokoomaroff method* - m RNA for pre-pro-insulin (109 amino acids) is isolated from islets of Langerhans cell.
- mRNA is reverse transcribed to form DNA and then it is inserted into pBR 322 plasmid in the middle of the gene for penicillinase.
- Then the recombinant plasmid is transformed into a suitable host i.e., E. coli cell
- The host produces penicillinase + pre-pro insulin
- Insulin is later separated by trypsin treatment.

Human Growth Hormone

- The gene for human growth hormone (hGH) is isolated from human pituitary gland.
- Insertion of whole hGH gene into plasmid vector and cloning into *E.coli* results into production of biologically inactive hormone because bacteria can translate the region of gene that are not translated in human thereby producing a pre hormone containing an extra 26 amino acids which might be difficult to remove.
- Hence the segment of gene that codes for the first 24 amino acids of hormone is constructed chemically from blocks of nucleotide.

Step I: Chemical synthesis of gene for first 24 aminoacids

- From the known amino acids sequence of hGH, gene for first 24 amino acids are constructed chemically.
- These genes are constructed in three small fragments and then they are joined by T4 DNA ligase to get whole gene for first 24 amino acids.

Step II: Isolation of mRNA for hGH

- In this step mRNA for hGH is isolated from human pituitary gland tissue.

Step III: Reverse transcription

- Using reverse transcriptase enzyme complimentary DNA (cDNA) is synthesized from mRNA
- The cDNA obtained by reverse transcription process, is the gene for hGH.
- The full gene is cut with restriction endonuclease enzyme to remove first 24 gene

Step IV: Joining of synthetic gene and cDNA

- In this step synthetic gene (gene for first 24 amino acids) and cDNA are joined in order to obtain full gene with its own initiation codon (AUG).
- T4 DNA ligase join these genes

Step V: selection of suitable vector and recombination

- Expression vector phGH407 derived from plasmid vector pBR322 is used as carrier vector.
- HGH gene is ligated into a restriction site just downstream of Lac; promotor/operator region of the expression vector.

Step VI: selection and recombination into suitable host cell

- E. coli is used as suitable host cell.
- The recombinant expression vector is then transformed into E.coli.
- The recombinant E.coli then starts producing hGH.
- The recombinant E. coli are isolated from the culture and mass production by fermentation technology to obtain hGH.

Uses of recombinant human growth hormone (hGH)

- 1. Treatment of children suffering from growth deficiency
- 2. Treat the patient with Turner's syndrome and chronic renal insufficiency
- 3. To treat patient with renal carcinoma
- 4. Bovine somatotropin hormone is used to increases milk production in lactating cows

Human Gene Therapy

- Gene therapy may be classified into the two following types:
  1. Germline gene therapy
  2. Somatic gene therapy
- **Germ line gene therapy** - In the case of germline gene therapy, Germ cells, i.e., sperm or eggs are modified by the introduction of functional genes, which are integrated into their genomes.
- The change due to therapy would be heritable and would be passed on to later generations.
- Should be highly effective in counteracting genetic disorders and hereditary diseases.
- It has not been attempted in humans due to technical and ethical problems.
- **Somatic gene therapy** - the therapeutic genes are transferred into the somatic cells, of a patient.
- Any modifications and effects will be restricted to the individual patient only, and will not be inherited to later generations.
- A wide range of disorders such as cancer, neurological disorders, heart diseases, and infectious diseases can be treated by somatic cell gene therapy.
- Based on the mode of treatment it is of
- 1. ***Ex vivo*** (out of the body) approach, here cells are removed from the body, incubated with a vector, and then returned to the body.
- This procedure 1s usually done with bone marrow cells.
- 2***. Insitu approach*** - The vector is applied directly to affected tissues
- Used to treat tumors by injecting into the tumor a vector bearing the gene for a toxin or a gene that would make the tumor susceptible to a chemotherapeutic agent.
- 3***. Invivo approach*** - the vector would be injected directly into the bloodstream.
- More than 4000 genetic diseases are known, many of which are fatal.
- Most are currently incurable.
- Diseases that result from a deficiency in adenosine deaminase (ADA) , Over 30 mutations in the ADA gene are associated with ***Severe Combined Immune Deficiency Disease*** (SCID), an autosomal recessive disorder.
- The progenitor stem cells can not differentiate into mature T and B lymphocytes of the immune system, in children suffering from SCID
- They are highly susceptible to bacterial and viral infections.
- Children suffering from this disease, therefore, have to be protected in a sterile environment (a bubble-like encasement) so it is also called *bubble baby syndrome*.

- The first authorized gene therapy in humans began on September. 14, 1990, with the treatment of a 4-year-old girl with ADA deficiency.
- The patient's peripheral blood T cells were cultured with appropriate growth factors.
- The ADA gene was introduced within these cells by retroviral - mediated gene transfer.
- The modified T cells carrying a normal ADA gene were the reintroduced to the patient by autologous transfusion.

Enzymes in detergents

- Enzymes have been used to improve the cleaning efficiency of detergents
- Most valuable ingredients of granular and liquid detergents, stain removers, and industrial cleaning products.
- Enzymes function optimally in detergents at a temperature between 20 and 60°C and within a pH range of 7 - 10.5
- Each enzyme exhibits a specific pH and temperature
- The major classes of detergent enzymes proteases, lipases, amylases, and cellulases
- Provide specific benefits for application in laundry and automatic dishwashing.
- Provides environmental benefits by reducing energy consumption through shorter washing times, lower washing temperatures, and reduced water consumption.
- Today proteases are joined by lipases and amylases, in improving detergent efficacy espeçially for household laundering at lower temperatures and, in industrial cleaning operations, at lower pH levels.
- Cellulases contribute to overall fabric care by rejuvenating or maintaining the new appearance of washed garments.
- Enzymes are produced by fermentation technologies that utilize renewable resources.
- Amylases contribute to increasing fabric whiteness by reducing the redeposition of starch-containing stains on co-washed garments.
- Another important class of detergent enzymes is alpha-amylase enzymes that solubilize starch and is present in stains such as baby food, gravy, chocolate, and other starch-thickened food.
- The removal of fatty food stains (e.g. butter, salad Oil, frying fat, lipstick), can be enhanced by the use of lipases.
- Cellulase - type enzymes enhance stain removal, brighten the colour and soften cotton containing garments.

Enzymes in leather industry

- The use of biotechnology by tanneries has increased in recent years.
- Enzymes can be applied during different steps of the leather production process: Soaking, dehairing, bating, dyeing, degreasing, or in effluent and solid waste treatment.
- The leather industry uses ***proteolytic and lipolytic*** enzymes in leather processing.

- Enzymes are used to remove unwanted parts.
- The concept of cleaner production has been used in tanneries to mitigate their impact and reduce the loss of chemicals, water, and raw materials.
- ***Proteases*** **-** hydrolyze the protein fraction of dermatan sulfate, making the collagen more accessible to water and reducing the attachment of the basal layer.
- They act in the removal of globular proteins
- ***Lipases*** - hydrolyze fats, oils, and greases present in the hypoderm
- ***Keratinases*** - hydrolyze the keratin of hair and epidermis and break down the disulfide bonds of this molecule.
- Enzymatic unhairing technologies are interesting because they can preserve the hair and contribute to a reduction in the organic load released into the effluent.
- These processes eliminate or reduce the dependence on harmful chemicals such as sulfide, lime, and amines.

Heterologous protein production

- The heterologous expression refers to the expression of a gene or part of a gene in a host organism that does not naturally have this gene or gene fragment.
- Insertion of the gene in the heterologous host is performed by recombinant DNA technology.
- After being inserted in the host, the gene may be integrated into the host DNA, causing permanent expression, or not integrated, causing transient expression.
- The heterologous expression can be done in many type of host organisms.
- The host organism can be a bacterium, yeast, mammalian cell, or plant cell.
- This host is called the ***expression system***.
- Homologous expression, on the other hand, refers to the overexpression of a gene in a system from where it originates.
- Protein production systems are used in the life sciences, and medicine.
- Molecular biology research uses numerous proteins and enzymes, many of which are from expression systems; particularly DNA polymerase for PCR, reverse transcriptase for RNA analysis, restriction endonucleases for cloning, and to make proteins that are screened in drug discovery as biological targets or as potential drugs themselves.
- Significant applications for expression systems in industrial fermentation, notably the production of biopharmaceuticals such as human insulin to treat diabetes and to manufacture enzymes.
- E. coli is one of the most widely used expression hosts, and DNA is normally introduced in a plasmid expression vector.
- Non pathogenic species of the gram-positive *Corynebacterium* are used for the commercial production of various amino acids.

- The *C. glutamicum* species is widely used for producing glutamate and lysine, components of human food, animal feed, and pharmaceutical products.
- Expression of functionally active human epidermal growth factor has been done in *C. glutamicum*, for industrial scale production of human proteins
- Yeasts expression systems using either *S. cerevisiae* or *Pichia pastoris* allow stable and lasting production of proteins that are processed similarly to mammalian cells, at high yield, in chemically defined media of proteins.

Biofiltration

- Biofiltration is the removal and oxidation of organic gases (volatile organic compound, or VOCs) from contaminated air by beds of compost or soil.
- It is a low cost and highly effective air pollution control technology.
- Billions of indigenous microorganisms inherent within the biofilter media convert the organic compounds to CO2 and water.
- These naturally occurring microorganisms consume the offending compounds in a safe, moist, oxygen-rich environment.

Biofilter

- It is an engineered bed of soil or compost under which lies a distribution system of perforated pipe and a layer of coarse distribution media.
- The biofilter media retains no residue from the original organic compound found in the contaminated air stream.
- This is due to extremely efficient microbial ecosystem that exists within the biofilter media.
- Some of the materials used as biofilter media are compost, soil, activated carbon, wood chips or bark, lava rock and inert plastic material.

Mechanism of biofiltration

- Contaminants + oxygen+ microorganism → microbial cells + CO2 + H2O
- Removal of contaminated material is a multistep process.
- At first contaminants are converted into liquid phase and transported to bacterial cell in the biofilm and transferred across the cell membrane, where the compound is degraded and used in cell metabolism.

Limitations

1. The rate of influents air flow is constrained by the size of the biofilter.

2. Fugitive fungi may be a problem.

3. Low temperature may slow or stop removal unless the biofilter is climate controlled.

4. Compounds that are resistant to biodegradation will not be converted to harmless products.

Bioremediation

- Bioremediation can be defined as any process that uses microorganisms or their enzyme to return the environment altered by contaminants to its original condition.
- Bioremediation application fall into two broad categories : **in situ or ex situ.**

Ex situ bioremediation

- Ex situ technique can be faster, easier to control.
- Used to treat a wide range of contaminants and soil types than in in situ techniques.
- Ex situ techniques include **slurry-phase** bioremediation and **solid-phase** bioremediation

Slurry Phase Bioremediation

- Slurry-phase bioremediation - contaminated soil is combined with water and other additives in a large tank called a "**bioreactor**" and mixed to keep the microorganisms which are already present in the in contact with the contaminants in the soil.
- Nutrients and oxygen are added, and conditions in the bioreactor are controlled to create the optimum environment for the microorganisms to degrade the contaminants.
- Upon completion of the treatment, the water is removed from the solids, which are disposed of or treated further if they still contain pollutants.
- Slurry-phase biological treatment can be a relatively rapid process compared to other biological treatment processes, particularly for contaminated clays.
- The success of the process is highly dependent on the specific soil and chemical properties of the contaminated material.
- This technology is particularly useful where rapid remediation is a high priority.

Solid Phase Bioremediation

- Solid-phase bioremediation - Solid-phase bioremediation is a process that treats soils in above-ground treatment areas equipped with collection systems to prevent any contaminant from escaping the treatment.
- Moisture, heat, nutrients, or oxygen are controlled to enhance biodegradation for the application of this treatment.
- Solid-phase soil treatment processes include landfarming, soil biopiles, and composting,

Landfarming

- Relatively simple treatment method, contaminated soils are excavated and spread on a pad with a built-in system to collect any "leachate" or contaminated liquids that seep out of contaminant soaked soil.

- The soils are periodically turned over to mix air into the waste.
- Moisture and nutrients are controlled to enhance bioremediation.

Solid biopiles

- Contaminated soil is piled in heaps several meters high over an air distribution system.
- Aeration is provided by pulling air through the heap with a vacuum pump.
- Moisture and nutrient levels are maintained at levels that maximize bioremediation.
- The soil heaps can be placed in enclosures

Composting

- Compost is used in gardening and agriculture, mixed in with the soil.
- It improves soil structure, increases the amount of organic matter, and provides nutrients.
- Compost is a common name for **humus**, which is the result of the decomposition of organic matter.
- Biodegradation is useful for many types of organic wastes and is a cost-effective, natural process.

In situ bioremediation

In situ Bioremediation of soil

- In situ techniques do not require excavation of the contaminated soils so may be less expensive create less dust and cause less release of contaminants than ex situ techniques.
- It is possible to treat a large volume of soil at once.
- It may be slower than ex situ techniques may be difficult to manage and are most effective at sites with permeable soil.
- The goal of aerobic in situ Bioremediation is to supply oxygen and nutrients to the microorganisms in the soil.
- Aerobic in situ techniques can vary in the way they supply oxygen to the organisms that degrade the contaminants.
- Two such methods are bioventing and injection of hydrogen peroxide.
- Oxygen can be provided by pumping air in to the soil above the water table (bioventing) or by delivering the oxygen in liquid form as hydrogen peroxide.
- It may not work well in clay's or in highly layered subsurface environments because oxygen can not be evenly distributed throughout the treatment area.

- It requires years to reach cleanup goals depending mainly on how biodegradable specific contaminants are.
- Less time may be required with easily degraded contaminants.

Bioventing

- Bioventing systems deliver air from the atmosphere in to the soil above the water table through injection wells placed in the ground where the contamination exists.
- The number location and depth of the wells depend on many geological factors and engineering considerations
- An air blower may be used to push or pull air in to the soil through the injection wells.
- Air flows through the soil and the oxygen in it is used by the microorganisms.
- Nutrients may be pumped in to the soil through the injection wells.
- Nitrogen and phosphorous may be added to increase the growth rate of the microorganisms.

Injection of Hydrogen peroxide

- This process delivers oxygen to stimulate the activity of naturally occurring microorganisms by circulating hydrogen peroxide through contaminated soils to speed the Bioremediation of organic contaminants.
- It involves putting a chemical in to the ground
- This process is used only at sites where the ground water is already contaminated.
- A system of pipes or sprinkler system is typically used to deliver hydrogen peroxide to shallow contaminated soils.
- Injection wells are used for deeper contaminated soils.

In situ Bioremediation of Ground water

- In situ bioremediation of ground water speeds the natural biodegradation processes that take place in the water soaked underground region that lies below the water table.
- For sites at which both the soil and ground water are contaminated this single technology is effective at treating both.
- This system consist of extraction well to remove ground water from the ground an above ground water treatment system.
- Nutrients and an oxygen source may be added to the contaminated groundwater and injection wells to return the conditioned ground water to the subsurface the microorganisms degrade the contaminants.

- Another frequently used method of in situ groundwater treatment is air spraying, means pumping air in to the groundwater to help flush out .

Bioleaching

- It is a method used for extraction of precious and base metals from hard to treat ore with the aid of bacterial microorganisms.
- It is used to recover copper, zinc, lead, arsenic, antimony, nickel, molybdenum, gold, silver, and cobalt.
- It is more efficient and ecological friendly process.
- It is a hydro metallurgical form of treatment.
- It does not produce no offensive gases
- It involves the bacteria feed the nutrients of the ore and separating the metal.
- The metal can then be collected from the bottom of the solution.
- It is possible because of the unique microorganism's ability to react and breakdown the mineral deposits in the ore.

Mechanism involved in bioleaching

- It refers to the use of bacteria – primarily *Thiobacillus ferrooxidance, Leptospirillum ferrooxidans and* Thermophilic species of *sulfobacillus, acidianus and sulfolobus* to leach metals of value such as copper, zinc, uranium, nickel, and cobalt from sulphide mineral ore.
- These bacteria tolerate acids and metabolize sulphur.
- The bacteria act as a catalyst to accelerate the natural processes inside the ore.
- The particular bacteria use a chemical reaction known as oxidation reaction- to convert metal sulphide crystals in to sulphates and sheer metals.
- Oxidation reaction is the reaction where a substances loses its electron.
- The elements parts of ore are separated in to precious metals and the unused sulphur and other acidic chemicals.
- At the end of the process, adequate materials build up at the bottom of waste solution- then filtered to get to the pure metal.
- Only those ore that contain sulphur can only be bioleached- since the bacteria feed on sulphur.
- For certain metals like copper- bioleaching process is the primary choice because it is very low in terms of costs and relatively slow process.
- Eg:- pyrite leaching
- Fe3+ ions are used to oxidize the ore.
- This step is entirely independent of microbes.

- The role of the bacteria is the further oxidation of the ore, but more importantly also the regeneration of the chemical oxidant Fe3+ from Fe2+

Advantages of bioleaching

1. Bioleaching is much better and cleaner than the heap leaching using cyanide.
2. Significant advantages is found in the pre treatment of refractory gold in ores and concentrate with high arsenic content.
3. It does not contaminate the environment.
4. It is eco-friendly process.
5. Operating the bioleaching plant is very simple , as bacteria do most of the work.
6. It is a relatively low cost procedure, compared to other options such as roasting or smelting.

Molecular farming

- **Molecular pharming** – genetically modified plants or animals to produce pharmaceutical products or industrial chemicals.
- Pharmaceutical production in this way is cheaper & would result in significantly increased availability & lowered drug costs & prices.
- The mammary gland is used to produce pharmaceutically important proteins in milk
- Mammary gland is used because milk is renewable, produced in substantial quantities & can be collected frequently without harm to the animal.
- Purification & separation of protein from milk is relatively easy.

Bioreactors

- **Bioreactors** – vessel which carried out chemical process which involves organisms.
- Commonly cylindrical made of stainless steels
- Size – ranges from litre to cube meters
- The vessels environmental conditions need to be closely monitored & controlled.

Molecular markers

RFLP

- Restriction Fragment Length Polymorphism
- RFLP is a difference in homologous DNA sequences detected by the presence of fragments of different lengths after digestion of the DNA sample in question with specific restriction endonuclease .

- When the genomic DNA of many individuals of one species separately cleaved by restriction enzyme passed through electrophoresis, blotted on nitrocellulose membrane and probed with a radio labeled DNA. Polymorphism arises in hybridization pattern of these DNA .
- This show difference in sequences between two individuals. After digestion with specific enzyme. Variation obtained in one DNA fragment with that enzyme is referred to as one RFLP.
- Eg:-Homologous human chromosomes differ in sequence ,on average every 1250 bp.

Applications

- Analysis of RFLP variation in genome was a vital tool in genome mapping and genetic disease analysis.
- RFLP analysis is the basis for early methods of genetic finger printing , useful in the identification of samples retrieved from crime scene ,in the determination of paternity and and in the characterization of genetic diversity.

RAPD

- **RAPD -** (Random Amplified Polymorphic DNA)
- PCR based method
- DNA profiling that involved amplification of sequence using random primers
- It generates a type of genetic fingerprint that can be used to identify individuals
- DNA amplification product is generated from a region flanked by a pair of 10 bp primary site
- Consequently, random sample of DNA markers is obtained, which is called RAPD
- Genome DNA of two individual produce different RAPD
- Random oligonucleotide primers are designed by using different combinations of nucleotide
- Theoretically many different gene loci can be analysed, because each random primer anneals to a different region of DNA

RAPD advantage over RFLP

- Data can be collected quickly
- Crude DNA preparation may be used for analysis of whole genome
- Only small amount of DNA is required to work with population
- It does not require blotting or hybridization

Applications

- Used as genetic markers to construct genetic maps
- Finger printing of individual organisms can be done
- Certain genetic markers may be tagged at specific region in the genome

Limitations of RAPD

- Nearly all RAPD markers are dominant, ie., It is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. Co-dominant RAPD markers, observed as different sized DNA segments amplified from the same locus ,are detected only *rarely*
- Mismatches between the primers and the template many result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, RAPD results can be difficult to interpret
- PCR is an enzymatic reaction, there for the quality and concentration of template DNA, concentration of PCR components, and the PCR cycling condition many greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocol to be reproducible.

VNTR

- VNTR – (Variable Number Tandem Repeats)
- The full genetic profiles of any two individuals (other than identical twins) reveal many differences.
- But since most human genes are the same from person to person.
- DNA typing relies on the stretches of DNA that tend to differ among different people.
- While the repeated sequences themselves are usually the same from person to person, the number of times they are repeated tends to vary.
- These stretches of repeats, known as Variable Number of Tandem Repeats or VNTRs, can be isolated from an individual's DNA.
- The number of repeats can be gauged by dividing the entire molecular weight of a given VNTR by the molecular weight of the repeated sequence.
- VNTRs are similar to Short Tandem Repeats, the difference being that in a VNTR, the repeated sequence is longer — about 10-100 base pairs long.
- A variable number tandem repeat (or VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length (number of repeats) among individuals. Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Their analysis is useful in genetics and biology research, forensics, and DNA fingerprinting.
- The repeats are in tandem – i.e. they are clustered together and oriented in the same direction.
- Individual repeats can be removed from (or added to) the VNTR via recombination or replication errors, leading to alleles with different numbers of repeats.

- Flanking regions are segments of non-repetitive sequence (shown here as thin lines), allowing the VNTR blocks to be extracted with restriction enzymes and analyzed by RFLP, or amplified by the polymerase chain reaction (PCR) technique and their size determined by gel electrophoresis.
- VNTR analysis is also being used to study genetic diversity and breeding patterns in populations of wild or domesticated animals.
- VNTRS are generally classified into two types
    1. Microsatellite DNA (STR)
    2. Minisatellite DNA

SNPs

- Single nucleotide polymorphisms (SNPs) are a type of polymorphism involving variations of a single base pair.
- Single nuçleotide polymorphism, variation in a genetic sequence that affects only one of the basic building blocks- adenine (A), guanine (G), thymine (T), or cytosine (C) in a segment of a DNA molecule and that occurs in more than l percent of a population.
- Single nucleotides may be changed (substitution), removed (deletions), or added (insertion) to a polynucleotide sequence.
- Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes.
- SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code.
- SNPs act as chromosomal tags to specific regions of DNA, and these regions can be scanned for variations that may be involved in human disease or disorder.
- SNPs can be used to identify the locations of genes on chromosomes.
- Scanning a genome to find where SNPs occur helps scientists to construct chromosome maps.

MODULE 4

INTRODUCTION AND METHODS IN MICROBIOLOGY

INTRODUCTION

Microbial Diversity

Archaebacteria

- Primitive bacteria
- Live in extreme environmental conditions
- Major part of earth's life & play roles in both carbon & nitrogen cycles
- Size ranges from 0.1 – 15 μm in diameter
- Occurs in various shapes
- Structurally similar to gram positive bacteria
- *Sulfolus* archae produces sulfuric acid as waste product – causes environmental damage
- *Methanogenic* archae play an important role in microbial decomposition of organic matter
- *Methanogens* contribute to green house gas emissions & global warming

Eubacteria

- Bacteria are a large domain of single celled prokaryote microorganisms.
- They are microscopic organisms not visible with the naked eye.
- Bacteria are everywhere,both inside and outside of our body.
- Bacteria can live in a variety of environment, from hot water to ice.
- Some are good for you while others can make you sick.
- The shapes varies from spheres to rods to spirals. Morphologically, most appear spherical, called cocci or rod-shaped, called bacilli.
- Many bacterial species exist simply as single cells, or in groups or clusters.
- Bacteria exhibit an extremely wide variety of metabolic types.
- The major type are: phototrophs, lithotrophs and organotrophs with energy sources, sunlight, inorganic compounds and organic compounds respectively.
- Carbon metabolism in bacteria is either heterotrophs or autotrophs.
- Bacteria are pathogenic and cause infection disease, such as, tuberculosis, cholera etc.
- Antibiotics are used to treat bacterial infections.
- In dairy industry, bacteria are used for the production of milk fermentation products like cheese, yogurt etc.
- Bacteria are the major agents in biotechnology for the production of antibiotics and other chemicals.
- In bacteria, the common method of asexual reproduction is binary fission.
- In bacteria, transfer of genetic material between cells can occur in main 3 ways; Transformation, transduction and conjugation.
- In biotechnology, understanding bacterial genetics and metabolism helps to genetically modified bacteria for the production of therapeutic proteins, such as insulin, antibodies.
- Bacteria can also be used in the biological control of pest

Prochlorophyta

- Prochlorophyta is a group of symbiotic prokaryotic algae like microbes associated with ascidians of tropical Pacific shores.
- They are bright green, generally spherical and about 10 - 20 m in diameter, and have no clearly delimited nucleus or plastids.
- They resemble blue green algae they were considered as cyanophytes.
- The cell wall is similar to that of the cyanophyta and contains a peptidoglycan layer.
- But, the DNA has a diffuse distribution and is not concentrated in the center of the cell as it is in the cyanophyta.
- Like the eukaryotic algae chlorophyta and euglenophyta, they contain two chlorophyll components, chlorophylls a and b, whereas no cyanophytes are known to contain chlorophyll b.

Algae

- Simple unicellular or multicellular.
- Eukaryotic and typically chlorophyll bearing non vascular plants.
- Algae are capable of photosynthesis and are the principal producers in aquatic environments.
- They lack various structures of land plants, such as leaves, roots etc.
- Chlorophylls a,b,c,d and e are present in different algal groups.
- They exhibit a wide diversity in morphology ranging from tiny unicellular forms with varying shapes to large complex thalloid structures.
- Many unicellular forms have motility by flagellar movement.
- Some forms symbiotic association with other organisms.
- Eg:- lichens, coral reefs, sponges,…
- Unicellular forms reproduce asexually by fission.
- Multicellular forms by fragmentation & sporulation.
- Used as fertilizers, soil conditioners & livestock feed.
- Used for sewage treatment.

Fungi

- Includes eukaryotic organisms like yeasts, moulds, mushrooms etc.
- The study on fungi is known as mycology
- Decomposers
- Saprophytes on diverse microhabitats in soil, water, spoiled food, dead matter etc;,
- Symbionts on plants, animals, or other fungi.
- The cells of most fungi grow as tubular, thread-like filamentous structures called hyphae.
- Hyphae may contain multiple nuclei and grow at their tips.

- The branching leads to the development of interconnected network of hyphae called mycelium.
- Hyphae are of two types; septate or coenocytic.
- Fungi are the only organisms that combine glucans and chitin molecules in their cell wall.
- Fungi reproduce both by asexual and sexual methods.
- Asexual reproduction is via vegetative spores (conidia) or through mycelial fragmentation.

Protozoa

- Unicellular eukaryotes
- Cell wall is absent.
- Animal like protists.
- Reproduce by sexual & asexual methods
- Sexual by conjugation & asexual by fission
- They range in size from 10 to 52 micrometers.
- Some members have protective covering of calcium carbonate called testa.
- The locomotor structures include flagella, cilia, pseudopodia etc;.
- Under adverse conditions many of them transform into cysts.
- They may live as free living, parasitic, saprophytic or as symbiotic forms.
- Examples include Amoeba, Paramecium, Trypanosoma, etc;.
- Some are pathogenic parasites.
- Protozoa cause several human diseases like malaria, leishmaniasis, sleeping sickness, dysentery etc;

Viruses

- Viruses are infectious intercellular obligate parasites.
- Sub-cellular level of organization.
- Without protoplasm,cell organelles ,metabolism and protein synthesis.
- They depend on the host cell for the raw materials for all functions.
- It consists of nucleic acid surrounded by a protective coat called ***capsid***.
- The capsid proteins are encoded in the viral genome and its shape determines the viral morphology.
- Based on the nature of packing of the capsomeres the following major types of symmetry can be recognised in viruses.
    1. Spiral symmetry
    2. Cubical symmetry
    3. Binal symmetry
    4. Complex symmetry

- Viral genome:- A virus has either DNA or RNA as its genetic material and irrespective of its type the nucleic acid is either single or double stranded, linear or circular.
- Viral multiplication:- viral multiplication cycle consists of six sequential and sometimes overlapping steps ,namely, penetration, uncoating, biosynthesis, maturation, release & attachment.
- Types of viruses:- Based on their natural hosts, viruses are classified into:
    1. Phytoviruses
    2. Zooviruses
    3. Mycoviruses
    4. Phycoviruses
    5. Bacterial viruses
    6. Mycoplasma viruses etc.
- Examples:- bacteriophages, tobacco mosaic viruses, retro viruses, human immunodeficiency viruses

Viroids

- Viroids are mainly sub viral pathogens consisting of a short stretch of circular, single stranded RNA without protein coat.
- The first viroid identified was the potato spindle tuber viroid (PSTVd).
- Viroid RNA doesn't code for any protein.
- The replication mechanism involves RNA polymerase II, which catalyses the synthesis of new RNA using the viroid's RNA as template.
- The only human disease known to be caused by a viroid is hepatitis D.
- The hepatitis D viroid can enter into human liver cell if it is enclosed in a hepatitis B virus capsid.
- For hepatitis D occur there must be simultaneous infection of cell with both the hepatitis B virus and hepatitis D viroid.
- The delta agent enter in to blood streams and that can be transmitted via or serum transfusion.
- There is the extensive sequence complementarity between the hepatitis D viroid RNA and human liver cell 7s RNA.
- Therefore it is suggested that hepatitis D viroids causes liver cell death via sequestering or cleaving the 7s RNA.

Prions

- Prions are subviral infectious agents formed of misfolded proteins.
- They are devoid of nucleic acid and are considered as the simplest living entities.
- Stanley B. Pursiner (1982) who first reported prions was awarded the Nobel Prize in physiology and medicine in 1997.
- Prions cause neurodegenerative diseases mediated by an entirely novel mechanism.

- Prion diseases involve modification of the prion protein, ,a constituent of normal. mammalian cells.
- P r p occur both in infectious and non infectious forms.
- The infectious prions modify host proteins and are responsible for many diseases in man and animals like the scrapie disease in sheep, Bovine spongiform encephalopathy in cattle, Kuru And Creutzfeldt-Jakob disease in humans etc.
- Prions cause diseases by aggregating extracellularly In the form of amyloid plaques in the central nervous system.
- Neurodegenerative symptoms include convulsions, dementia, ataxia, and behavioural or personality changes.
- When prions enters a healthy organism, it induces normal prion proteins to convert into the disease – associated, infections form.
- This altered structure accumulates in infected tissue, causing tissue damage and cell death.
- Prions are resistant to denaturation by chemical and physical agents, making disposal and containment of these particles difficult.
- The endogenous, property -folded, form is denoted as PRPC while the disease- linked, misfolded forms is denoted PRPSC.
- While the incubation period for prion diseases is generally quite long, once symptoms appear the diseases progresses rapidly, leading to brian damage and death.

Mycoplasma

- Mycoplasma is a genus of parasitic or saprotrophic bacteria that lack cell wall.
- A. B Frank in 1889 first identified them as fungi.
- The cell size ranges between 0.1-0.3 in diameter, with typically about 10% of the volume of an Escherichia coli cell.
- The cell are pleomorphic as the cell shapes are variable due to the absence of a cell wall.
- Most are pseudococcoidal, whereas species like M. fastidious are rod-shaped.
- Some species like *M. Pneumoniae,* possess a polar extension protruding from the cell body that function as an attachment organelle for adherence to host cells and for movement along solid surface.
- Mycoplasma are unusual bacteria in that most required sterols for the stability of their cytoplasmic membrane.
- Mycoplasma possess both DNA and RNA and the genome is relatively small with 0.58 - 1.38 mega bases, with reduced biosynthetic capabilities indicating their dependence host cell.
- Mycoplasma causes various disease in man, animals and plants.
- Mycoplasma pneumoniae as well as at least 7 other mycoplasma species have now been linked as a direct cause or significant co- factor to many chronic disease including, rheumatoid arthritis, Alzheimer's, multiple sclerosis, fibromyalgia, chronic fatigue, diabetes, etc;
- They are not affected by many common antibiotics such as penicillin that target cell wall synthesis

Rickettsias

- Rickettsias are a group of bacteria living as pathogenic, intracellular parasites.
- It is found in ticks ,fleas ,lice etc.
- It is non- motile ,Gram-negative, non-spore forming, highly pleomorphic cells that appear in spherical, rod shaped or thread-like forms.
- The cells are small with the size varying from 0.1 micrometer-0.5 micrometer in diameter and 0.8 micrometer – 10 micrometer in length.
- Rickettsias and their survival depend on entry, growth, & replication within the cytoplasm of the eukaryotic host cells.
- After entering the host cell the pathogenic microbe multiplies, the host cell then lysis and releases the rickettsial progeny to initiate a new infection cycle.
- Rickettsia causes several diseases such as Typhus, Rickettsial pox, Boutonneuse fever, African tick bite fever, Rocky Mountain spotted fever, Flinders Island spotted fever etc.
- Earlier it was positioned b/w viruses & true bacteria, recent authors include them under eubacteria.

METHODS IN MICROBIOLOGY

Sterilization: Physical and Chemical methods

- All apparatus used must be sterilised by heat (glassware 160°C for 2 hrs) or exposure to radiation.
- Aseptic techniques must be used to reduce the likelihood or microbial contamination.
- This involves disinfection of working areas, minimizing possible access by bacteria from the air to exposed media, and use of flames to kill the bacteria which might enter vessels as they are opened.

Dry and Moist Heat

- Dry heat sterilization is used for moisture-sensitive materials and employs high temperatures in the range of l60°C-180°C.
- Common dry heat sterilization techniques employed are incineration, flaming, use of hot air ovens, etc.
- ***Incineration***: It is one of the commonest sterilization techniques used for effective sterilization of disposable materials and biological wastes.
- It burns the materials and physically destroys all the microbes in them.
- ***Flaming***: This is one of the oldest methods where holding an instrument in an open flame for short period destroys all the microbes present in it.
- Inoculation loops or needles are sterilized by heating to red hot in Bunsen burner or spirit lamp flame.
  Hot air ovens: Dry heat ovens are instruments used to sterilize objects which are damaged by, or impenetrable to moist heat. Higher temperatures in the range of 16Q 180°C and exposure time up to 2 hours are employed for effective sterilization based on the object to be treated. Good penetrability and non-corrOSIve nature of dry heat

Pasteurization

- Pasteurization is a form of microbial control for food products that are easily affected by higher temperatures.
- Developed by Louis Pasteur
- Commonly used to kill the heat sensitive pathogens in milk and other food products.
- The original pasteurization technique used was the application of a relatively low temperature of 60°C for 30 minutes - batch pasteurization
- Nowadays flash pasteurization by two methods is commonly employed especially for pasteurizing milk.
- ***High temperature short time (HTST)*** pasteurization, exposes milk to a temperature of 72°C for 15 Seconds, which lowers bacterial numbers while preserving the quality of the milk.

- ***Ultra high-temperature (UHT)*** pasteurization where the milk is exposed to a temperature of 138°C for 2 or more seconds which preserves the milk for longer shelf life even without refrigeration.

Radiation

- Various forms of radiation can be used to kill microbes or inhibit their growth.
- Exposure to sunlight is one of the most common and ancient methods for controlling microbial growth through irradiation.
- Radiations can be ionizing (X-rays, gamma rays, high energy electron beams) or non-ionizing (UV rays).
- Ionizing radiations are highly penetrative and cause direct damages to the DNA leading to cell death.
- Because of this high penetrative power, ionizing radiation is used to sterilize packaged materials, non autoclavable materials such as plastic petri dishes, disposable plastic inoculating loops, gloves, intravenous tubing, and other heat-sensitive materials like pharmaceutiçal drugs, and medical equipment.
- Non-ionizing radiation which is less penetrative is also used to control microbial growth as they induce indirect damages to the DNA through the formation of reactive groups within the cell.
- They are less penetrative and use less energy than ionizing radiation.
- Ultraviolet lamps are used to sterilize workspaces and tools used in microbiology laboratories and health care facilities as a supplement to other sterilization methods.

Ultrasonication

- This is a method that utilizes sound waves of ultrasonic frequencies (>20 kHz) for controlling the growth of microorganisms.
- Ultrasonication generates alternating low pressure and high pressure waves in liquids, leading to the formation and violent collapse of small vacuum bubbles.
- The treatment of microbial cells in suspension with inaudible ultrasound results in their inactivation and disruption.

Disinfection

- Disinfection means reducing the number of viable microorganisms present in a sample
- Not all disinfectants are capable of sterilizing, but, of course, all disinfectants are employed with the hope of disinfecting

Sanitization

- Sanitization is the cleaning of pathogenic microorganisms from public eating utensils and objects such as that done by the kitchen of a restaurant

Antiseptics

- Typically an antiseptic is a chemical agent that is applied to living tissue to kill microbes.
- Not all disinfectants are antiseptics because an antiseptic additionally must not be so harsh that it damages living tissue.
- With this constraint imposed on antiseptics, in general antiseptics are either not as cheap or not as effective at killing microbes as disinfectants

Sterilants

- These are chemicals applied to inanimate objects which kill all microorganism including their endospores and viruses.
- They can be used to treat heat sensitive critical items.
- Ethylene oxide and glutaraldehyde are examples of sterilants.

Fumigation

- It is the process of gaseous sterilization done in enclosed areas by introducing chemicals that will destroy microbes in the air and surfaces like walls, floors, etc.
- Here the gaseous fumigants employed will suffocate or poison the microbes and even pests within.
- This method can be adopted for a wide range of areas from small chambers or instruments to large enclosures like buildings. Formaldehyde,

Preparation of culture media

Natural or empirical media

- Earlier natural substances like urine, milk, vegetable juices, meat extracts etc were used as microbial culture media.

Synthetic or defined media

- These are chemically pure media containing known composition of organic and inorganic nutrients.
- Such media are prepared in the laboratories and are of different types such as; a) General purpose media used for majority of microbes, b)Selective media for a selected microbe or c) Differential media for the isolation of different microbes in the same sample
- For the preparation of a bacteriological defined media, a carbon source like glucose or dextrose and peptones for nitrogen are often provided.

- Minerals and vitamins may also be provided, according to the growth requirements of the bacteria
- Buffers are used to keep the pH stable.
- For the culture of most bacteria, pH of the medium is adjusted to between 7.2 and 7.4.
- Measured amounts of the concentrates are added to water, and dissolved to constitute the media.
- These media must then be sterilized by heating in an autoclave at 121°C for 15 minutes, which kills all living organisms, including spores.
- After sterilization the medium is transferred into petri dishes or into conical flasks or glass tubes and allowed to cool.
- For preparing an agar slant the tubes are kept in a slanting position until the agar sets into a gel.
- The slant provides a larger surface for the growth of microorganisms.
- Microbial cultures are maintained on agar for saving space and materials and for sorting the cultures for extended periods.

Living media

- Living cells, tissues, organs.. are used for the culture
- Chick embryos are used for virus cultures.

Selective media

- lt is a media that selectively inhibit the growth of all but a few organisms.
- Such a medium contains substances that either facilitate or inhibit the growth of a particular group of bacteria.
- Thus it enables the isolation of certain bacteria from a mixed population.
- Mac Conkey agar is used as a selective medium for the isolation of intestinal gram-negative rods.
- The addition of two inhibitory compounds, bile salts which inhibit non-intestinal bacteria, and crystal violet which inhibit gram-positive bacteria, ensures that only gram-negative rods are growing in the medium.
- Similarly mannitol salt agar (MSA) is a selective medium for Gram-positive bacteria and Deoxycholate citrate agar (DCA) is selective for enteric bacilli, such as Salmonella spp. and Shigella spp.

Enrichment media

- These are liquid media providing conditions that specifically enrich the growth of one type of organism in a mixed population.
- Thus it enables isolation of bacteria from natural sources, especially where they are present in relatively small numbers, which cannot be done effectively by the plating techniques.

- In such cases adjusting the pH or temperature of the medium or addition of a nutrient substance that helps in the growth of the particular microbial population will increase the chances of its selective isolation.
- *Escherichia coli* present in feces, tend to overgrow pathogenic ones in stool specimens.

Differential media

- It is also known as indicator media.
- This is a medium that enables to distinguish the microorganisms growing on them by their growth characteristics.
- A typical example is the blood agar medium which is a nutritional medium that enables to distinguish hemolytic bacteria from non hemolytic ones.
- The hemolytic bacteria has an enzyme hemolysin which causes lysis of RBCs in the blood agar medium which can be distinguished by a colour change around the colonies.
- Eosin methylene blue (EMB) differential for lactose and sucrose fermentation
- Mannitol salt agar (MSA) differential for mannitol fermentation
- MacConkey agar, differential for lactose fermentation are some other examples.

Plating techniques and Isolation of pure colonies

Isolation

- Natural habitats such as soil, water, sewage, infected tissues etc. harbour millions of microorganisms.
- Samples collected natural microbial habitats are serially diluted many times with sterile water, for the separation of individual cells.
- Pathogenic microorganisms from infected tissues are collected with a cotton swab or syringe.
- The serially diluted samples are broth cultured and tested for the separation and purity of microbes with the ***pour plate or streak plate method***.

Pour plate method

- It is a method used for preparing bacterial cultures for enumeration and isolation purposes.
- Here the medium is maintained in a molten state at 45°C while introducing the microbial sample.
- 1 ml of appropriately diluted microbial sample is poured into a sterile Petri plate and about 10-20 ml of the molten agar medium is added.
- The contents are mixed well by clockwise and counter clockwise rotation of the plate.
- The media is allowed to solidify and incubated in an inverted position.

Spread plate method

- It is a technique used to plate bacteria in suspensions so that they are easy to count and isolate.
- A successful spread plate will have a countable number of isolated bacterial colonies evenly distributed on the plate.
- In this method, approximately 0.1 ml of the sample containing microbes is added directly to the center of a solidified agar plate and evenly spread on the surface of the plate with the help of a glass spreader or by rotating the plate.
- The plate is incubated in an inverted position for the required period.

Streak plate method

- This method is a rapid qualitative isolation method where the inoculum is streaked over the surface of an agar plate in such a way that it "thins out" the bacteria.
- This method does not require serial dilution to be performed as proper streaking will separate out the individual bacterial cells well spaced from each other.
- Hence direct isolation of individual bacterial colonies is possible with the help of an inoculation loop.
- This is one of the most preferred methods for isolation of bacteria into pure cultures from mixed populations.

Staining

- To observe the structure, colourless microbes are stained with stains such as, methylene blue, crystal violet etc,.
- Good staining needs skill and experience.
- Staining procedures are adopted to improve the contrast for observation.
- The cells are first fixed by physical or chemical means so that internal and external features of cells remain in fixed position.
- The commonly used chemical fixatives are ethanol, acetic acid, glutaraldehyde etc.
- A smear is then made on a slide is then heated gently over a small flame until the water evaporates.
- Staining may be simple or differential - in the former a single stain is used and in the latter a combination of stains is used.
- Differential staining is done to differentiate between the cell components or microbial groups.
- In bacteriology the conventional differential staining technique for the preliminary grouping is the Gram staining technique, developed by Christian Gram (1884) which separates bacteria into two groups namely
  - Gram positive and Gram negative bacteria.
- For this, bacterial smear is first stained with crystal violet (primary stain).
- The smear is then treated with a mordant made up of iodine in potassium iodide solution.

- It helps in strong adherence of the stain.
- The smear is then destained in ethanol or acetone.
- Gram positive bacteria retain the crystal violet dye while gram negative bacteria lose it.
- In order to stain the destained group a basic dye saffranin is used for counterstaining.
- Gram-positive bacteria appear dark blue or purple
- Gram-negative appear reddish or pinkish.

Simple staining

- In simple staining, a single stain is used to colour the microbes.
- This is adopted mainly to study morphology and spatial arrangement of the cell.
- Basic dyes with a positive charge are used in this staining procedure as many of the cellular components including nucleic acids and proteins are negatively charged and will attract a positive dye.
- Any of the basic dyes can be used depending on our needs.
- Methylene blue, crystal violet, carbol fuchsin, saffranine, etc. are the commonly used dyes for simple staining

Negative staining

- Negative staining, which use acidic dyes having a negative charge, produces an outline of the organisms.
- Since both the dye and cell surface have the same charge, the dye is repelled by the cell and the colourless cell is visible against coloured background.
- Commonly used acidic dyes for this procedure include acid fuchsin, eosin, rose bengal, etc.

Gram staining

- In bacteriology the conventional differential staining technique for the preliminary grouping is the Gram staining technique, developed by Christian Gram (1884) which separates bacteria into two groups namely
  - Gram positive and Gram negative bacteria.
- For this, bacterial smear is first stained with crystal violet (primary stain).
- The smear is then treated with a mordant made up of iodine in potassium iodide solution.
- It helps in strong adherence of the stain.
- The smear is then destained in ethanol or acetone.
- Gram positive bacteria retain the crystal violet dye while gram negative bacteria lose it.
- In order to stain the destained group a basic dye safranin is used for counterstaining.
- Gram-positive bacteria appear dark blue or purple
- Gram-negative appear reddish or pinkish.

Culture preservation techniques

Refrigeration

- One usual method is keeping sealed agar slant cultures in small culture tubes under refrigeration in which evaporation is reduced and metabolic activity remains low.
- Microorganisms can be preserved in this condition for 2-3 months and recovered by inoculating freshly prepared agar slants

Deep freezing

- Reducing the temperature well below 0°C considerably increases the storage time of microbial culture.
- This can be achieved with a deep freezer at temperatures ranging from 20°C to 30°C or in an ultra deep freezer at 80°C.
- The formation of ice crystals and concentration of electrolytes are the disadvantages of these systems.

Freezing under liquid Nitrogen

- Cryopreservation
- lt is the preservation of culture media the microorganisms of the culture are rapidly frozen in liquid nitrogen at 196°C.
- The presence of stabilizing agents such as glycerol or dimethyl sulfoxide (DMSO) prevent cell damage due to the formation of ice crystals and promote cell survival.
- This helps the survival of pure cultures for long storage times of nearly 10 to 30 years without changing their characteristics.

Lyophilisation

- For the maintenance of bacterial cultures for years, a technique called ***lyophilization*** is followed, in which nutrient broth culture is taken in small vials and frozen at - 60 to -78°C.
- The vials are then connected to high vacuum suction tubes in a freeze drying instrument called ***lyophilizer.***
- The moisture content quickly sublimed under high vacuum so that temperature does not rise.
- This process dehydrates the bacteria with a minimum damage to the cell structure.
- The mouths of the vials are then sealed so that vaccum is maintained in the vial.

MODULE 5

BASIC CONCEPTS IN BACTERIOLOGY AND VIROLOGY

Bacteria: Structure of a typical Bacterium

Based on the shape of cells

1. ***Cocci*** - spherical or ellipsoidal forms. Eg:- *Staphylococcus, Lactococcus* etc.
2. ***Bacilli*** – rod shaped or cylindrical forms. e.g. *Bacillus, Clostridium* etc.
3. ***Vibrio*** - slightly curved or comma-shaped forms. e.g. *Vibrio cholera*
4. ***Spirilla*** - twisted, rigid and wavy forms. e.g. *Azospirillum, Aquaspirillum*
5. ***Spirochaetes*** - tightly coiled, cork-screw-shaped forms. e.g. *Treponenma, Leptospira etc.*
6. ***Stalked forms*** - cell characterized by a small stalk. e.g. *Caulobacter*.
7. ***Mycelial bacteria*** - appear in the form of fungal filaments. e.g. *Actinomycetes*
8. ***Pleomorphic forms*** - lack cell wall and the cells without definite shape. e.g.*Mycoplasmas*

Based on temperature

- Psychrophiles – can survive at very low temperature
- Mesophiles – temperature ranges between 20 - 40°C
- Thermophiles – grow best at temperatures above 45°C

Based on pH

- Acidophilic – pH – 3.3 – 7.2 – eg:- Lactic acid
- Basophilic – pH – 4.8 – 10.6 – eg:- *Enterococcus spp*
- Neutrophilic – pH – around 7

Cell wall

- Bacterial cell is protected by the cell wall.
- It provides structural integrity, shape & mechanical support to the cell.
- Cell wall is made of ***peptidoglycan.***
- It is a muco polysaccharide formed of ***N acetyl glucosamine (NAG) & N acetyl muramic acid (NAM)***
- They are linked by β – 1,4 glycosidic linkage
- There are two main types of bacterial cell wall - ***Gram positive and Gram negative***.
- In Gram positive bacteria the peptidoglycan 1ayer is much thicker than that the Gram negative bacteria.
- In addition it contains some proteins and teichoic acid.
- Gram negative cell wall is comparatively thin and more complex with three 1ayers, an inner thin peptidoglycan layer, a middle lipoprotein layer and an outer lipopolysaccharide (LPS) layer.

- In between the lipoprotein layer and peptidoglycan layer is a space called periplasmic space.
- The cell wall of Gram negative bacteria prevents the penetration of antibiotics into the cell interior making the control of such pathogenic forms difficult.

Cytoplasmic membrane

- The cytoplasmic membrane or plasma membrane lies immediately beneath the cell wall.
- It is approximately 7.5 nm thick and formed of phospholipids and proteins.
- It has the fluid mosaic organization, structurally consisting of a continuous phospholipid bilayer in which globular proteins are embedded.
- It carries several enzymes for the synthesis of proteins, amino acids, cell wall and permease enzymes for the membrane transport system.
- In aerobic bacteria the plasma membrane also carries enzymes of the electron transport chain and oxidative phosphorylation.

Glycocalyx

- Some bacteria are surrounded by an extra cellular polymeric coat called the glycocalyx.
- These polymers are usually composed or polysaccharides or polypeptides or both.
- They are relatively impermeable structures that cannot be stained with dyes.
- They prevent dessication of the cell, protects it from the attachment of bacteriophages, phagocytosis by other cells,...
- When the surface coat is in the form of a loose layer it is called a ***slime layer*** and when it is organized as a thick covering around the cell wall then it is called a ***capsule.***

Flagella

- Flagella are hair-like appendages protruding from the bacterial cell wall and are responsible for bacterial motility.
- The common patterns of flagellar arrangement are:
  A) Monotrichous - a single polar flagellum is present in bacterium
  B) Lophotrichous - a cluster of polar flagella
  C) Amphitrichous - flagella present at both ends of the bacterium occurring singly or in cluster.
  D) Peritrichous - flagella occurring evenly on the entire cell surface of the bacterium.
- A typical bacterial flagellum has three parts
- A ***basal body*** for attaching itself to the cytoplasmic membrane
- A long whip like part called ***filament***
- A short ***hook*** connecting the basal body with the filament.

Fimbriae and pili

- Many bacteria have numerous hollow hair like short protein tubes called fimbriae distributed over the entire bacterial cell surface.
- Fimbrae usually function to facilitate cell to cell or cell to surface attachment.
- Pili are similar in structure to fimbriae but are much longer and present in low numbers.
- Pili are formed of the protein pilin and involved in the process of bacterial conjugation.

Chromosome

- DNA in the bacterial cell is confined to the central ribosome free region called the nucleoid or the nuclear region.
- Bacterial chromosome is not packaged using histones to form chromatin as in eukaryotes, but instead exists as a highly compact supercoiled structure.

Plasmids & episomes

- Small extra nuclear independent circular DNA molecules called plasmids and episomes.
- They carry extra genetic information
- P1asmids replicate independently
- Episomes may get integrated with the chromosome and replicate as part of it.
- Some of the functionally different kinds of plasmids are:
    1. Sex or fertility (F) factor/plasmids - carry genes that confer the ability for genetic transmission from one strain of bacterium to the another.
       Bacterial cells having f-factors are designated as F+ strains.
    2. Resistance (R) factor/plasmids - carry genes coding for proteins that inactivate specific antibiotics thus making the bacterial strain antibiotic resistant.
    3. Col (R) factor/plasmids - carry genes for the production of bacterial toxins or bacteriocins.
    4. Metaboloic plasmids carry genes for bacterial metabolism.

Mesosomes

- These are localised infoldings of the plasma membrane in the form of vesicles, tubules or lamellar whorls.
- It is believed that they play some role in initiating DNA synthesis, cell division, cell wall synthesis etc.

Ribosomes

- Most numerous intracellular structure is the ribosome, the ***site of protein synthesis***
- Ribosomes lie free in the cytoplasm and give a granular appearance to the cytoplasm under electron micrography.
- Group of ribosomes bound to an mRNA molecule like beads on a thread called ***polysome or ergosome or polyribosme.***

- All prokaryotes have 70 S ribosomes while eukaryotes contain larger 80 S ribosomes.
- The 70S ribosome is made up of 50 S & 30 S subunits.
- The 50 S subunit contains the 23 S and 5 S rRNA while 30 S subunit contains the 16 S rRNA.

Nutrient storage structures

Cytoskeleton

- Cytoskeletal elements play essential roles in cell division, protection, shape determination, and polarity determination in various prokaryotes.

Gas vesicles

- Gas vesicles are spindle shaped structures found in some planktonic bacteria that provides buoyancy to these cells by decreasing their overall cell density.

Carboxysomes

- Carboxysomes are intracellular proteinaceous structures found in many autotrophic bacteria.
- They resemble phage heads in morphology and contain the enzymes of carbon dioxide fixation.

Magnetosomes

- Magnetosomes are intracellular organelles found in magnetotactic bacteria that allow them to sense and align themselves along a magnetic field

Spores and cysts

- These are metabolically dormant forms of certain species of bacteria.
- Spores may form within the cell (**endospores**) or external to the cell (**exospores**).
- Cysts are formed by the transformation of vegetative cells.
- Both spores and cysts are desiccation resistant and germinate under favourable conditions.

Different types of bacterial culture (Batch, Synchronous, Arithmetic)

- In laboratories, bacterial cultures are maintained in containers holding a suitable medium.
- They can be grown either in broth (the liquid form of the medium contained in a tube or a flask) or on an agar plate depending on the purpose of the culture.
- These cultures are considered as closed systems as nutrients are not added to or waste materials are not removed from such systems after the inoculation of microorganisms into them.
- Such a culturing system is called a ***batch culture***.
- Batch cultures generally show a typical pattern of microbial growth where all the stages in a typical bacterial growth curve are exhibited.

- Hence such cultures are unable to support microbial growth indefinitely
- Repeated sub culturing in freshly prepared media is required in a batch culture to maintain cells viable for longer periods.
- ***Continuous cultures*** are systems that maintain bacteria in a continuous state of growth.
- This can be achieved in an open system where nutrients continually added and waste products removed from the system.
- This maintains bacteria in a state ot continuous exponential growth.
- A continuous culture maintained in the laboratory with the help of a chemostat or turbidostat.
- ***Synchronous growth*** of a bacterial population is one in which all the bacterial cells of the population are logically identical and in the same stage of the cell division cycle at a given time.
- In such cultures, instead of studying a single cell, the study of the particular stages of the division cycle and their interrelations can be done by analysing the culture characteristics.
- But synchronous growth of population rarely occurs in nature.
- Synchronous culture techniques can be obtained either by manipulating environmental conditions such as by repeatedly changing the temperature, or by adding fresh nutrients to the culture

Bacterial growth: Growth phases

- In bacteria the term growth is generally used to indicate increase in number of the total population rather than the size of individual organism.
- The nutritional requirements of bacteria include macronutrients like carbon, nitrogen, phosphorous, sulphur etc., micronutrients like, manganese, copper, Zinc etc., and a wide range of compounds like vitamins, nitrogen bases, amino acids etc.
- The nutritional requirements and physical conditions of growth like temperature, pH, gaseous environment etc. vary widely among bacteria.

Phases of Growth

- The cell mass starts increasing and doubles after every cell division.
- The pattern of growth can be studied by plotting time along X axis and number of cells along the Y axis.
- Typically, a bacterial growth curve has four phases as; lag phase, log phase, stationary phase and death or autolytic phase.

1. Lag phase

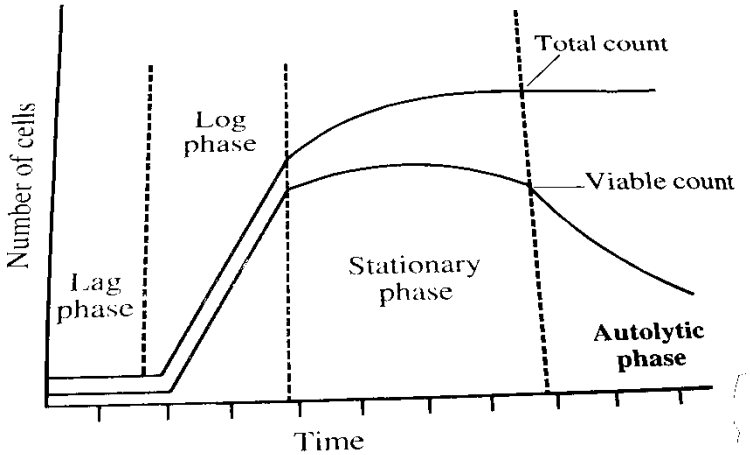
- After inoculation, individual bacterial cells require some time for adapting to the new environment and the uptake of ingredients present in the medium.

- This is the establishment phase or lag phase of the culture during which there is no appreciable increase in the population size.

2. ***Log phase***
   - Log phase or logarithmic phase is the period of active multiplication and growth
   - After the establishment phase the cell mass starts increasıng and doubles after every cell division.
   - The time taken for doubling is known as the generation time.
   - lt varies from about 20 minutes to a few hours for most bacteria.

3. ***Stationary phase***
   - After the logarithmic phase the growth rate falls, due to limitation of one or more constituents in the medium, accumulation of toxic or unwanted chemicals, changes in pH etc.
   - During this period, increase in cell mass is almost balanced by cell death, so that the population size remains more or less static.

4. ***Death or autolytic phase***
   - The final stage of growth of bacteria in a culture medium
   - Cells die faster than new cells are produced
   - resulting drastic decline in the population size.
   - During this phase the number of viable cells decline exponentially due to the depletion of nutrients_and accumulation of inhibitory products.



Bacterial growth curve

Methods of growth determination

- Bacterial growth can be determined by various methods based on measuring any of the common parameters of growth; the cell mass or cell number or cell activity

A. Cell mass

- The cell mass of bacterial culture can be determined by taking the weight or measuring the turbidity of the medium.
- The weight is determined after separating the cells from the medium by filtration or centrifugation.

B. Cell number

- Different techniques are used to determine the cell number in a bacterial culture

1. *Direct microscopic count*

- The cell number can be determined accurately by counting the number of cells after transferring a uniformly suspended medium to a standard counting chamber like Neubour's chamber.

2. *Plate count method*

- Here, a known volume of bacterial culture is spread on a Petri dish, agar medium is then added to it and incubated after mixing thoroughly.
- The number of colonies developed is counted to determine the bacterial population in the inoculum'

3. *Membrane filter count*

- In this method a bacterial culture is filtered through membrane filters to trap bacteria and the filter is placed on special counting pad saturated with appropriate medium.
- After incubation the number of colonies developed is counted to determine the bacterial population.

4. *Electronic enumeration*

- In electronic enumeration bacterial suspension is placed inside an electronic counter and counting is done as the cells pass through a tiny orifice.

C. Cell activity

- The biochemical activity of the culture is estimated and it is considered as an index of bacterial growth.
- For example, production of a metabolic end product like an organic acid is estimated and is considered as an index of growth.
- The assumption is that, the amount of the product estimated is proportional to the magnitude of growth.

Basic Concepts of Virology

General characteristics

1. Viruses are extremely simple, ultra microscopic and sub-cellular organisms, without protoplasm, cell, cell organelles, and energy generating and protein synthesising molecular machineries.
2. They are formed of proteins and DNA or RNA.
3. Viruses are not free living organisms, but are host specific intracellular obligate parasites, infecting bacteria, protists, fungi, plants and animals.
4. Viruses can multiply only in living cells and so they cannot be cultured in artificial or synthetic media.
5. The genetic material of viruses (viral genome) consists of either DNA or RNA and never both. So, there are two groups of viruses, DNA viruses and RNA viruses.
6. Viral nucleic acid may be single-stranded or double-stranded.
7. Viral nucleic acid is encased by a shell or coat of proteins, called ***capsid.***
8. The sub units of the capsid are called ***capsomeres.***
9. Viral multiplication is different from the reproduction of other organisms. It occurs only within the host cell and does not involve fission. It takes place by the replication of the nucleic acid and the synthesis of capsid proteins, making use of the raw materials and the molecular machinery of the host cell.
10. There is an "***eclipse phase***" in the life cycle of viruses during which the virus particle disintegrates to its molecular constituents. Towards the end of the eclipse phase, progeny viruses are formed.
11. Viruses can only replicate, but cannot grow
12. Viruses are transmitted from one host to another by mechanical means or by biological vectors.

Classification of viruses

- Viruses have been classified in diverse ways.
- Earlier, based on their natural hosts, viruses were classified into
    1. Phytoviruses (plant viruses)
    2. Zooviruses (animal viruses)
    3. Mycoviruses (mycophages or fungal viruses)
    4. Phycoviruses (phycophages or algal viruses)
    5. Bacterial viruses (bacteriophages)
    6. Mycoplasma viruses.
- The International Committee on Taxonomy of Viruses (ICTV) classified viruses based on the following three major aspects.
  (i) The kind of nucleic acid present (DNA or RNA).
  (ii) Strandedness of the nucleic acid (single-stranded or double stranded).
  (ii) Presence or absence of additional envelope. i.e., enveloped or non-enveloped.

According to this scheme, viruses are grouped under eight clusters as follows.

1. Enveloped and single-stranded DNA viruses
2. Non-enveloped and single-stranded DNA viruses.
3. Enveloped and double-stranded DNA viruses.
4. Non-enveloped and double-stranded DNA viruses
5. Enveloped and single-stranded RNA viruses
6. Non-enveloped and single-stranded RNA viruses.
7. Enveloped and double-stranded RNA viruses.
8. Non-enveloped and double-stranded RNA viruses.

Bacteriophages: Diversity

- Bacteriophages or bacterial viruses are the viruses which parasitise bacteria.
- They are widely distributed in nature, and are especially found in large abundance in enteric bacteria which inhabit the intestine of man and other animals.
- They are especially adapted for adsorption and penetration through the rigid cell wall of bacteria.
- Bacteriophages fall under four major groups, namely single-stranded DNA (ssDNA) phages, double-stranded DNA (dsDNA) phages, single-stranded RNA (ssRNA) phages and double-stranded RNA (dsRNA) phages.

Lytic and lysogenic Phages (Lambda and P1 Phage)

- Based on the multiplication cycle, bacteriophages are classified into two groups : lytic (virulent) phages and lysogenic (non-virulent or temperate) phages.
- The ***lytic cycle*** occurs in the lytic phages where the viral genome multiplies inside the bacterial cell and the progeny viruses are released through the host cell lysis.
- In the ***lysogenic cycle***, the virus does not undergo multiplication to produce viral particles, instead, they are integrated as a part of the bacterial genome.

Lytic cycle

- Lytic cycle is considered as one of the main methods of viral replication as it results in the release of a large number of mature viruses.
- They are called virulent phages as they induce pathogenicity in the host by increasing fatality rates in the host cell by leading to cell lysis.
- Eg:- T4 phage
- The lytic cycle has the following stages
- **1. *Adsorption*** : Here the phages adsorb on specific receptors on the bacterial wall with the help of its tail fibers and basal plate.
- **2. *Injection of genetic material*** : The genetic material in the phage head is injected through the opening in the tail region.

- **3. *Viral protein synthesis***: Soon after entry into the host cell, viral DNA takes control of the protein synthetic machinery of the host cell thereby arresting host gene expression. It then mediates transcription and translation of the viral genome leading to the production of viral RNAs and proteins.
- **4. *Viral genome synthesis*** : Here some of the viral proteins synthesized in the host cell are used for mediating the viral DNA replication. Several copies of viral DNA are synthesized within a few minutes.
- **5. *Maturation and release*** : Assemblage and release of mature viral progeny occur within 15 minutes after the initiation of the lytic cycle. Newly synthesized viral DNAs get wrapped in coat proteins to form mature phages. At some critical point, the wall of the host bacterium lyses, and the mature phages are released through the destruction of the host cell.

Lysogenic cycle

- It occurs in temperate phages which integrate their genome into the host bacterium's chromosome.
- The host bacteria in which the cycle is completed are called ***lysogens or lysogenic strains***.
- The transformation of a normal bacterium to a lysogen by a viral infection called ***lysogenation***.
- The close association between the viral genome and the bacterial genome in the lysogenic cycle is called ***lysogeny***
- Lysogenic cycles can be commonly exhibited by **phage λ** which infects *E.coli.*
- The phage gets adsorbed on the host cell and injects the genome into the cytoplasm.
- The phage DNA may integrate itself into the host cell chromosome in the lysogenic pathway
- The integrated vial genome is called a ***prophage***.
- The prophage along with the bacterial genome replicates as a single unit through the normal cell cycle & is passed to the daughter cells.
- However, quite occasionally the association breaks, and the viral genome is released into the bacterial cytoplasm & undergo lytic cycle.
- **Phage P1** is a temperate bacteriophage that infects E.coli and some other bacteria
- Unlike other phages that integrate into the host DNA in a lysogenic cycle, the P1 phage genome exists as a plasmid in the bacterium.
- P1 can exist within the bacterial cell they infect in two different ways.
- *In lysogeny,* P1 can exist within a bacterial cell as a circular DNA, where it replicates as if it were a plasmid and does not cause cell death.
- In its *lytic phase,* P1 can promote cell lysis during replication resulting in host cell death.
- By alternating between these two modes of infection P1 can survive during extreme nutritional conditions that may be imposed upon the bacterial host in which it exists

Applications of bacteriophages

- Bacteriophages are natural predators of bacteria
- ***Phage therapy*** is a branch where phages are used as therapeutic agents in humans.
- Even therapeutic uses of phages in combination with antibiotics are expected to have a great future.
- Phages have been used as agents of bacterial transduction.
- Virus mediated gene transfer in between bacterial strains results in the production of a bacterium with recombinant genes. They also serve as ***cloning vectors***
- Phages can be used as bio control agents in the agriculture and food industry where phage mediated killing of the infection causing bacterial population is carried out.
- They are also used as vehicles for vaccines, for the detection of pathogenic bacterial strain, etc.

Oncogenic Viruses

- During the viral replication process, some of the viral genetic material affects the host cell's genes in ways that may cause it to become cancerous – ***oncoviruses***
- The common human cancer causing by
  are hepatitis B virus (HBV), hepatitis C virus (HCV), human papilloma virus (HPV) Epstein Barr Virus (EBV), human herpesvirus 8 (HHV8), Merkel cell poliomavirus (MCPyV), and HTLV-1.
- ***Proto oncogenes*** are genes that code for proteins which activate transcription of genes playing a major role in regulating normal cell growth and development.
- Oncogenic viruses produce mutation in the proto oncogenes and thereby altering their function. This causes abnormal cell growth leading to the formation of tumors.
- Oncogenic viruses fall into 2 groups : the ***DNA tumor viruses*** that contain either linear or circular double stranded DNA, and the RNA-containing tumor viruses (also called ***retroviruses***).
- Retroviruses are the most common cause of tumors in animals.
- They transform the host cells by inserting viral genes called ***oncogenes*** into the genome of the host cell.
- The oncogene inserted now disrupts the normal function of proto oncogenes thus leading to tumor formation
- The process from the initial viral infection to tumor formation is slow and inefficient.
- Only a small percentage of viral infections progress to cancer years or decades after the initial infection.
- Other factors may increase the chance of cancer including immune system complications, cell mutations, exposure to cancer-causing agents, and hereditary susceptibility.

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Prevention and control of Viral diseases

- The spread of many viral diseases can be prevented by implementing hygienic factors such as efficient sanitation facilities, effective waste disposal, clean water, and personal cleanliness.
- In addition to the hygienic factors, the use of antiviral compounds, viral vaccines, etc. is found to effectively control viral infections in host cells.

Antiviral compounds

- Antiviral compounds refer to a class of chemicals that act by arresting the viral replication cycle at various stages inside a cell.
- Antiviral therapy aims to minimize symptoms and infectivity as well as to shorten the duration of illness.
- Their action helps to prevent the viral load of an infected person from increasing to a point where it could cause pathogenesis.
- At lower viral loads the body's innate immune mechanisms can neutralize the virus and remove them from a person's body within a few days.
- An antiviral agent must act at one of the five basic steps in the viral replication cycle to inhibit the virus
- 1. Attachment & penetration of the virus into the host cell
- 2. Uncoating of Virus
- 3. Synthesis of new viral components by the host cell as directed by the viral DNA
- 4. Assembly of the components into a new virus and
- 5. Release of the virus from the host cell.
- Currently, antiviral therapy is available only for a limited number of infections.
- Most of the antiviral drugs currently available are used to treat infections caused by HIV, herpes Viruses, hepatitis B and C and Influenza A and B viruses.

Interferons

- Interferons are proteins normally synthesized by the cells of vertebrates, including humans, either intrinsically or in response to certain viral infections, chemicals, or immune reactions.
- They are secreted from infected cells and activate an innate immune response that promotes not only cytokine production but also natural killer cell functions and antigen presentation.
- The interferon response represents an early host defense, one that occurs prior to the onset of the immune response against viral or bacterial infection.
- Due to their ability to modulate immune responses, they have become attractive therapeutic options to control chronic viral infections.

- Interferons are classified into three groups based on the structure of their receptors on the cell surface. They are ***Type I, II, and III*** interferons.
- Type I IFNs have been part of the standard treatment for HCV and HBV infections, and the appearance of IFN resistant viral subpopulations has not been observed

Viral vaccines

Attenuated viral vaccines

- These are the preparations of organisms, weakened by various methods, including genetic alterations.
- They produce only a mild, sub-clinical infection which can provide strong resistance and protection.
- These vaccines are not as safe as inactivated vaccines, because their reversion to virulent state is possible and so they can cause diseases in immunologically weak individuals.
- Oral polio vaccine, mumps vaccine, measles vaccine, whooping cough vaccine, etc. are live attenuated vaccines.
- Attenuation is the process of reducing the virulence of a pathogen.
- Attenuated vaccines induce both humoral and cell-mediated immunity.
- Many of these vaccines require a booster dose for effective disease prevention.
- Booster dose is a dose of antigen given after the primary dose to stimulate accelerated production of large amounts of antibody (e.g., polio vaccine).

Inactivated viral vaccines

- These are the vaccines in which the microorganisms are killed by various methods.
- They are safer than live attenuated vaccines.
- Examples are cholera vaccine, injectable polio vaccine, pertussis vaccine, etc.
- Inactivated vaccines are produced by the inactivation of virulent pathogens by chemical treatment or irradiation with gamma rays.
- They require multiple boosters.
- They are more stable than attenuated vaccines, and so they will not revert to virulent forms.
- These vaccines produce mainly humoral immunity.
- They may produce a general toxic effect in the organism, and sometimes induce allergies.

Toxoid vaccines

- Some viruses release toxins (poisonous proteins) when they attack the body.
- The immune system recognizes these toxins in the same way that it recognizes other antigens on the surface of viruses, and is able to mount an immune response to them.
- Some vaccines are made with inactivated version of these toxins - Toxoids.

- They look like toxins but are not poisonous.
- They triggers a strong immune response.
- ***Toxoid*** is an inactivated toxin whose toxicity has been suppressed either by chemical or heat treatment, while other properties typically immunogenicity are maintained.
- Toxins are secreted by viruses, whereas toxoids are altered form of toxins which are not secreted by bacteria.
- ***Toxoid vaccines*** - Vaccines made from a toxin (poison) that has been made harmless but that elicits immune response against toxin.
- Booster shots may be required
- Eg;- diphtheria, tetanus

mRNA vaccines

- These vaccines use proteins to trigger an immune response.
- It has many benefits
- 1. Shorter manufacturing time
- 2. Do not contain a live virus – no risk of causing disease
- Eg :- Moderna, Pfizer,..

Viral vector vaccines

- They use a modified version of a different virus as vector to deliver protection
- Eg:- Sputnik, AstraZeneca,…

Biosynthetic vaccines

- They contain manmade substances that are very similar to pieces of virus or bacteria
- Eg:- Hepatitis B

MODULE 6

INDUSTRIAL AND MEDICAL MICROBIOLOGY

INDUSTRIAL MICROBIOLOGY

Bioengineering of microorganisms for industrial purposes: Microbial production of industrial products (micro-organisms involved, media, fermentation conditions, downstream processing and uses)

Citric acid

- Commercially used in foods, soft drinks, certain pharmaceuticals and some other industries like dyes, ink, etc.
- It is produced by aerobic fermentation using fungi such as ***Aspergillus niger, Aspergillus wentii*** etc.
- Typical medium of citric acid fermentation contains molasses, ammonium nitrate, magnesium sulphate,etc.
- Temperature maintained between 25 and 30 C and pH between 5.6 - 6.0.
- Fermentation is aerobic.
- Citric acid is recovered as calcium citrate crystals.

Ethanol

- Ethanol (ethyl alcohol) is a clear, colorless, flammable liquid with a characteristic pleasant odour.
- Ethyl alcohol is the intoxicating component in beer, wine, and other alcoholic beverages.
- It is also widely used in industry as a solvent for dyes, oils, waxes, explosives, cosmetics, used as a disinfectant in commercial and household uses, an important solvent in laboratories, and also used as a fuel.
- ***Saccharomyces cerevisiae*** js the most preferred organism for the industrial produçtion of ethanol.
- Any material containing appreciable amounts of sugar or substances that can be converted to alcohol can be used for the industrial production of ethanol.

Wine

- Wine is a kind of undistilled alcoholic beverage mainly prepared from fruit juices.
- Here microorganisms like yeast and bacteria consume sugars, acids, amino acids, and other compounds under anaerobic conditions leading to the formation of a microbiologically safe, stable, and enjoyable beverage.
- Quality of wine products depends on maintaining proper microbiological control during the transformation of the grape juice into the finished wine.
- ***Oenology*** is the science that studies wine and wine making.

- The spontaneous wine fermentation is carried out by indigenous microbiota.
- Species of ***Metschnikowia, Candida, Hanseniaspora, Pichia*** and ***Saccharomyces*** are often present at the initial stages of wine fermentations.
- Wine production starts with the harvesting of appropriate variety of berries that contains a high amount of fermentable sugars.
- The fruits are crushed an extracted mechanically to yield a mass known as ***Must***.
- It is then inoculated with 10% of inoculum and fermentation is carried out under optimum temperature.
- Throughout the fermentation process contact with air must be restricted to prevent oxidation during fermentation.
- This fermentation is caused by enzymes produced by certain lactic acid bacteria which may add additional flavors to the wine.
- Removal of the suspended material may be required after fermentation by a process called ***clarification***.
- The major procedures involved are fining (adsorption of protein content and yeast cells), filtration, centrifugation, refrigeration, ion exchange, and heating.
- Many wines improve in quality during barrel and bottle storage by a process called aging.

Penicillin

- Penicillins are a group of antibiotics produced by molds in the *Penicillium* family.
- Their antibiotic activity was discovered in 1928 by Alexander Fleming
- It is a commonly used antibiotic for treating a number of bacterial infections, especially for infections caused by Streptococcus and other gram-positive bacteria
- Penicillin was the first important commercial product produced by anaerobic, submerged fermentation.
- The process industrial production of penicillin is carried out in batch fermenters with a fed batch fermentation process that increases the yield.
- The fermentation usually requires glucose, phenoxy acetic acid, additional nitrogen source . lactic acid, inorganic ions and growth factors as substratum.
- Fermentation is carried out at a temperature ranging between 25-27°C and pH 5.5 - 7.5.
- Downstream processing is relatively easy since penicillin is secreted into the medium.
- A purified end product is obtained by dissolving and precipitating as a potassium salt to separate it from other substances in the medium.
- The resulting penicillin can be chemically and enzymatically modified to make a variety of penicillins with slightly different properties.

Glutamic Acid

- Japanese researchers isolated the glutamic acid producing bacterium ***Corynebacterium glutamicum***.
- Glutamic acid is widely used in the production of monosodium glutamate (MSG) which is commonly known as the 'seasoning salt'.
- Monosodium glutamate is a condiment and flavor enhancing agent, it finds its greatest use as a common ingredient in convenient food stuffs.
- The raw materials used for fermentation include carbohydrates, peptone, inorganic salts, and biotin.
- α - ketoglutaric acid serves as the precursor of glutamic acid production.
- The conversion of the α - ketoglutaric acid to glutamic acid occurs in presence of enzyme glutamic acid dehydrogenase.
- Biotin concentration in the fermentation medium also has a significant influence on the yield of glutamic acid.
- The optimal temperature is 30 - 35°C and a high degree of aeration is necessary.
- Fermentation completes within 2 - 4 days and, at the end of the fermentation, the broth contains glutamic acid in the form of its ammonium salt.
- In a typical downstream process, the bacterial cells are separated and the broth is passed through a basic anion exchange resin.
- Glutamic acid anions get bound to the resin and ammonia is released.
- This ammonia can be recovered via distillation and reused in the fermentation.

Riboflavin

- Vitamins are organic compounds and are requisite to the normal growth of man and animals.
- Commonly known as vitamin B2
- It is the central source of all biologically important flavins, and is a yellow colored organic compound.
- Their deficiency may lead to an increased risk of cardiovascular disease, impairment of iron metabolism, and night blindness.
- The industrial strains used for production of riboflavin are mainly derived from the bacterium ***Bacillus subtilis*** and the fungus ***Ashbya gossypii***.
- Biosynthesis begins from fermentation of fatty acids and glycerol by the microbes.
- Fermentation of riboflavin for *A. gossypii* is performed at the optimum temperature range of 26 - 30°C in fed - batch fermenters.
- The initial pH of the culture medium is maintained between 6.5 – 7.5 and fermentation is continued in aerobic conditions for 6-8 days.

- Downstream processing begins with pasteurization of the broth to remove all viable cells in the final product.
- Due to low solubility of riboflavin in neutral aqueous solvents, they can be easily crystallized as needle - like crystals in a crystallizer by evaporation of some water.
- Subsequent washing of crystals with hot diluted acids (hydrochloric or sulfuric acid) and separation (via decantation, filtration, or centrifugation) followed by purification and drying (vacuum/spray drying) allows acquisition of a final product.

Enzymes

- Several commercially important enzymes are produced by microbes.
- Microbial system of enzyme production are preferred over the other chemical and biological systems of production due to many advantages like cost-effectiveness, ease in control, easier extraction as the products are directly released to the media.
- Availability of biotechnologically modified strains of microbes provides a higher yield.
- Amylase, protease, lipase, glucose isomerase, glucose oxidase, cellulases, etc. are commonly synthesized enzymes by the industrial process.

Amylase

- These are common hydrolysis enzymes that act by cleaving glycosidic bonds in starch molecules.
- Hence they are also called glycoside hydrolases
- They are widely used in the food industry for the production of corn syrups, maltose syrups, glucose syrups, juices, alcohol fermentation and baking.
- They are also used for making detergents, pharmaceuticals, and in the paper and textile industry.
- A wide range of bacterial species like *Bacillus, Corynebacterium gigantea, Chromohalobacter sp. Lactobacillus fermentum* and fungal species belonging to genus *Aspergillus* and *Penicillium* are used for the industrial production of amylases.
- Microbial strains isolated from starch or amylase rich environments naturally produce higher amounts of enzymes
- Amylase substrates are widely available from cheap plant sources
- Two kinds of fermentation processes have been followed***: submerged fermentation and solid state fermentation***.
- ***Submerged fermentation*** employ free flowing liquid substrates such as molasses and broths where the products are directly yielded into the medium.
- This method is suitable for microorganisms like bacteria that require high moisture content for their growth.
- In ***solid-state fermentation***, nutrient-rich materials like bran, paper pulp etc. can be used.

- This method requires less moisture content and microbes adapted for such conditions can be used here.
- Purification can be accomplished by ion-exchange chromatography, hydrophobic interaction chromatography, gel filtration, immune precipitation, polyethylene glycol / Sepharose gel separation.

Cellulase

- Cellulases are the enzymes that hydrolyze β -1,4 linkages in the biopolymer cellulose to release glucose units.
- They are produced by a large number of microorganisms including fungi, bacteria, protozoans, plants, and animals who are capable to thrive on cellulose rich medium.
- Microbial cellulases have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture.
- Microbes belonging to genera *Clostridium, Cellulomonas, Thermomonospora, Trichoderma, Aspergillus,* etc. are identified as potential producers of the enzyme.
- The commonly used substrates are willow, banana peel, coconut coir pith, paddy straw, fruit waste, groundnut shell, wheat straw, sugar beet, etc.
- Cellulase production is done either using ***solid-state fermentation*** mode or submerged fermentation for industrial production Pre treatment steps such as enzymatic, chemical, or thermal treatments for removal of lignin and pectin are performed mostly by biological, chemical, or physical means, to increase cellulase production.
- Downstream processing mainly involves extraction, purification, and recovery steps.
- Sonication, solid/liquid extraction, aphron flotation, diafiltration, etc. are some of the methods adopted for extraction.
- Recovery is done by any of the methods like the use of the polyethylene glycol (PEG)/salt system, affinity precipitation of cellulase, membrane fltration, etc

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Protease

- Proteases are a group of hydrolytic enzymes that catalyze the cleavage of peptide bonds in protein molecules.
- They have a vital role in many physiological processes like zymogen activation by proteolysis, blood coagulation, transport of secretory protein across membranes, tumor growth, protein catabolism, inflammation, cell growth, tissue arrangement, and morphogenesis in development.
- They are present in a wide range of sources such as animals, plants, and microorganisms.
- Proteases of microbial sources have found commercial applications in the detergents industry, tannery, leather industry, peptide synthesis, dairy processing, brewing, tenderization of meat, baking, and pharmaceutical industry.

- In the food industry, they are an invaluable tool for efficiently enhancing nutritional value, digestibility, palatability, flavor, and reducing allergenic compounds as well as in the management of domestic and industrial wastes.
- In industrial production, proteases can be isolated and purified in a relatively shorter period by using the fermentation process utilizing microbial species.
- Bacteria belonging to the genus, *Bacillus* are the most active and dynamic extracellular alkaline protease producer.
- Additionally, several fungi belonging to *Aspergillus, Penicillium, Rhizopus, Thermomyces*, etc. are also used for microbial production.
- Both solid substrate and submerged fermentation are used for the cost effective production of microbial proteases.
- Substrates like wheat bran, cow dung, agro industrial waste, groundnuts, etc. can be remarkable for the production.
- After the production of enzymes, purification of these enzymes is a very complex process.
- The uses of precipitation, ion exchange, gel filtration chromatography, ultrafiltration, etc. are some of the methods adopted for the isolation of the enzymes

Lipase

- Lipases are enzymes that catalyze the hydrolysis of long chain triglycerides.
- Liapases catalyze the hydrolysis of ester bonds in lipid substrates and play a vital role in digestion, transport and processing of dietary lipid substrate.
- They have been widely used in the detergent industry, dairy, food and beverage, animal feed, cleaning biofuel, pharmaceuticals, textile, cosmetic, perfumery, flavor industry, biocatalytic resolution, fine chemicals production, agrochemicals, biosensor, and bioremediation.
- Microorganisms like ***Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus,*** and ***Bacillus subtilis*** are the best sources or lipase enzymes.
- The microbial lipase is mostly extracellular and secreted into the production medium.
- Submerged fermentation and solid state are the fermentation methods commonly adopted.
- The substrates used are oily compounds rich in fatty acids like mustard seed oil, olive oil, palm oil, and other vegetable oils or combination of different oils.
- The extracellular microbial lipases from the culture broth are isolated through ammonium sulfate precipitation, ultrafiltration or extraction with organic solvents, and gel filtration.

Glucose Isomerase

- It is an enzyme that catalyzes the reversible isomerization of glucose to fructose and that of xylose to xylulose.
- Glucose isomerase forms an important enzyme used in the food industry for the production of fructose rich food products especially high fructose corn syrup a food sweetener.

- It also indirectly plays an important role in the ethanol fermentation of plant biomass hydrolysates in biofuel production.
- Hemicellulose, an abundant source of xylose in nature, is a cheap substrate for the production of glucose isomerase by microorganisms capable of growing on xylan - containing materials
- Bacterial genera ***Streptomyces, Bacillus, Clostridium, Anoxybacillus, Acidothermius*** and yeasts like ***Aspergillus*** are preferred organisms for microbial fermentation in industries.
- Fermentation is carried out at 30°C for a period of 24 to 48 hours.
- For isolation of glucose isomerase from the culture, the process of immobilization is adopted as it does not require rigorous purification and concentration processes.
- Adsorption to insoluble materials, entrapment into polymeric gels and encapsulation in membranes, cross linking with bi functional reagents, or covalent linking on to insoluble carriers are the common methods used for immobilizátion.

Glucose Oxidase

- lt is an enzyme that catalyzes the oxidation of β -D- glucose by molecular oxygen and produces gluconic acid and hydrogen peroxide.
- It is now utilized in various fields of the food industry, such as in baking products, dry egg powder, beverages, and gluconic acid production where it improves the color, flavor & shelf life of food products.
- Additionally, it is also used in bioelectronic devices, particularly biofuel cells, and biosensors.
- Glucose oxidase can be produced from various fungal sources, mainly of the ***Aspergilluus, Penicillium*** and ***Saccharomyces*** species, of which ***A. niger*** is the most commonly utilized one for industrial production.
- The culturing is carried out in a media containing glucose, sucrose, or molasses as the carbon source.
- The addition of calcium carbonate to the growth medium is found to increase the production.
- Oxygen for growth and production in fungal cultures is ensured by the aeration and agitation of the mycelial culture.
- The release of the enzyme from mycelium may be facilitated by mechanical and physical forces, e.g. agitation or sonication.
- Precipitation with chemicals like ammonium sulphate is used for purification followed by chromatographic separation techniques such as ion exchange chromatography for final extraction.

Bioinsecticides (Bt)

- These are insecticides derived from such natural materials as animals, plants, bacteria, and certain minerals obtained from biological agents.
- The pathogenicity of microbes against different pest species has been widely utilized in the synthesis of bioinsecticides.
- Many of the bacteria, viruses, and fungi are highly specific for particular pest species and have served as the fundamental agents for several integrated pest management programs.
- The bacterial agent ***Bacillus thuringiensis*** has been used for the control of lepidopterans
- ***Agrobacterium radiobacter*** for treating Crown Gall disease
- Viruses like ***Nuclear polyhedrosis virus*** for controlling hymenopterans and lepidopterans
- Fungi like ***Neozygites floridana*** for controlling cassava green mite
- ***Entomophaga praxibulli*** for controlling grasshoppers.

Advantages of using biopesticides

1. Biopesticides are usually inherently less toxic than conventional pesticides.
2. Biopesticides generally affect only the target pest and closely related organisms.
3. Biopesticides often are effective in very small quantities and often decompose quickly, resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides.
4. When used as a component of Integrated Pest Management (IPM) programs biopesticides can greatly reduce the use of conventional pesticides, while crop yields remain high.

- Currently, bioinsecticides constitute only up to 2-3% of the insecticide market share because of their narrow spectrum of toxicity and sensitive degradation by solar radiation.
- A great variety of commercial bioinsecticides are based on the entomopathogenic bacterium ***Bacillus thuringiensis (Bt).***
- It is a rod-shaped, gram-positive bacterium that forms spore and is found in the soil and on plants.
- This bacterium was discovered to have pesticidal properties if consumed by the larvae of specific insects.
- These insecticides represent more than 90% of the microbial insecticide market and are primarily applied in organic agriculture.
- Bt. insecticides rely on the activity of ***Cry proteins*** that disrupt the insect's midgut epithelium causing larval death.
- They do not affect adult insects.
- Bt. Insecticides may contain different Bt. strains expressing different Cry genes.
- Susceptible larvae that ingest the toxin are not killed immediately but die over the next few days.

Steroid biotransformation

- Biotransformations constitute an important methodology in organic chemistry where an organic compound is modified into a reversible product by biological agents.
- This involves simple, chemically defined reactions catalyzed by enzymes present in the cell.
- When the transformation is carried out by microorganisms then it is termed to be ***microbial transformation***.
- Microbiological transformations are an effective tool for the preparation of various compounds, which can be difficult to obtain by conventional chemical methods.
- This has been widely used in the bioconversion of steroids.
- Naturally occurring steroids possess remarkable hormonal properties which are of immense therapeutic importance to the wellbeing of human beings.
- The microbiological transformations of steroids have been an essential chemical tool used for the preparation of many intermediaries and in the generation of new drugs.
- The type of chemical reaction in transformations includes oxidation, reduction, hydrolysis, isomerization, and other actions like amination esterification, etc.
- Currently, there is a wide variety of steroids used as diuretics, anabolic, anti- inflammatory, anti androgenic, anti contraceptive, antitumor agents.
- Several fungi of the genera ***Rhizopus, Aspergillus, Curvularia, Cunninghamella,*** and ***Streptomyces*** with high yields are important chemical transformators in many synthesis schemes of new steroids with important biological activity.

MEDICAL MICROBIOLOGY

Normal microflora of the human body: skin, throat, gastrointestinal tract and urogenital tract. Diseases caused by: (with reference to causative agent, symptoms and mode of transmission).

a) Bacteria: anthrax, tuberculosis, typhoid, whooping cough, pneumonia, cholera, gonorrhea, and syphilis.

Sl. No.	Disease	Pathogen	Symptoms	Mode of infection
1	Anthrax	*Bacillus anthracis*	• Ulceration of skin • Head ache • Nausea • Vomiting	• Direct contact with infected animals • Through wound • Inhaling of endospores.
2	Tuberculosis	*Mycobacterium tuberculosis*	• Slowly progressive disease affecting the lungs & other organs. • Spread of the disease is chronic, involving destruction of lung tissues.	• Inhalation of air carrying particle. • Spray from sputum of infected individuals • Milk containing *M. bovis* from bovine infection
3	Typhoid	*Salmonella typhi*	• Fever • Head ache • Rose spots on abdomen • Diarrhea • Intestinal perforation • haemorrhage	• Through contaminated food & water
4	Whooping cough	*Bordetella pertusis*	• Produces an exotoxin which damages host cells • Spasmodic cough	• Droplets produced during coughing or sneezing.
5	Pneumonia	*Streptococcus pneumonia*	• Affects respiratory tracts & lungs causing inflammation.	• Inhalation of virus • Air borne droplet infection • Through blood

6	Cholera	*Vibrio cholera*	• Nausea • Vomiting • Diarrhea • Abdominal pain	• Contaminated food & water
7	Gonorrhea	*Neisseria gonorrhoeae*	• Painful symptoms in male • Mild symptoms in female	• Sexually transmission
8	Syphilis	*Treponema pallidum*	• Form lesions • Skin rashes	• Sexually transmission

b) Viruses: polio, chicken pox, herpes, hepatitis, rabies, dengue, AIDS and chikungunya.

Sl. No.	Disease	Pathogen	Symptoms	Mode of infection
1	Polio	*Human polio virus*	• Fever • Sore throat like ordinary flu • Headache • Fever • Vomiting • Stiffness in the neck • Paralysis	• Contaminated water
2	Chicken pox	*Varicella zoster virus*	• Itchy rash with small fluid filled blisters on the skin • Lesions may form in the throat, eyes, mucus membranes,.. • Fever • Loss of appetite • Head ache • Tiredness	• Spread 48 hours before the rash appears & the virus remains contagious until all broken blisters have crusted over.
3	Herpes	*Herpes simplex virus*	• Painful blisters or ulcers at the site of infection	• Oral oral contact • Oral genital contact • Sexual contact
4	Hepatitis	*Hepatitis virus*	• Jaundice • Dark urine • Extreme fatigue • Nausea • Vomiting • Abdominal pain	• Contaminated food & water • Physical contact • Sexual contact

5	Rabies	*Rabies virus*	• Fever with pain • Unusual tingling • Burning sensation • Cerebral dysfunction • Anxiety • Confusion • Hyper activity • Excitable behaviour • Hydrophobia • Aerophobia	• Bites or scratches of a rabid animal through contaminated saliva
6	Dengue	*Dengue virus*	• Head ache • Pain behind the eyes • Muscle & joint pains • Nausea • Vomiting • Swollen glands • Rashes • Rapid breathing • Fatigue • restlessness	• Spread by mosquitoes, mainly *Aedes aegypti* • Maternal transmission
7	AIDS	HIV	• Influenza • Immunosuppression • Lung disease • Skin tumours • Fungal infections • Diarrhea,	• Sexual intercourse • Transfusion of contaminated blood. • Parental transmission from infected mother to baby through transplacental circulation and breast-feeding. • Sharing of hypodermic injection needles. • Organ transplantation and artificial insemination. • Contact of the mucous membrane or bloodstream of a normal person with an HIV-containing bodily fluid, such as blood, semen, vaginal fluid, seminal fluid, or breast milk, from an infected person.
8	Chikungunya	*Chikungunya virus*	• Fever • Joint pain • Muscle pain	• Mosquito bite (*Aedes aegypti & A. albopictus*)

			• Joint swelling • Head ache • Nausea • Fatigue • Rash	

c) Protozoa: malaria, kala-azar and toxoplasmosis

Sl. No.	Disease	Pathogen	Symptoms	Mode of infection
1	Malaria	*Plasmodium*	• fever and chills. • Anaemia	• Mosquito bite (female *Anopheles*)
2	Kala azar	*Leishmania donovani*	• Fever • Loss of appetite • Weight loss • Enlargement of spleen & liver • Dry & scaly skin • Anaemia • Discolouration of skin	• Bites of sandflies
3	Toxoplasmosis	*Toxoplasma gondii*	• Flu like symptoms	• Eating undercooked meat of animals harbouring cysts • Contaminated food & water • Blood transfusion • Organ transplantation • Transplacental transmission

d) Fungi: dermatomycoses and opportunistic mycoses

Sl. No.	Disease	Pathogen	Symptoms	Mode of infection
1	Dermatomycoses	*Microsporum, Trichophyton*	• Scaling • Redness • Discoloration of skin • Thickening & discoloring of nails	• Direct or indirect contact with infected animals
2	Opportunistic mycoses	*Candida, Aspergillus, Mucur*	• Redness • Itching • Discomfort	• Direct or indirect contact

Bacterial drug resistance

- Antibiotics are medicines used to prevent and treat bacterial infections.
- The discovery of antibiotics had made a great advancement in the field of medicine and treatment of diseases.
- Evolution of microbial drug resistance has become a great hindrance in the treatment of diseases using drug therapy.
- Antibiotic resistance happens when germs like bacteria and fungi develop the ability to defeat the drugs designed to kill them.
- Bacterial resistance can be either innate or acquired.
- Antimicrobial drug resistance can be due to their spontaneous mutation which alters existing genes or leads to the acquisition of newer ones.
- The occurrence of such mutations leading to drug resistance is accelerated by the misuse and overuse of antibiotics, as well as poor infection prevention and control.

MODULE 7

CELLS & ORGANS OF IMMUNE SYSTEM

Introduction

Immunity

Immunity is the resistance of the body against antigens & their biological products.

Immunity is classified into two major classes

1. Innate (Natural) immunity
2. Aquired immunity

Innate (Natural) immunity

- Inborn or naturally inherited.
- Native immunity or natural immunity
- Non specific immunity
- Provides immediate defence against infection

Mechanism of innate immunity

1. Physical & anatomical barrier

- Skin & respiratory mucosa
- Prevents the entry of pathogens into the body
- First line defence
- Skin serves as mechanical & chemical barrier
- Secretions of sweat glands & sebaceous glands make the skin acidic & unfavourable for invading bacteria.
- Lysozyme in tear & sweat, destroys bacteria.
- Acids & other metabolic wastes by resident bacteria inhibits the growth & multiplication of invading pathogens.
- During exfoliation infectious agents landed on the skin are also removed.
- Cilia & mucus in the respiratory tract trap & ingest the bacteria present in the inhaled air.

2. Chemical barrier

- Saliva & gastric juice
- Ingested pathogens are killed by salivary lysozyme & gastric HCL.

3. Physiological barrier

- Temperature, pH, saliva, gastric juice, lysozyme, interferon, complement proteins,..
- Fever responses inhibit the growth of pathogens.
- Acidity kills the ingested microorganisms

4. Phagocytic barrier

- Second line defence
- Major phagocytic cells – neutrophils, monocytes & tissue macrophages
- Ingest & digest invading antigens & microbes

5. Inflammatory barrier

- Inflammation – tissue response to injury or infection
- Clinical symptoms – localized fluid accumulation, swelling, redness, heat & pain
- Physiological changes – histamine & pyrogen production, dilation & increased permeability of blood vessels

Aquired immunity

- Adapted immunity
- Specific immunity
- Developed by an individual during life time
- Develops in response to artificial or natural stimulation
- Mediated by specific antibodies or sensitized lymphocytes
- Capable of recognizing & selectively eliminating specific microbes, antigens, toxins,..
- Individual specific

Four characteristic attributes

1) ***Diversity*** – ability to recognize a variety of foreign molecules
2) ***Specificity*** – ability to discriminate different kinds of foreign molecules
3) ***Self non-self recognition***
4) ***Immunological memory*** – retain the information about the foreign molecules of previous encounters

Active & passive

Kinds of acquired immunity

- Two types –
  Active immunity
  Passive immunity

Acquired active immunity

- Elicited against natural or artificial antigenic stimulation
- Two kinds – Antibody mediated – humoral immunity
  Cell mediated – cellular immunity
- Both involves immunological memory
- May be natural or artificial

a) Naturally acquired active immunity

- Developed against by the natural infection by bacteria, viruses,..
- Life-time immunity – small pox, measles, mumps
- Short living immunity – bacterial dysentery

b) Artificially acquired active immunity

- Developed against the antigens introduced artificially by vaccination
- Vaccines – preparations of live attenuated or killed micro organisms or their products (toxoids)

Aquired passive immunity

- Ready made resistance, passively transferred to a recipient
- Transferred from an immune individual to a non immune individual
- Two types – natural & artificial

Advantages

- No antigenic stimulation
- No antibody production
- No latent period

Disadvantages

- Immunity developed is temporary
- No memory cell formation
- Decreases with repeated administration

a) Naturally acquired passive immunity

- Acquired through natural methods
- Transfer of immunity from mother to foetus before the immune system of the foetus becomes functional
- Occurs through transplacental transfer of antibodies
- Enables the foetus to defend against infections until it is able to produce antibodies
- IgG , IgA ,.. obtain from mother body

b) Artificially acquired passive immunity

- Aquire through artificial methods
- Transfer from immunized donor to a non immune recipient by transferring antibodies or immunized lymphocytes
- Administration of specific antibodies or sera which contain specific antibodies
- Used therapeutically for the treatment of tetanus, diphtheria, snake bite,..
- Covalescent serum – serum collected from the patients recovering from a particular infectious disease, which contain high amount of antibodies
- Combined immunization – combination of active & passive methods of immunization.

Immunization

- The process of producing a state of immunity in an individual
- Active immunization - vaccination
- Passive immunization – acquisition of immunity by receipt of preformed antibodies rather than by active production of antibodies after exposure to antigen.

Vaccines principles of vaccination

- The aims of vaccination are to induce memory in T & B lymphocytes through the injection of a non virulent antigen preparation

Attenuated bacterial or viral vaccines

- These are the preparations of organisms, weakened by various methods, including genetic alterations.
- They produce only a mild, sub-clinical infection which can provide strong resistance and protection.
- These vaccines are not as safe as inactivated vaccines, because their reversion to virulent state is possible and so they can cause diseases in immunologically weak individuals.
- Oral polio vaccine, mumps vaccine, measles vaccine, whooping cough vaccine, etc. are live attenuated vaccines.
- Attenuation is the process of reducing the virulence of a pathogen.
- Attenuated vaccines induce both humoral and cell-mediated immunity.
- Many of these vaccines require a booster dose for effective disease prevention.
- Booster dose is a dose of antigen given after the primary dose to stimulate accelerated production of large amounts of antibody (e.g., polio vaccine).

Inactivated viral or bacterial vaccines

- These are the vaccines in which the microorganisms are killed by various methods.
- They are safer than live attenuated vaccines.
- Examples are cholera vaccine, injectable polio vaccine, pertussis vaccine, etc.
- Inactivated vaccines are produced by the inactivation of virulent pathogens by chemical treatment or irradiation with gamma rays.
- They require multiple boosters.
- They are more stable than attenuated vaccines, and so they will not revert to virulent forms.
- These vaccines produce mainly humoral immunity.
- They may produce a general toxic effect in the organism, and sometimes induce allergies.

Toxoid vaccines

- Some bacteria release toxins (poisonous proteins) when they attack the body.
- The immune system recognizes these toxins in the same way that it recognizes other antigens on the surface of bacteria, and is able to mount an immune response to them.
- Some vaccines are made with inactivated version of these toxins - Toxoids.
- They look like toxins but are not poisonous.
- They triggers a strong immune response.
- ***Toxoid*** is an inactivated toxin whose toxicity has been suppressed either by chemical or heat treatment, while other properties typically immunogenecity are maintained.
- Toxins are secreted by bacteria, whereas toxoids are altered form of toxins which are not secreted by bacteria.
- ***Toxoid vaccines*** - Vaccines made from a toxin (poison) that has been made harmless but that elicits immune response against toxin.
- These vaccines are used when a bacterial toxin is the main cause of illness.
- Eg :- Crotalus atrox toxoid is used to vaccinate dogs against rattle snake bite.
- Toxoid vaccines are used to protect against
  1. Diphtheria
  2. Tetanus
- A Toxin is both toxic and immunogenic.
- A Toxoid is no longer toxic but immunogenic as the toxin from which it was derived.
- Some bacterial pathogens produces Exotoxins.
- Vaccines can be produced by purifying these exotoxins with the help of chemicals like formaldehyde to form a Toxoid.
- Toxoid vaccines induce anti-toxoid antibodies, which are also capable of binding to the toxin and neutralizing its effects.
- Eg:- diphtheria vaccines and tetanus vaccines.

Advantages

- Toxoid vaccines are safe because they cannot cause the disease.
- They prevent and there is no possibility of reversion to virulence.
- They are stable as they are less susceptible to change in humidity and light.

Disadvantages

- Toxoid vaccines tend not to be highly immunogenic unless large amounts or multiple doses are used.
- One problem with using larger doses is that tolerance can be induced to the antigen

Cells of the immune system

- Five major groups
- Lymphocytes, antigen presenting cells, granulocytes, agranulocytes & mast cells
- Lymphocytes play the key role in the control & co-ordination
  Recognition of infected or heterologous cells

B – lymphocytes

- B cell
- Uninucleate & non granular
- Produce antibodies – responsible for humoral immunity
- Having a large nucleus & a ring of marginal cytoplasm.
- Found in blood, lymph, lymph nodes & spleen.
- ***Surface markers*** – unique proteins or antibodies found in B cell surface membrane, serve as receptors for antigens.
- Derived from haematopoetic stem cells of red marrow
- Migrate to lymph nodes & spleens for their action.
- An antigenic stimulation occurs, B cells divides to plasma cells & memory cells.
- ***Plasma cells*** – secrete antibodies – bring about primary immune responses – short life.
- ***Memory cells*** – store information – bring about secondary immune responses - long life.

Plasma cells

- Found in lymph nodes & spleen.
- Rarely seen in blood plasma.
- Eccentrically placed nucleus & sparse cytoplasm
- Antibody synthesizing machinery is well developed – densely packed endoplasmic reticulum & ribosomes.
- Devoid of surface receptors & surface immunoglobulins.

- Russel's bodies – immunoglobulins are localised in the spaces of the endoplasmic reticulum.

T – lymphocytes

- Uninucleate & non granular.
- Bring about cell mediated immunity.
- Origin in bone marrow & maturation in thymus
- Have membrane receptors for antigens
- Four major sub populations of T cells are

1) ***Helper T cells***
   - Activated by small quantities of antigens
   - Activated T_H cells secrete lymphokines – increases the response of B & T cells.
   - MMIF – Macrophage Migration Inhibition Factor – lymphokine which causes the accumulation of macrophages & activates phagocytosis.
2) ***Suppressor T cells***
   - Suppress the activity of B cells & other T cells.
   - Inhibit antibody production.
   - Responsible for immune tolerance – limiting the ability of immune system to attack own boy tissues.
3) ***Cytotoxic T cells***
   - Killer T cells
   - Kills microbes or body's own tissues.
   - After the binding of receptor proteins with antigens causes the swelling of TC cells – release cytotoxic substances into the target cells – lysosomal enzymes.
   - Distructive to virus infected cells & tissues.
   - Play an important role in destroying cancer cells & allografts.
4) ***Delayed type hypersensitivity T cells***
   - Bring macrophages to areas where delayed hypersensitivity reactions occur.
   - Secretes macrophage chemotoxin & MMIF.

NK cells

- ***Natural killer cells – null cells***
- Have no surface markers
- Large granular lymphocytes
- Display cytotoxic activity against tumour cells
- Important role in host defence against tumour cells & the cells infected with viruses.

Monocytes

- Largest type of leukocyte
- Monocytes circulate in the blood stream for about 8 hrs
- They enlarge and migrate into tissue spaces to differentiate into macrophages & myeloid lineage dendritic cells
- Process adaptive immunity

Macrophages

- Large mononuclear phagocytic leucocytes.
- Derived from monocytes.
- Crowded in lymphoid organs.
- Immobile at rest.
- Actively mobile when stimulated by lymphokines.
- ***Kupffer cells*** – found in liver
- ***Dust cells*** – lungs
- ***Histiocytes*** – connective tissues
- ***Osteoclasts*** – bones
- ***Microglia*** – nervous system
- ***Mesangial*** cells - kidney

Neutrophils

- They have a multilobed nuclei and granulated cytoplasm
- It is often called as polymorphonuclear cells or leukocyte (PMNs).
- After their production in the bone marrow, they are released into peripheral blood, where they circulate 7-10 hrs before they migrate into the tissue where they have 3 day life span.
- Neutrophils are the first cells to arrive at the site of inflammation.
- They are capable of entering the tissue spaces or the site of infection by penetrating through the wall of the blood vessel and the phenomenon is called extravasation.
- This process involves the following steps.
  Neutrophils first adhere to the endothelial wall of the blood vessel.
- Penetrate the wall of the blood vessel through the gap between adjacent endothelial cell linings.
- Penetrate the vascular membrane, moving out into the tissue spaces.
- Several chemotactic factors that is responsible for the accumulation of neutrophils at the site of inflammation.
- The chemotactic factors such as IL-1, IL-8, and transforming growth factor B, are the cytokines secreted by activated macrophages, and several cytokines secreted by activated T_H, cells are all responsible for neutrophil infiltration into infected area.

- Neutrophils also show phagocytosis, they contain lytic enzymes and bactericidal substances in the primary and secondary granules.
- The primary granules are large, denser, contain lytic enzymes, peroxidase and hydrolytic enzymes.
- Neutrophils have receptor for Fc region of lgE antübody, so they show antibody mediated cytotoxic reaction against the parasites.

Basophils

- Non phagocytic granulocytes
- Responsible for release of pharmacologically active mediators found in their cytoplasmic granules.
- It play major role in Type I hypersensitive reaction.
- Antigen sensitized IgE bind to basophil through their Fc receptor and stimulate the release of pharmacological mediators.
- The main mediators are histamine, Heparin, ECF, NCF and cytokines (IL-8, IL-5)
- The principal effects are vasodilatation, increase in vascular permeability, increase in secretions and smooth muscle contraction.
- When large number of mast cells and basophils are stimulated to degranulate, severe anaphylactic responses can occur, which in their mildest form give rise to the allergic symptoms.

Eosinophils

- They are also mobile phagocytes showing extravasation from the blood vessels to tissue spaces.
- The major role is against parasitic infections.
- They are known to secrete basic proteins, sulfotransferases and sulfatases, which are stored in the granules.
- They also have Fc receptor for IgE and show antibody dependent cell mediated cytotoxic reaction against parasites.
- They respond to eosinophil chemotactic factor (ECF) secreted by mast cells sensitized against parasites.

Mast cells

- They are found in connective tissues throughout the body, close to blood vessels and particularly in the sub - epithelial areas of the respiratory, urinogenital and gastrointestinal tracts.
- They also store pharmacologically active mediators in their cytoplasm as granules and release these mediators by degranulation when an antigen specific IgE binds to the Fc receptor on the membrane of mast cells.

- Mediators, activators and biological effects are the same as that of basophils.
- Like basophils they take part in allergic reactions.

Dendritic cells

- Antigen Presenting Cell (APC)
- Very active in stimulating T & B cells.
- Essential for primary immune response.
- Process antigen & present it on the surface of other immune cells.
- Present in skin & the internal lining of nose, lungs, stomach & intestine.
- Found in blood as immature state.
- Once activated, migrate to lymph nodes & spleen

Organs of the immune system

- Immune system is formed of different tissues & organs.
- Immune organs can be categorized into two main groups, namely *primary lymphoid organs & secondary lymphoid organs*

Primary lymphoid organs

Thymus

- Bilobed gland.
- Located above heart & behind the top of the sternum.
- Centre of the chemical modification & functional maturation of T cells.
- Thymic hormones govern T cell maturation .
- It progressively grows in size, reaches maximum size at puberty, then gradually shrinks.
- Two parts – cortex & medulla.
- Cortex – outer part – contains immature T lymphocytes & reticular cells.
- Medulla – inner part – consists of vascular tissue, reticular epithelial cells & scattered lymphocytes.
- T cells are non functional inside thymus.

Bone marrow

- Soft tissues within the cavities of bones.
- Origin of B & T cells & maturation of B cells.
- Divisible into two regions – vascular adipose region & the haemopoietic region.
- Vascular adipose region – contains blood vessels – supplies nutrients & oxygen – removes wastes.

- Haemopoietic region – haemopoiesis – red marrow – contains multipotent stem cells.
- Major site of antibody synthesis.

Secondary lymphoid organs

Spleen

- Large, ovoid, located in left abdominal cavity below pancrease.
- Specialized for filtering blood & trapping blood borne antigens.
- Concerned with destruction of old, dead & worn out RBC's.
- Reserve site for haematopoiesis

Lymphnodes

- Small, capsulated, solid, rounded or bean shaped bodies.
- Located on lymphatic vessels.
- Numerous in arm pits, groins, mesenteries,..
- Filter out foreign material from lymph.

MODULE 8

ANTIGENS, ANTIBODIES, IMMUNITY AND MHC

ANTIGENS

Types

Exogenous antigens

- These antigens enters the body or system and start circulating in the body fluids and trapped by the APCs (Antigen processing cells such as macrophages, dendritic cells, etc.)
- The uptakes of these exogenous antigens by APCs are mainly mediated by thephagocytosis
- Examples: bacteria, viruses, fungi etc.
- Some antigens start out as exogenous, and later become endogenous (e.g., intracellular viruses)

Endogenous antigens

- These are body's own cells or sub fragments or compounds or the antigenic products that are produced.
- The endogenous antigens are processed by the macrophages which are later accepted by the cytotoxic T- cells.
- Endogenous antigens include xenogenic (heterologous), autologous and idiotypic
- These antigens should not be under normal conditions, the target of the immune system, but, due mainly to genetic and environmental factors, the normal immunological tolerance for such an antigen has been lost in these patients.
- E.g.,Nucleoproteins, Nucleic acids,…

Complete Antigen or Immunogen

- Posses antigenic properties denovo, i.e. they are able to generate an immune response by themselves.
- High molecular weight (more than 10,000)
- May be proteins or polysaccharides

Incomplete Antigen or Hapten

- These are the foreign substance, usually non-protein substances
- Unable to induce an immune response by itself, they require carrier molecule to act as a complete antigen.
- The carrier molecule is a non-antigenic component and helps in provoking the immune response.
- Example: Serum Protein such as Albumin or Globulin.
- Low molecular weight

Factors for immunogenicity

1. ***Nature of immunogen***
    - **Foreigness**
      Molecule must be recognized as non self by the biological system
    - **Molecular size**
      Active immunogens – 100000 Da.
      Poor immunogens – 5000 – 10000 Da.
    - **Chemical composition & heterogeneity**
      Chemical complexity contributes to immunogenicity
    - **Lipids as antigens**
      Appropriately presented lipoidal antigens can induce B cell & T cell responses.
    - **Susceptibility to antigen processing & presentation**
      Larger molecules are more readily phagocytosed & processed.
2. ***Biological systems***
    - Genotype of recipient animal
    - Immunogen dosage & route of administration
    - Adjuvants

Adjuvants

- Immunologic adjuvants are substances added to vaccines to stimulate the immune system's response to the target antigen.
- Aluminum salts are common adjuvants in vaccines sold in the United States and have been used in vaccines for over 70 years.
- ***Freund's adjuvant*** is a solution of antigen emulsified in mineral oil and used as an immunopotentiator (booster).
- The *complete form* is composed of inactivated and dried inactivatedMycobacteria (usually *M. tuberculosis*).
- *Incomplete form* lacks the mycobacterial components (hence just the water in oil emulsion). It is named after Jules T. Freund.
- Freund's complete adjuvant is effective in stimulating cell-mediated immunity
- Its use in humans is forbidden by regulatory authorities, due to its toxicity.
- Even for animal research there are currently guidelines associated with its use, due to its painful reaction and potential for tissue damage.
- Injections of CFA should be subcutaneous or intraperitoneal, because intraderrmal injections may cause skin ulceration and necrosis; intramuscular injections may lead to temporary or permanent muscle lesion, and intravenous injections may produce pulmonary lipid embolism.

Haptens

- Low molecular weight compounds.
- Antigenic not immunogenic.
- ***Hapten – carrier conjugate*** – chemical coupling of hapten with a carrier protein results in hapten – carrier complex.
  Serve as immunogen.
- A hapten can combine with an antibody, but cannot induce antibody formation & immune response.
- Some substances act as haptens in some animals, where as they act complete antigens.
- Eg:- Pneumococcal polysaccharide
  Antigen in man
  Hapten in rabbits

Epitopes

- ***Epitopes*** – immunologically active regions of an immunogen that bind to antigen specific membrane receptors on secreted antibodies.
- B cells recognize soluble antigens when it binds to membrane bound antibodies.
- They bind antigen that is free in solution.
- B cells recognize folded amino acid sequences.
- T cells recognize only peptides combined with MHC molecules on the surface of APC's & altered self cells.
- T cell recognizes linear amino acid sequences.

Antigen – antibody reaction

- Bimolecular association similar to enzyme – substrate interaction.
- Interaction between the chemical groups on the surface of the epitope & paratope.

Precipitation reaction

- These are the precipitate-forming antigen-antibody interactions.
- The reaction between soluble antigens and antibodies results in precipitate formation.
- Antibodies that are capable of precipitate formation on reacting with antigens are called ***precipitins***.
- The antibody must be bivalent, i.e, with more than one Fab site for reacting with antigen,
- The antigen should also be bivalent or polyvalent, with two or more similar or dissimilar epitopes for reacting with antibodies.

- Precipitate formation occurs when antigen and antibody are present in equal concentration.

Agglutination reaction

- These are the antigen-antibody reactions.
- Clumping or agglutination of antigen molecules.
- The antibodies which can bring about the agglutination of antigen molecules are called ***agglutinins***.
- Occurs when antigens and antibodies exist in equal concentrations.
- The inhibition of the agglutination of antigens by high concentrations of antibody is called ***prozone effect***.
- The antibodies which cannot agglutinate antigen molecules are sometimes called ***incomplete antibodies.***
- When bacterial antigens are agglutinated, the reaction is called ***bacterial agglutination*** - diagnose bacterial infections.
- The agglutination of red blood cells is termed ***haemagglutination***.

Agglutination – inhibition reaction

- A modification of the agglutination reaction, called ***agglutination inhibition***.
- Provides a highly sensitive assay for small quantities of an antigen
- Agglutination inhibition assays can be used to determine if an individual is using certain types of illegal drugs such as cocaine or heroin.
- A urine or blood sample is first incubated with antibody specific for the suspected drug.
- Then red blood cells (or other particles) coated with the drug are added.
- If the red blood cells are not agglutinated by the antibody, it indicates the sample contained an antigen recognized by the antibody, suggesting that individual was using the illicit drug.
- Agglutination inhibition assays are widely used in clinical laboratories to determine whether an individual has been exposed to certain types of viruses that cause agglutination of red blood cells.
- If an individual's serum contains specific antiviral antibodies, then the antibodies will bind to the virus and interfere with hemagglutination by virus.
- This technique is used in premarital testing to determine the immune status of women with respect to rubella virus.

IMMUNOGLOBULINS

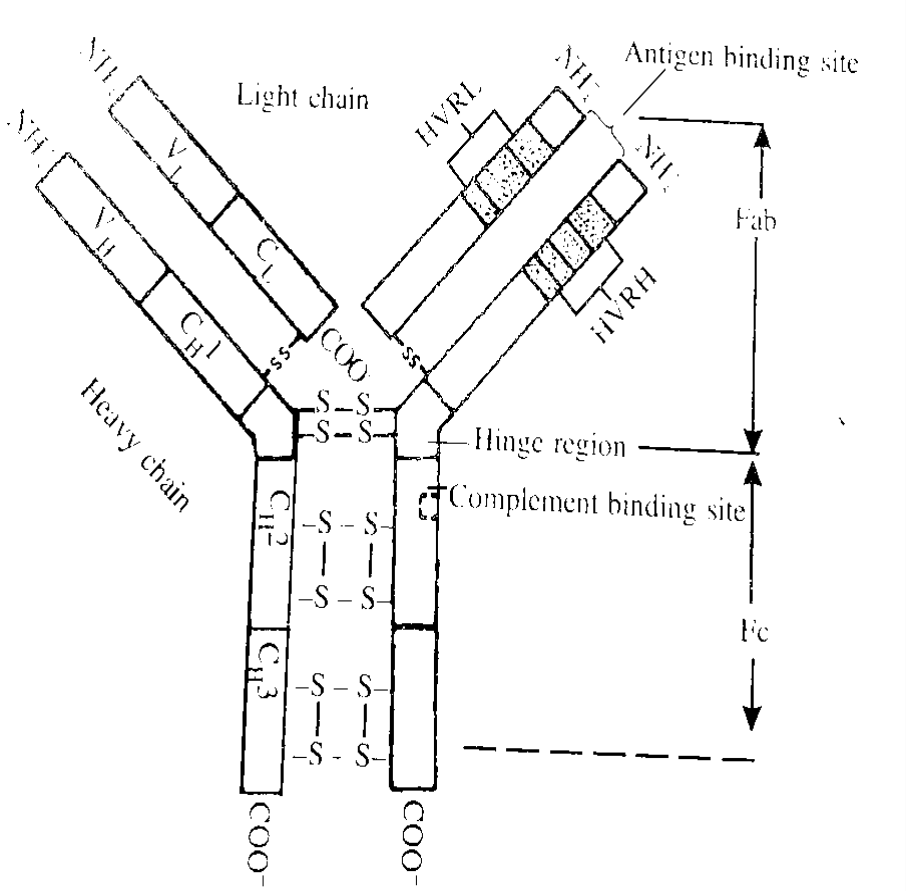
- Synthesized by plasma cells
- Present on B lymphocyte membrane
- Responsible for humoral immunity
- Recognize & inactivate specific antigens
- Immunoglobulin – represents the resting or non reacting state.
- Antibody – represents the active or reacting state.

Structure

- Y – shaped
- Symmetrical & tetrameric glycoprotein.
- It has a tail & two arms
- Arm has specific antigen binding properties – Antigen Binding Fragment (Fab).
- Tail – crystallizable – crystallizable fragment (Fc).
- Antibody consist of four polypeptide chains – two heavy chains & two light chains.
- Heavy chains are longer than light chains
- Each chain has two terminals :
  1. Amino terminal – N
  2. Carboxyl terminal –C
- These chains are held together by disulphide bond, salt linkages, hydrogen bonds & hydrophobic interactions.
- Each chain has two distinct regions – variable & constant region.
- Variable region of H chain – V_H
- Variable region of L chain – V_L
- Constant region of H chain – C_H
- Constant region of L chain - C_L
- Amino acid sequence of V region is highly variable, different in different antibodies.
- Amino acid sequence of C region is almost same.
- C region of H chain has three domains – C_H1, C_H2 & C_H3.
- Hinge region – between C_H1 & C_H2, contains 12 amino acids – proline rich – gives flexibility for the movement of Fab.
- Two types of light chains – λ & κ.
- Five types of heavy chains - Gamma, alpha, mu, delta, epsilon.

Based on function five kinds of immunoglobulins –

1. Neutralins – anti toxins
2. Agglutinins – clumping antigens
3. Precipitins – precipitate antigens
4. Opsonins – inactivate antigens by opsonization
5. Lysins – disintegrate antigens.



Different classes & functions

Ig class	Heavy chain	No. of paratopes	Major functions	Location
IgG	Gamma (γ)	2	Activation of complement system & macrophages, agglutination serves as anti-toxin	Blood, tissue fluid Can cross placenta
IgA	Alpha (α)	2 or 1	Prevents bacterial growth & multiplication on mucosa, inhibits bacterial adhesion to host cells	Saliva, intestinal secretions, urino genital secretions, bronchial mucus, tear, milk,..
IgM	Mu (μ)	10	Activation of complement system, agglutination of RBC's, neutralization of viral infections	Blood, tissue fluid
IgD	Delta (δ)	2	Not definitely known	Lining of thoracic & abdominal tissues
IgE	Epsilon (ε)	2	Activation of mast cells to release histamine, defence against allergens & parasitic worms	Tissues

Mention immunoglobulin gene families – κ and λ light chain families and the heavy chain family.

- The light and heavy chain of the immunoglobulin consists of multigenes which contribute towards the diversity.
- The λ (lambda) and κ (kappa) light chains and the heavy chains are encoded by separate multigene families situated on different chromosomes.
- Each of these multigene families contains several coding sequences, called gene segments separated by non coding regions.
- During B - cell maturation, these gene segments are rearranged and brought together to form functional immunoglobulin genes.
- The λ & κ light-chain families contain V, J, and C gene segments
- The rearranged VJ segments encode the variable region of the light chains.
- The heavy chain family contains V, D, J, and C gene segments
- The rearranged VDJ gene segments encode the variable region of the heavy chain.
- In each gene family, C gene segments encodes the constant regions.

λ chain multigene family

- Encoded by two gene segments
- Functional λ variable region gene contains two coding segments - a 5'V segment and a 3'J segment which are separated by a noncoding DNA sequence in unrearranged germline DNA.
- The multigene family in the mouse germ line contains three VL gene segments, four JL gene segments, and four CL gene segments.
- In humans, the lambda locus is more complex. There are 31 functional VL gene segments, 4 JL segments, and 7 CL segments.
- In additional to the functional gene segments, the human lambda complex contains many VL, JL, and CL pseudogenes.

κ -chain multigene family

- The κ - chain multigene family in the mouse contains approximately 85 V κ gene segments
- There are five J κ gene segments and a single C κ gene segment
- As in the λ multigene family, the V κ and J κ gene segments encode the variable region of the κ light chain, and the C gene segment encodes the constant region.
- The κ chain multigene family in humans, which has an organization similar to that of the mouse, contains approximately 40 V κ gene segments, 5 J κ Segments, and a single C κ segment.

Heavy chain multigene family

- The organization of the immunoglobulin heavy chain genes is similar to, but more complex than, that of the λ and κ light chain genes.
- An additional gene segment encodes part of the heavy chain variable region.
- The VH gene segment was found to encode amino acids l to 94 and the JH gene segment was found to encode amino acids 98 to 113
- Neither of these gene segments carried the information to encode amino acids 95 to 97.
- When the nucleotide sequence was determined for a rearranged myeloma DNA and compared with the germ line DNA sequence, an additional nucleotide sequence was observed between the VH and JH gene segments.
- This nucleotide sequence corresponded to amino acids 95 to 97 of the heavy chain. This segment is called DH segment.
- The heavy chain multigene family on human chromosome 14 has been shown by direct sequencing of DNA to contain 51 VH gene segments located upstream from a cluster of 27 functional DH gene segments.
- As with the light chain genes, each VH gene segment is preceded by a leader sequence a short distance upstream.

- Downstream from the DH gene segments are six functional JH gene segments, followed by a series of CH gene segments.
- Each CH gene segment encodes the constant region of an immunoglobulin heavy chain isotype.
- The CH gene segments consist of coding exons and non coding introns.
- Each exon encodes a separate domain of the heavy chain constant region.
- The conservation of important biological effector functions of the antibody molecule is maintained by the limited number of heavy chain constant region genes.

IMMUNITY

Types of Immunity

Humoral response

- This is the ***antibody-dependent*** immune response.
- Mediated by B-cells.
- For the recognition of antigens, B-cells have specific receptors on their surface.
- When an antigen invades the body, antigen-presenting macrophages will ingest them and carry them to the location of B-cells.
- The naive B-cell, having receptors for the invading antigen, would recognize, ingest and process the antigen - activate the B-cell.
- The activated B-cell would undergo rapid multiplication and produce several clones -This is called ***clonal expansion***.
- The cells of these clones differentiate into ***effectors, helpers, suppressors and memory cells.***
- ***Effector B-cells*** soon undergo rapid multiplication and produce ***plasmablasts*** which, in turn mature and transform to active antibody-producing ***plasma cells***.
- ***Helper B-cells*** promote the multiplication of all kinds of T cells.
- ***Suppressor B cells*** inhibit the rapid proliferation of effector B cells.
- ***Memory B-cells*** are functionally similar to memory T-cells - can store informations about the antigens they have encountered.
- Plasma cells do not normally leave the lymphatic system, but secrete specific immunoglobulins which serve as the arsenals of the chemical weaponry of immunity.
- They are carried to infected areas by blood or lymph.
- Antigen-antibody complexing and antigen-antibody interaction follow - inactivates or destroys the antigen by agglutination or precipitation, promotes their phagocytosis by macrophages.

Cell mediated immunity

- ***Antibody – independent*** immune mechanism.
- Mediated by T cells.
- Effective against fungi, parasites, intracellular viral infection,…
- There are many thousand kinds of competent T-cells.
- Each of them is specific to a particular antigen.
- When an antigen invades the body - antigen-presenting cells (APC), rush to it, ingest and degrade it into antigenic fragments and then bring it to T-cells – Presentation of antigen.
- A specific type of T-cell, especially sensitive to it, gets activated - ***sensitized T-cell***.
- Sensitized T-cell undergoes rapid and repeated proliferation and forms 1arge number of clones or colonies - This is known as ***clonal amplification***.

- Each clone differentiate into several sub-populations, such as ***effector cells and memory cells***.
- Effector cells include ***helper T-cells, cytotoxic T-cells, suppressor T-cells, initiator T-cells, amplifier T-cells and regulator cells.***
- Some effector T-cells soon undergo rapid division and form ***killer cells***.
- Memory T-cells stay in the lymphatic station.
- All other cells leave the lymphatic organ and move to the site of infection or inflammation.
- Killer cells bind with the antigen or virus-infected target cells and secrete ***lymphokines and some activating factors***.
- ***Lymphokines*** are powerful toxins – directly destroy the antigen or kill the pathogen.
- Activating factors are of three main groups, ***transfer factors, chemostaxis factors and macrophage-activating factors (MAF).***
- ***Transfer factors*** - transforms non-sensitised T-cells to active killer cells.
- ***Chemostaxis factors*** - promotes inflammatory changes and attracts macrophages.
- ***MAF*** – Stimulates macrophages and promotes their phagocytic activity.
- On receiving appropriate cytokines, helper T-cells differentiate into two groups – T_H1 cells and T_H2 Cells.
- ***T_H1 cells*** activate macrophages and stimulate the differentiation and maturation of cytotoxic T cells.
- ***T_H2 cells*** are mainly involved in the activation and differentiation of B-cells into antibody producing plasma cells.
- Mature T_C cells will directly destroy virus-infected cells.
- ***Initiator T-cells*** are concerned with the recruitment of killer T-cells for quick action.
- ***Amplifier T-cells*** stimulate the activity of all kinds of T-cells, B-cells and lymphoid stem cells.
- ***Suppressor T-cells*** suppress or inhibit all kinds of immune responses by other lymphocytes.
- ***Regulator T-cells*** regulate the functioning of helpers and suppressors.
- Those regulators which inactivate suppressor T-cells are called ***counter suppressors***.
- ***Memory T-cells*** are concerned with storing the information about antigens.

Primary response

- It is characterized by the production of antibody secreting plasma cells and memory B cells from the first contact of an individual with exogenous antigen.
- The primary response begins with a lag phase, during this phase naive B cell undergo clonal selection in response to antigen and differentiate in to plasma cells and memory cells.

- The lag phase lasts for 3 to 4 days and during this period no antibody is detected in the serum.
- The lag phase is immediately followed by logarithmic phase, where increase in serum antibody levels is observed for 4 to 10 days, which reaches a peak and then plateaus for a variable time and then declines.
- The early primary response is characterized by the presence of 1gM followed by 1gG.

Secondary response

- This occurs upon exposure to the same antigen, weeks, months or even years later.
- The memory B cells formed during the primary response and had entered into Go phase are now stimulated by the presence of same antigen.
- They undergo rapid proliferation and differentiation into plasma cells.
- There is a short negative phase before the secondary response starts, which is characterized by activation, proliferation and differentiation of memory B cells.
- The higher levels of antibody coupled with the overall higher affinity provide an effective host defense against reinfection.

Generation of cytotoxic T- cells (CTLs)

- Cytotoxic T lymphocytes, generated by immune activation of cytotoxic (T) cells.
- These effector cells have lytic capability and are critical in the recognition and elimination of altered self-cell (e.g., virus-infected cells and tumor cells) and in graft-rejection reactions.
- In general, CTLs are CD8+ and are therefore class I MHC restricted, although in rare instances CD4+ class II-restricted T cells have been shown to function as CTLs.
- Since virtually all nucleated cells in the body express class 1 MHC molecules, CTLs can recognize and eliminate almost any altered body cells.
- The CTL - mediated immune response can be divided into two phases.
- The first phase activates and differentiates naive T cells into functional effector CTLs.
- In the second phase, effector CTLs recognizes antigen- class 1 MHC complexes on specific target cells, which leads them to destroy the target cells.

NK cell mediated cytotoxicity

- NK cells appear to kill tumor cells and virus-infected cells by processes similar to those by CTLS.
- NK cells bear Fas L on their surface and readily induce death in Fas- bearing target cells.
- The cytoplasm of NK cells contains numerous granules containing perforin and granzymes.
- NK cells are constitutively cytotoxic, always having large granules in their cytoplasm.

- After an NK cell adheres to a target cell, degranulation occurs with release of perforin and granzymes at the junction of the interacting cells.
- The roles of perforin and granzymes in NK-mediated killings of target cells by apoptosis are believed to be similar to their roles in the CTL-mediated process.
- NK cells differ from CTLs in several ways. First, NK cells do not express antigen-specific T-cell receptors or CD3.
- In addition recognition of target cells by NK cells is not MHC restricted and NK cell activity does not increase after a second injection with the same tumor cells.

ADCC

- Antibody Dependent Cell Mediated Cytotoxicity
- A number of cells have cytotoxic potential express membrane receptors for the Fc region of the antibody molecule
- When Ab is bound to a target cell, these receptor bearing cells can bind to the antibody Fc region, and thus to the target cells, and subsequently cause lysis of the target cell.
- These cytotoxic cells are nonspecific tor antigen, the specificity of the antibody directs them to specific target.cells - This type of cytotoxicity is referred to as ADCC
- Cells that can mediate ADCC are NK cells, macrophages, monocytes, neutrophils, and eosinophils.
- When macrophages, neutrophils, or eosinophils bind to a target cell by way of the Fc receptor, they become more active metabolically
- The lytic enzymes in their cytoplasmic lysosome granules increase.
- Release of these lytic enzymes at the site of the Fc-mediated contact may result in damage to the target cell.
- Activated monocytes, macrophages & NK cells have been shown to secrete tumor necrosis factor (TNF) which may have a cytotoxic effect on the bound target cell.
- Since both NK cells and eosinophils contain perforin in cytoplasmme granules, their target cell killing also may involve perforin- mediated membrane damage similar to the mechanism described for CTL – mediated cytotoxicity.

Cytokines

- Low - molecular weight chemical messengers, or Immunomediators.
- Secreted by white blood cells.
- Functionally they are regulatory proteins or glycoproteins which mediate immune responses.
- They bind to specific receptors on the target cell membrane and induce signal transduction which ultimately triggers gene expression in target cells.

- The cytokine secreted by a particular cell binds to the receptor of the same cell and stimulates that particular cell, it is called ***autocrine***.
- The cytokine secreted by a particular cell acts on a target cell in the immediate vicinity, it is called ***paracrine***.

Functions of cytokines

- Accomplish the activation, proliferation and differentiation of B-cells and T cells.
- Promote the proliferation and differentiation of haematopoietic stem cells.
- Mediate the chemotaxis and tissue access of macrophage, neutrophils, eosinophils, etc, at the site of infection.
- Stimulate the growth, differentiation and activation of granulocytes.
- Mediate inflammation and innate immunity.
- Cytotoxic action against foreign antigens, foreign tissue grafts and cancer cells.

Examples of cytokines

1. **Interleukins**

- Produced by T cells
- Participate in the production of immune cells, interaction between leucocytes & inflammatory responses.
- Different classes of interleukins are IL-1, IL-2, IL-3,IL-4,…

2. **MCSF**

- Monocyte colony stimulating factor
- Secreted by macrophages, endothelial cells,..
- Acts on progenitors that are already committed to develop to monocytes.

3. **GCSF**

- Granulocyte colony stimulating factor
- polypeptide
- Produced by monocytes, endothelial cells,..
- Promotes the maturation of neutrophils.

4. **GM – CSF**

- Granulocyte monocyte colony stimulating factor
- Glycoprotein
- Secreted by activated T cells
- Promotes macrophage activity.

MAJOR HISTOCOMPATIBILITY COMPLEX

MHC

- The major histocompatibility complex is a collection of genes arrayed within a 1ong continuous stretch of DNA on chromosome 6 in humans and on chromosome 17 in mice.
- The MHC is referred to as the HLA complex in humans and as the H- 2 complex in mice.

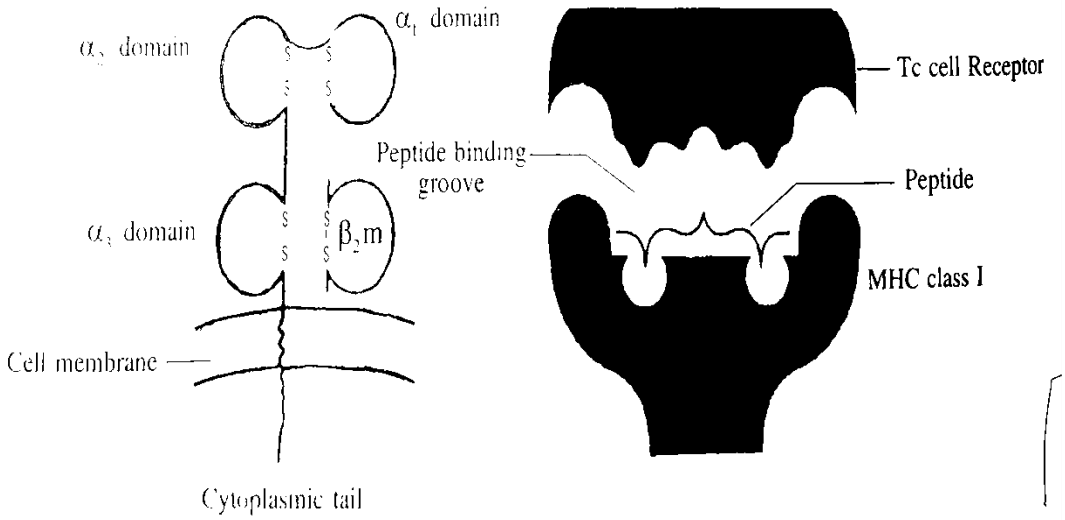
HLA

- Human Leucocyte Antigen
- Antigens that evoke an immune response associated with graft rejection are referred to as ***transplantation antigens or Histocompatibility antigens.***
- T'hese antigens are cell - surface molecules encoded by Histocompatibility genes (H genes) situated at a Histocompatibility locus (H locus).
- Different alleles encoding allelic forms of Histocompatibility antigens exist at each H locus in different individuals the antigens are also referred to as alloantigens.
- In humans, the MHC that is located on chromosome 6 is known as HLA since it contains genes encoding these antigens.
- The human HLA gene complex consists of several closely linked genes (loci), known as ABC (MHC - Class I) and DP, DQ, DR, (MHC- Class II).
- Each locus has many different alleles.
- The particular combination of alleles at closely linked loci on the same chromosome is termed the haplotype; two haplotype, one from each parent, constitute genotype of the individual.
- The haplotype is the genotype of linked loci on each of the parental chromosomes.
- The Class I MHC molecule consists of an α - chain encoded by the MHC and β_2 microglobulin, encoded on chromosome no: 15
- The A B and C loci in humans code for the α chain (heavy chain) of MHC Class I molecules, which contain a variable region.
- The DP, DQ, DR, genes of the D regions in humans code for Class II molecules that may be expressed on the surface of such cells as B Iymphocytes, monocytes, Langerhans cells of the skin, and certain endothelial cells.
- Each class II molecule consists of an α and a β chain.
- The human Major Histocompatibility locus, Class I and Class II human leukocyte antigens (HLA) are encoded by three (ABC) and six genes (DP, DQ, DR), respectively.

Class I MHC

- Class I MHC genes encode glycoprotein expressed on the surface of nearly all nucleated cells

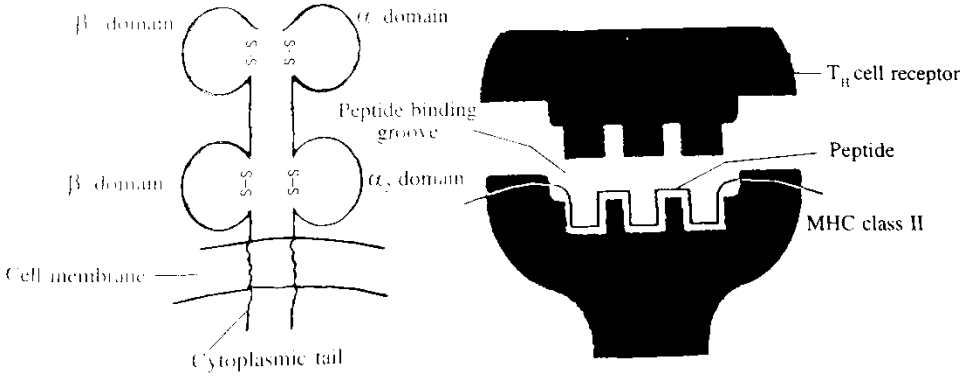
- The major function of the class I gene products is presentation of peptide antigens to T_C cells.
- MHC class I gene codes for a trans membrane glycoprotein of approximate molecular weight 43 kda, which is referred to as the α or heavy chain
- It comprises three extra cellular domains, α_1, α_2 & α_3.
- Every MHC class I molecule is expressed at the surface of a cell in non-covalent association with an invariant small peptide called β_2 microglobulin
- β_2 microglobulin has a structure homologous to single Ig domain, and indeed β_2 m is also a member of the Ig super family.
- The cell surface complex of MHC class I and β_2 m appears like a four domain molecule, with the α_3 domain of the class I molecule and β_2 m juxtaposed closest to the membrane.
- All MHC class I molecules from the human and mouse have the same general structure.
- The most striking feature is that the part of the molecule farthest from the membrane contains a ***deep groove or cleft.***
- This cleft is made up of parts of the α_1 and α_2 domains.
- The peptide bound in this cleft and parts of the MHC class I molecule interact with the variable regions, V α, & Vβ, of a T_C cell receptor.
- A single MHC molecule can bind to a variety of peptides but binds preferentially to peptides with certain motifs



Structure of class I MHC

Class II MHC

- Class II MHC genes encode glycoproteins expressed primarily on antigen presenting cells (macrophages, dendritic cells, and B-cells)
- They present processed antigenic peptides to T_H Cells.
- MHC class II α and β genes encode for chains of α & β of approximate molecular weight 35000 and 28000 Da respectively
- MHC class II molecules, are trans membrane glycoprotein molecules with cytoplasmic tails and extra cellular Ig like domains.
- Theses domains are referred to as α_1 & α_2 , and β_1 and β_2.
- MHC class II molecules are also members of the immunoglobulin super family.
- One of the key features of the MHC class II molecule crystal structure is peptide binding groove or cleft at the top of the molecule, structurally analogues to the MHC class I groove
- In the MHC class II molecule the cleft is formed by interactions between domains of different chains, α_1 & β_1 domains.
- The floor of the MHC class groove comprises eight β -pleated sheets, with the α_1& β_1 domains each contributing four helical sections of the α_1& β_1 domains each comprise one wall of the cleft.
- In contrast to the class I groove the class II groove is open at both ends, allowing larger peptides to bind.
- MHC class groove binds peptides varying in length from 12 to 20 amino acids in linear array, with the ends of the peptides outside the groove.
- The MHC class II molecule interact with variable regions of Vα, Vβ of T_H receptor.



Structure of class II MHC

Class III MHC

- Class III MHC genes generally encode various secreted proteins that have immune functions, including components of the complement system and molecules involved in inflammation (C4, C2, Factor B and TNF).

MODULE 9

AUTOIMMUNE & IMMUNODEFICIENCY DISEASES , TUMOUR & TRANSPLANTATION IMMUNOLOGY

Autoimmune diseases

When immune system recognize & respond against the self components of the body – ***autoimmunity.***

Divided into two categories

1. Organ specific diseases
2. Systemic autoimmune diseases

Systemic

- In this type of autoimmune disease the autoantibodies are directed against broad range of antigens and target organs.
- It is due to defective immune regulation of T & B-lymphocytes, which leads to wide spread tissue damage from cell mediated and humoral antibodies as well as with immune complex mediated response.

SLE

- Systemic Lupus Erythematosus
- Autoimmune disease.
- The immune system of the body mistakenly attacks healthy tissue.
- Prevalent in female between 20 and 40 years of age
- It is characterized by butterfly shaped rashes on face, whole body rash, arthritis and kidney dysfunction.
- Individuals affected with this disease may produce autoantibodies against wide variety of self antigens, such as DNA, thrombocytes, RBC, leukocytes, clotting factor etc.
- The amount of circulating neutrophils decreases and occlusions of small blood vessels occur. These occlusions can cause vasculitis and widespread tissue damage.
- It can affect the skin, joints, kidneys, brain, and other organs.

Causes

1. Genetic
2. Environmental
3. Hormonal
4. Certain medicines

Signs and symptoms

1. Fatigue
2. Skin rashes
3. Fevers
4. Pain or swelling in the joints

Treatment

- Immunosuppressive drugs that inhibits the activity of the immune system.
- Hydroxychloroquine and corticosteroids (e.g., prednisone) are often used to treat SLE.

Multiple sclerosis

- It is an autoimmune disease that affects the central nervous system and cause neurological disability.
- Individuals affected with this disease produce auto reactive T cells that involve in inflammatory lesions along the myelin sheath of nerves.
- Cerebrospinal fluid tapped from these will contain auto activated T lymphocytes, which destroy myelin sheath - this leads to various neurological disorders.

Rheumatoid arthritis

- It is a systemic autoimmune disease most common among women and affects them between 40 to 60 years of age.
- Most common symptom is inflammation of joints and painful joints accompanied by other symptoms like cardiovascular, hematologic, and respiratory system.
- Affected individuals produce autoantibodies called rheumatoid factors that are reactive in the Fc region of IgG.
- A classic rheumatoid factor is IgM antibody, which reacts with IgG to form IgM-IgG complex and deposits in the joints.
- These complexes activate complement reaction leading to Type III hypersensitivity reaction and chronic inflammation of the joints.
- In rheumatic fever, Group A Streptococci infection proceeds and protective antibodies are being generated.
- These antibodies attack on antigen – antibodies cross react with heart muscle antigen leading to damage & Rheumatic fever.

Organ specific

- In this autoimmune disease, the immune response is directed against a specific antigen corresponding to a single organ.
- The target organ as result of autoimmune attack by humoral or cell mediated mechanism may suffer severe damage or the function of the organ may be enhanced due to autoimmune attack resulting in *hyper functioning*, or the auto antibodies may block the function performed by the cell or organ and result in *failure of the particular function.*

Hashimoto's thyroiditis

- The disease is due to the production of autoantibodies and sensitized T cells, that are specific against thyroid antigens.
- Frequently affects middle aged women.
- The sensitized T-cells involved in it are concerned with delayed type hypersensitivity (DTH) reactions (T_{DTH} cells).
- DTH response results in severe inflammatory reactions.
- The thyroid gland gets infiltrated by lymphocytes, macrophages and plasma cells.
- This leads to the formation of lymphocytic follicles and germinal centers, that are characteristic of lymph nodes, as a result thyroid develops ***goitre.***
- Autoantibodies are also produced against thyroid globulin and thyroid peroxidase.
- Binding of autoantibodies to these thyroid protein products severely ***impairs iodine uptake, it results in hypothyroidism.***

Graves disease

- Autoimmune disease.
- Due to the production of agonistic autoantibodies.
- Characterized by ***Goitre*** : due to the autoantibodies which stimulate the growth of thyroid gland.
- ***Hyperthyroidism*** : due to the autoantibodies which stimulate the excessive production of thyroid hormone.
- ***Exophthalmus*** : due to the antibodies which stimulate the retro -orbital tissue around the eyeball.
- TSH from pituitary gland - stimulate the production of thyroid hormone.
- In grave's disease - autoantibodies are produced against TSH. Binding of these receptors by this autoantibodies mimics the action of TSH which stimulate the production of excess thyroid hormone.
- No feedback regulation

- ***Long- acting thyroid stimulating antibodies*** (LATS) : autoantibodies continuously stimulate thyroid gland for hormone secretion.

Myasthenia gravis

- Myasthenia gravis is a serious autoimmune disease.
- Characterized by muscular weakness and a strong tendency for fatigue which comes on suddenly.
- It is caused by antagonistic autoantibodies, which bring about negative reaction by blocking the receptor of the neurotransmitter, acetyl choline.
- Acetyl choline receptors are abundant in skeletal muscles.
- Binding of autoantibodies with these receptors inhibits acetyl choline binding and induces complement-activated muscle lysis, resulting in progressive weakening of skeletal muscle response.
- In myasthenia, the body develops autoantibodies which interfere with the functioning of neuromuscular junctions and thereby block the transmission of impulses from nerves to muscles.
- In myasthenia, the autoantibodies inhibit binding of acetyl choline with the receptors on muscles.
- This blocks impulse transmission, with the result that depolarisation of muscles fails to occur and muscular contraction does not take place.

TUMOUR IMMUNOLOGY

Malignant transformation of cells

- Treatment of normal cultured cells with chemical carcinogens, irradiation, and certain virus can alter their morphology and growth properties
- Such cells are said to have under gone malignant transformation and often exhibit properties in vitro similar to cancer cell
- These cells when injected into animal cause tumours.
- Induction of malignant transformation with chemical or physical carcinogens (mutagens) involves two distinct phases, initiation and promotion.
- Initiation involves changes in the genome but does not itself lead to malignant transformation.
- Promoters stimulate cell division and lead to malignant transformation.
- Eg., Xeroderma pigmentosum.
- A number of DNA and RNA virus have been shown to induce malignant transformation e.g., sv 40 and polyoma.

Tumour antigens

- Tumour antigens or neoantigens are those antigens that are presented by MHC class I or MHC Class II molecules on the surface of tumour cells.
- These antigens can sometimes be presented by tumour cells and never by the normal ones ***- tumour specific antigens (TSAs)***
- More common are antigens that are presented by tumour cells and normal cells ***- tumour associated antigens (TAAs).***
- Cytotoxic T lymphocytes that recognize these antigens may be able to destroy the tumour cells before they proliferate or metastasize.
- Tumour antigens can also be on the surface of the tumour in the form of, a mutated receptor, in which case they will be recognized by B cells.
- ***TAAs (Tumour associated antigens)-*** Antigens that do not stimulate immunologic response in the host are called tumour- associated antigens (TAAs).
- Important for diagnosis and possible treatment of cancers.
- There are 3 classes of TAA's family
- *A.Oncofoetal antigens*
- *B. altered glycoprotien and gycolipid antgen*
- *C. Tissue specific (dijferentiation) antigens on tumours*

Immune response to tumour antigens

a) Antibodies

- lf a tumour evades the surveillance system, it might be then recognized by the specific immune systems
- In models of chemically or virally induced tumours, the tumour associated antigens are immunogenic and trigger specific cellular and antibody responses against the tumour
- This immunity may be protective and can be passively transferred with immune cells.
- In tumour bearing patients it is possible to demonstrate anti-tumour antibody, whích may mediate some tumour cell lysis.

b) T – lymphocytes

- Anti - tumour immune responses develop in tumour bearing patients in much the same way as they do to pathogens or foreign antigens.
- Thus anti-tumour antibodies and T cells are generated and, along with more non-specific immune mechanisnms, play a role in tumour immunity.
- TSA and TAA are associated with tumour cells and, after their intracellular synthesis are processed and presented in association with Class I molecules, making them potential targets for cytotoxic T cells (CTLs).
- The CD4 cells are known to secrete tumour necrosis factor (TNF) and (IFNγ) on activation by tumour antigens which can increase tumour cell class I MHC expression and render the cells sensitive to lysis by CTLs.

c) NK cells

- NK cells kill tumour cells not expressing MHC Class 1.
- The mechanism of lysis of tumour cells by NK cells is the same as that of CTLs, but they do not express T cell antigen receptors, and they kill targets in an MHC unrestricted manner.
- Tumouricidal activity of NK cell is enhanced by cytokines such as IFNγ, IL-2, TNFα and IL-12.

d) Macrophages

- Activated macrophage is known to secrete the cytokine TNFα which is capable of selectively killing tumour cells.
- Antibody coated tumour cells can be killed by complement activation, by macrophage and PMN mediated phagocytosis or by ADCC.

- Overall, the potential effector mechanisms which may be involved in human tumour cell lysis in vivo are the same as those used in microbial immunity.

TRANSPLANTATION IMMUNOLOGY

Transplantation Antigens

- Antigens that evoke an immune response associated with graft rejection are referred to as ***transplantation antigens or Histocompatibility antigens***
- MHC

Liver transplantation

- Liver malfunction can be caused by damage to the organs form viral diseases such as hepatitis or by exposure to harmful chemicals as in chronic alcoholism.
- The liver tissue does not degenerate, damage may be fatal.
- The majority of liver transplants are used as therapy for congenital abnormalities of liver.
- The immunology of liver transplantation is interesting because the organ appears to resist rejection by hyper acute antibody mediated mechanisms.

Precautions

1. Matching of blood and histocompatibility groups.
2. Detailed tissue typing must be used to ascertain that the patient has no antibodies or active cellular mechanisms against.
3. Manifestation of GVHD (Graft Vs Host Diseases) have occurred in liver transplants.

Problems

- Leukocytes within the donor organ together with anti-blood antibodies can mediate antibody depended haemolysis of recipient red blood cells if there is a mismatch of the blood groups.
- In addition manifestation of GVHD have occurred in liver transplants even when donor and recipient are blood group compatible these reactions are obviously caused by donor lymphocytes carried by the transplanted liver.

Kidney transplantation

- The most commonly transplanted organ is kidney.
- Many diseases such as diabetes, nephritis, result in kidney failure that can be alleviated by transplantation.
- Kidneys can be obtained not only from cadavers but also from living relatives or volunteers
- Kidney transplantation is simpler than liver or heart

Precautions

- Matching of blood and histocompatibility groups because the kidney is heavily vascularised.
- Detailed tissue typing must be used to ascertain that the patient has no antibodies or active cellular mechanisms against.

Problems

1. The short supply of the organs.
2. Increasing number of sensitized recipients.
3. In many case patients can never again find a match after one or two rejection episodes. The transplanted kidney must remain functional for the rest of the patient's life, for which immunosuppressive therapy should be administered.
4. This gives rise to complications, including risk of cancer and infection as well as side effects such as hypertension and metabolic disease.

Heart transplantation

- Heart transplantation is an option for patients who have any of the following and who remain at risk of death and have intolerable symptoms despite optimal use of drugs and medical devices:
    - End-stage heart failure
    - Coronary artery disease
    - Arrhythmias (improper heart beating)
    - Hypertrophic cardiomyopathy
    - Congenital heart disease

- All donated hearts come from brain-dead donors, who are usually required to < 60 years and have normal cardiac and pulmonary function and no history of coronary artery disease or other heart disorders.
- Donor and recipient must have compatible ABO blood type and heart size.

Procedure

- Donor hearts are preserved by hypothermic storage.
- They must be transplanted with in 4 to 6 hours
- The recipient is placed on a bypass pump, recipient heart is removed preserving the posterior right atrial wall in situ.
- The donor heart is then transplanted orthotopically (in its normal position) with aortic, pulmonary artery and pulmonary vein anastomoses; a single anastomosis joins the retained posterior atrial wall to that of the donor organ.
- Immunosuppression is started soon after surgery.

- Most immunosuppressive protocol use a 3-drug regimen consisting of a calcineurin inhibitor (CNI) (Cyclosporine or tacrolimus), an antimetabolite agent (mycophenolatemofetil or azathioprine), and tapering doses of corticosteroids over the first year post transplantation.

Complications

- Bleeding from suture lines
- Hyperacute rejection
- Infection
- Psychiatric disturbances from steroid therapy
- Cardiac rejection

Skin transplantation

- Most skin transplantation is done with autologous tissue.
- In cases of severe burn, grafts of foreign skin thawed from frozen deposits in tissue banks may be used.
- True allogenic skin grafting need immunosuppressive therapy.
- This is not desirable because often burn victims are at high risk of infection, and immunosuppressive therapy accentuates it.
- Skin grafts may be autografts or allografts
- Skin autografts use the patient' s own intact skin as the source.
- Skin allografts use donor skin (typically from cadavers).
- Skin allografts are used for patients with extensive burns or other conditions causing such massive skin loss that the patient does not have enough undamaged skin to provide the graft.

Problems

- Bleeding
- Chronic pain (rarely)
- Infection
  Loss grafted skin (the graft not healing, or the graft healing slowly)
- Reduced or lost skin sensation, or increased sensitivity
- Scarring
- Skin discoloration
- Uneven skin surface
- Graft rejection when allotype graft is used.

Xenotransplantation

- It is the transplantation of an organ of another species to human beings
- A major problem with xenotransplants is that immune rejection is often quite vigorous, even when recipients are treated with immunosuppressive drugs
- The major response involves the action of humoral antibody and complement, leading to the development of hyper acute rejection.
- One possible approach to limiting the destruction mediated by antibody and complement to produce transgenic donor pigs that express human decay accelerating factor (DAF), which dissociates C3 convertase and prevents activation of C3 and C5.
- The presence of human DAF on the cells of implanted organ should reduce complement activity and decrease the severity of the hyper acute rejection to the graft.

Why pigs are used as potential donors than primates?

- They breed rapidly, have large litters, and can be housed in pathogen free environment, and share a considerable number of anatomic and physiologic similarities with humans.