Unit-4

Pharmaceutical Biotechnology

B.Pharma 6th Sem Notes

Unit: 4

- Immuno-blotting techniques- ELISA, Western blotting, Southern blotting.
- Genetic organization of Eukaryotes and Prokaryotes.
- Microbial genetics including transformation, transduction, conjugation, plasmids and
- transposons.
- Introduction to Microbial biotransformation and applications.
- Mutation: Types of mutation/mutants.

Immuno-blotting Techniques

Immuno-blotting techniques are laboratory methods used to detect and identify specific proteins or nucleic acids in a sample. These techniques combine separation methods with antibody-based or probe-based detection.

ELISA (Enzyme-Linked Immunosorbent Assay)

Definition:

ELISA is a plate-based technique used to detect and measure the presence of antibodies or antigens in a sample using enzyme-linked antibodies.

Principle:

- Antigen or antibody is immobilized on a solid surface (microplate well)
- Specific antibody binds to the target
- Enzyme-linked secondary antibody is added
- Substrate is added which produces a color change
- Color intensity is measured and is proportional to the amount of target present

Types of ELISA:

1. **Direct ELISA:** Antigen is coated directly on the plate; enzyme-labeled antibody binds to antigen
2. **Indirect ELISA:** Antigen is coated; unlabeled primary antibody binds; enzyme-labeled secondary antibody binds to primary
3. **Sandwich ELISA:** Capture antibody is coated; antigen binds; detection antibody (enzyme-labeled) binds
4. **Competitive ELISA:** Sample antigen and labeled antigen compete for limited antibody binding sites

Applications:

- Detection of antibodies in patient serum (HIV, hepatitis testing)
- Hormone level measurement (pregnancy tests, thyroid hormones)
- Detection of allergens
- Quality control in pharmaceutical industry

Western Blotting (Immunoblotting)

Definition:

Western blotting is a technique used to detect specific proteins in a sample by separating them based on size and then identifying them using antibodies.

Steps in Western Blotting:

1. **Sample Preparation:** Proteins are extracted from cells or tissues

2. **Gel Electrophoresis:** Proteins are separated by molecular weight using SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)
3. **Transfer:** Separated proteins are transferred from gel to a membrane (usually nitrocellulose or PVDF)
4. **Blocking:** Membrane is blocked with protein (like BSA or milk) to prevent non-specific binding
5. **Antibody Binding:** Primary antibody specific to target protein is added, then enzyme-linked secondary antibody
6. **Detection:** Enzyme substrate is added producing a signal (chemiluminescence or color) at the protein band

Applications:

- Confirmation of protein expression
- HIV diagnosis (confirmatory test)
- Detection of disease markers
- Research in protein studies and drug development

Southern Blotting

Definition:

Southern blotting is a technique used to detect specific DNA sequences in a sample by separating DNA fragments and identifying them using labeled probes.

Steps in Southern Blotting:

1. **DNA Extraction:** DNA is isolated from cells or tissue
2. **Restriction Digestion:** DNA is cut into fragments using restriction enzymes
3. **Gel Electrophoresis:** DNA fragments are separated by size on agarose gel
4. **Denaturation:** Double-stranded DNA is converted to single-stranded using alkali treatment
5. **Transfer:** DNA is transferred from gel to membrane (nylon or nitrocellulose) by capillary action
6. **Hybridization:** Membrane is incubated with labeled DNA probe (radioactive or fluorescent) that binds to complementary sequences
7. **Detection:** Signal from labeled probe is detected (autoradiography or chemiluminescence)

Applications:

- Detection of specific gene sequences
- Genetic fingerprinting and forensic analysis

- Detection of gene mutations and rearrangements
- Diagnosis of genetic disorders
- Study of gene structure and organization

Genetic Organization of Eukaryotes and Prokaryotes

Genetic Organization in Prokaryotes

Features:

- **Nucleoid Region:** No membrane-bound nucleus; DNA is located in the nucleoid region
- **Circular DNA:** Single, circular chromosome (usually 1-10 million base pairs)
- **No Histones:** DNA is not wrapped around histone proteins (except in some Archaea)
- **Gene Organization:** Genes organized in operons (clusters of genes with related functions controlled together)
- **No Introns:** Genes lack introns (non-coding sequences); continuous coding sequences
- **Plasmids:** Small, circular extrachromosomal DNA molecules present
- **Polycistronic mRNA:** Single mRNA can code for multiple proteins
- **Gene Density:** High gene density; little non-coding DNA (about 85-95% is coding)

Example Operon (Lac Operon):

- Contains genes for lactose metabolism
- Single promoter controls three genes (lacZ, lacY, lacA)
- Regulated by repressor and activator proteins

Genetic Organization in Eukaryotes

Features:

- **Nucleus:** DNA is enclosed within a membrane-bound nucleus
- **Linear Chromosomes:** Multiple linear chromosomes (humans have 46)
- **Histones:** DNA is tightly wrapped around histone proteins forming nucleosomes
- **Chromatin Structure:** DNA organized as chromatin (euchromatin - active; heterochromatin - inactive)
- **Individual Genes:** Each gene has its own promoter and regulatory elements
- **Introns and Exons:** Genes contain introns (non-coding) and exons (coding); splicing required
- **Monocistronic mRNA:** Each mRNA codes for only one protein

- **Gene Density:** Lower gene density; large amounts of non-coding DNA (only 1-2% is coding in humans)
- **Telomeres and Centromeres:** Chromosome ends (telomeres) and central regions (centromeres) present

Gene Expression Regulation:

- Chromatin remodeling
- Transcription factors
- Epigenetic modifications (DNA methylation, histone modifications)
- RNA processing and splicing

Comparison Table: Prokaryotes vs Eukaryotes

Feature	Prokaryotes	Eukaryotes
Nucleus	Absent (nucleoid)	Present (membrane-bound)
DNA Shape	Circular	Linear
Histones	Usually absent	Present
Introns	Absent	Present
Gene Organization	Operons	Individual genes
mRNA Type	Polycistronic	Monocistronic

Microbial Genetics

Microbial genetics studies how genetic information is transferred, expressed, and regulated in microorganisms. Bacteria can exchange genetic material through three main processes:

Transformation

Definition:

Transformation is the process by which a bacterial cell takes up free DNA from its surroundings and incorporates it into its own chromosome.

Process:

1. Bacterial cell becomes competent (able to take up DNA)
2. Free DNA (from dead bacteria) binds to cell surface
3. DNA passes through cell membrane
4. One strand is degraded; other strand integrates into chromosome
5. Cell replicates with new genetic trait

Requirements:

- Competent cells (naturally or artificially induced)

- Free DNA in the environment
- DNA must have homology with recipient chromosome for integration

Significance:

- First discovered by Frederick Griffith (1928) in Streptococcus pneumoniae
- Used extensively in genetic engineering to introduce recombinant DNA into bacteria
- Natural mechanism of antibiotic resistance spread

Transduction

Definition:

Transduction is the transfer of bacterial DNA from one bacterium to another via a bacteriophage (bacterial virus).

Types of Transduction:

1. Generalized Transduction:

- During lytic cycle, phage DNA is replicated and bacterial chromosome is broken down
- By mistake, bacterial DNA fragment is packaged into phage head instead of phage DNA
- When this phage infects another bacterium, it injects the bacterial DNA
- Any bacterial gene can be transferred
- Example: P1 phage in E. coli

2. Specialized Transduction:

- Occurs with temperate phages (lysogenic cycle)
- Phage DNA integrates into specific site in bacterial chromosome (prophage)
- During excision, sometimes nearby bacterial genes are taken along
- Only genes near the integration site can be transferred
- Example: Lambda phage in E. coli

Significance:

- Important in bacterial evolution and diversity
- Used in gene mapping
- Can transfer virulence factors and antibiotic resistance genes

Conjugation

Definition:

Conjugation is the transfer of genetic material from one bacterium to another through direct cell-to-cell contact via a pilus (sex pilus).

Process:

1. **Donor cell (F+ or Hfr)** has F (fertility) plasmid with genes for pilus formation
2. **Recipient cell (F-)** lacks F plasmid
3. Donor extends pilus and attaches to recipient
4. Pilus retracts bringing cells together; mating bridge forms
5. F plasmid DNA replicates via rolling circle mechanism
6. Single-stranded copy passes through bridge to recipient
7. Both cells synthesize complementary strand; both become F+

Types of Cells:

- **F+ cells:** Have F plasmid as separate entity; can donate F plasmid
- **F- cells:** Lack F plasmid; act as recipients
- **Hfr cells (High frequency recombination):** F plasmid integrated into chromosome; can transfer chromosomal DNA in linear fashion
- **F' cells:** F plasmid excised from chromosome carrying some bacterial genes

Significance:

- Most important method of horizontal gene transfer
- Spreads antibiotic resistance (R plasmids)
- Used in gene mapping and bacterial genetics research
- Discovered by Lederberg and Tatum (1946)

Plasmids

Definition:

Plasmids are small, circular, double-stranded DNA molecules that exist independently of the bacterial chromosome and can self-replicate.

Characteristics:

- Size: Typically 1-200 kilobase pairs (kb)
- Extrachromosomal: Separate from main chromosome
- Self-replicating: Have their own origin of replication (ori)
- Not essential: Carry non-essential but advantageous genes
- Copy number: Can be present in 1-100+ copies per cell
- Transferable: Can move between cells via conjugation

Types of Plasmids:

1. **F plasmids (Fertility):** Contain genes for conjugation and pilus formation

2. **R plasmids (Resistance):** Carry antibiotic resistance genes; major clinical concern
3. **Col plasmids (Colicinogenic):** Produce colicins (proteins that kill other bacteria)
4. **Virulence plasmids:** Carry genes for toxins and disease-causing factors
5. **Degradative plasmids:** Enable breakdown of unusual organic compounds
6. **Ti plasmids (Tumor-inducing):** Found in Agrobacterium; used in plant genetic engineering

Applications in Biotechnology:

- Cloning vectors for recombinant DNA technology
- Production of insulin, growth hormone, vaccines
- Gene therapy vectors
- Plant genetic modification
- Study of gene function and regulation

Transposons

Definition:

Transposons (also called "jumping genes") are mobile DNA sequences that can move from one location to another within a genome or between genomes.

Structure:

- Inverted terminal repeats (ITRs) at both ends
- Transposase gene (enzyme that catalyzes movement)
- May carry additional genes (antibiotic resistance, toxins)
- Size ranges from hundreds to thousands of base pairs

Types of Transposons:

Simple (Insertion Sequences - IS elements):

- Only contain genes for transposition
- Smallest transposons (700-1500 bp)
- Example: IS1, IS2 in E. coli

Composite Transposons:

- Central region with additional genes flanked by IS elements
- Often carry antibiotic resistance genes
- Example: Tn10 (tetracycline resistance)

Replicative Transposons:

- Create a copy of themselves during transposition
- Original stays in place; copy moves to new location

- Example: Tn3 (ampicillin resistance)

Mechanism of Transposition:

5. Transposase recognizes inverted terminal repeats
6. Cuts transposon from original location (or makes a copy)
7. Inserts into new target site
8. Creates short duplications (direct repeats) at insertion site

Significance:

- Cause mutations by inserting into genes
- Spread antibiotic resistance rapidly
- Drive bacterial evolution and adaptation
- Used as genetic tools for mutagenesis
- Barbara McClintock discovered transposons in corn (Nobel Prize 1983)

Microbial Biotransformation

Definition:

Microbial biotransformation is the use of microorganisms or their enzymes to chemically modify organic compounds. The microbe acts as a biocatalyst to convert one chemical compound into another through enzymatic reactions.

Principle:

Microorganisms possess diverse enzymes that can perform specific chemical modifications on substrates. These reactions are often:

- Stereospecific (produce specific stereoisomers)
- Regiospecific (modify specific positions on molecule)
- Carried out under mild conditions (normal temperature and pressure)
- Environmentally friendly (no toxic chemicals)
- Cost-effective compared to chemical synthesis

Types of Biotransformation Reactions:

1. **Oxidation:** Addition of oxygen or removal of hydrogen (e.g., hydroxylation, epoxidation)
2. **Reduction:** Addition of hydrogen or removal of oxygen (e.g., reduction of ketones to alcohols)
3. **Hydrolysis:** Breaking bonds by adding water (e.g., ester hydrolysis)
4. **Isomerization:** Rearrangement of molecular structure
5. **Methylation/Demethylation:** Addition or removal of methyl groups
6. **Acetylation/Deacetylation:** Addition or removal of acetyl groups
7. **Decarboxylation:** Removal of carboxyl group releasing CO2

Applications in Pharmaceutical Industry:

1. Steroid Transformations:

- 11-hydroxylation of progesterone (using Rhizopus) to produce cortisone precursors
- Production of hydrocortisone and prednisolone
- Key step in corticosteroid manufacturing

2. Antibiotic Production:

- Conversion of penicillin G to 6-APA (6-aminopenicillanic acid) using penicillin acylase
- Semi-synthetic antibiotic production
- Modification of cephalosporins

3. Vitamin Production:

- Vitamin C (ascorbic acid) from glucose via 2-keto-L-gulonic acid
- Vitamin B12 production by fermentation
- Riboflavin (Vitamin B2) production

4. Drug Metabolite Studies:

- Predicting human drug metabolism
- Producing metabolites for toxicity testing
- Generating reference standards

5. Resolution of Racemic Mixtures:

- Production of optically pure drugs
- Separation of D and L isomers
- Example: Production of L-ephedrine

Advantages of Microbial Biotransformation:

- High specificity and selectivity
- Mild reaction conditions
- Environmentally friendly (green chemistry)
- Can perform complex multi-step reactions
- Cost-effective for large-scale production
- Reduces need for chemical protecting groups

Common Microorganisms Used:

- **Fungi:** Aspergillus, Rhizopus, Cunninghamella
- **Bacteria:** Pseudomonas, Bacillus, Streptomyces
- **Yeast:** Saccharomyces cerevisiae, Candida species
- **Actinomycetes:** Nocardia, Mycobacterium

Mutations

Definition:

A mutation is a permanent change in the DNA sequence of an organism. Mutations are the source of genetic variation and drive evolution.

Types of Mutations (Based on Molecular Changes)

1. Point Mutations (Single Base Changes):

a) Substitution Mutations:

- **Transition:** Purine to purine (A↔G) or pyrimidine to pyrimidine (C↔T)
- **Transversion:** Purine to pyrimidine or vice versa (A/G↔C/T)

Effects of substitutions:

- **Silent mutation:** No change in amino acid (due to degeneracy of genetic code)
- **Missense mutation:** Different amino acid is coded
- **Nonsense mutation:** Creates a stop codon, truncating the protein

2. Frameshift Mutations:

- **Insertion:** Addition of one or more nucleotides
- **Deletion:** Removal of one or more nucleotides
- **Effect:** Shifts the reading frame, altering all downstream amino acids (usually severe)
- Exception: Insertion or deletion of 3 nucleotides (one codon) maintains frame

3. Large-Scale Mutations:

- **Deletion:** Loss of a chromosome segment
- **Duplication:** Extra copy of a chromosome segment
- **Inversion:** Segment is reversed end-to-end
- **Translocation:** Segment moves to different chromosome

Types of Mutants (Based on Effect)

1. **Auxotrophic Mutants:**
   - Cannot synthesize essential nutrients
   - Require supplementation in growth medium
   - Example: Mutant lacking enzyme for histidine synthesis needs histidine added
2. **Prototrophs:**
   - Wild-type; can synthesize all required nutrients
   - Grow on minimal medium

3. **Conditional Mutants:**
   - Express mutant phenotype only under certain conditions
   - **Temperature-sensitive:** Normal at permissive temperature, defective at restrictive temperature
   - Useful for studying essential genes
4. **Resistant Mutants:**
   - Resistant to antibiotics, toxins, or viruses
   - Major clinical concern (antibiotic resistance)
5. **Lethal Mutants:**
   - Mutation causes death of organism
   - Can only be maintained if conditional or heterozygous
6. **Loss-of-Function Mutants:**
   - Gene product loses its normal function
   - Can be partial or complete loss
7. **Gain-of-Function Mutants:**
   - Gene product acquires new function or enhanced activity
   - Often dominant mutations

Causes of Mutations

1. Spontaneous Mutations:

- DNA replication errors (despite proofreading)
- Spontaneous DNA damage (depurination, deamination)
- Tautomeric shifts (rare forms of bases pair incorrectly)
- Slippage during replication (especially in repeat regions)

2. Induced Mutations (Mutagens):

a) Physical Mutagens:

- **UV radiation:** Causes thymine dimers
- **X-rays and gamma rays:** Cause strand breaks and base damage
- **Heat:** Increases spontaneous mutation rate

b) Chemical Mutagens:

- **Base analogs:** 5-bromouracil, 2-aminopurine (incorporated during replication)
- **Alkylating agents:** EMS, MMS (add alkyl groups to bases)
- **Intercalating agents:** Acridine dyes, ethidium bromide (slip between bases causing frameshift)
- **Deaminating agents:** Nitrous acid (converts cytosine to uracil)
- **Hydroxylating agents:** Hydroxylamine (modifies cytosine)

c) Biological Mutagens:

- Transposons (jumping genes)
- Viral insertions

Detection and Screening of Mutants

1. **Replica Plating:** Transfer colonies from master plate to test plates to identify auxotrophs
2. **Ames Test:** Tests mutagenic potential of chemicals using histidine auxotrophs
3. **Enrichment Techniques:** Select for mutants by killing wild-type (e.g., penicillin enrichment)
4. **Direct Selection:** Mutants grow under selective conditions (antibiotic resistance)

Significance of Mutations

- **Evolution:** Provide raw material for natural selection
- **Genetic Research:** Study gene function by observing loss of function
- **Strain Improvement:** Enhance antibiotic production, enzyme activity
- **Disease:** Many diseases result from mutations (cancer, genetic disorders)
- **Antibiotic Resistance:** Major clinical challenge from bacterial mutations
- **Biotechnology:** Create mutants for industrial processes

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