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PHARMACEUTICAL MICROBIOLOGY

UNIT 3

TOPIC :

- Study of morphology, classification, reproduction/replication and cultivation of Fungi and Viruses.
  Classification and mode of action of disinfectants
  Factors influencing disinfection, antiseptics and their evaluation.
  For
  bacteriostatic and bactericidal actions
  Evaluation of bactericidal & Bacteriostatic.
  Sterility testing of products (solids, liquids, ophthalmic and other sterile
  products) according to IP, BP and USP.

Fungi

- Fungi are eukaryotic microorganisms that include microbes such as *Yeasts, Moulds, and Mushrooms*.
  They are distinct from plants, animals, and bacteria in their structural and nutritional characteristics.
- Cell Wall:
  The cell wall of fungi is mainly composed of chitin, glucans, mannans, and glycoproteins, which provide rigidity and protection.
- Nutrition:
  They are heterotrophic in nature, meaning they cannot synthesize their own food.
  - They obtain nutrients by absorbing organic material from dead or living organisms.
  - Modes of nutrition: saprophytic (from dead matter), parasitic (from host organisms), and symbiotic (mutual relationship with other organisms).
- Cellular Organization:
  - Unicellular forms: e.g., Yeasts
  - Multicellular forms: e.g., Moulds, Mushrooms
- Reproduction:
  Fungi reproduce by both:
  - Asexual methods: via spores (conidia, sporangiospores, budding in yeast).
  - Sexual methods: via fusion of gametes or nuclei, producing zygospores, ascospores, or basidiospores depending on fungal group.
- Branch of Study:
  The study of fungi is known as Mycology.

Morphological Classification of Fungi

According to morphology, fungi are mainly classified into four types:

1. Yeasts

- Unicellular fungi (oval or spherical in shape).
- Reproduce mainly by budding or fission.
- Example: *Saccharomyces cerevisiae* (Brewer's yeast, used in fermentation).

2. Yeast-like Fungi

- Exist mainly as unicellular organisms but can form elongated chains of budding cells (pseudohyphae) under certain conditions.
- They resemble both yeasts and moulds.
- Example: *Candida albicans* (causes candidiasis).

3. Moulds

- Multicellular filamentous fungi.
- Composed of hyphae (thread-like structures) which collectively form a mycelium.
- Reproduce by producing various types of spores (asexual and sexual).
- Examples: *Aspergillus*, *Rhizopus*, *Penicillium*.

4. Dimorphic Fungi

- Fungi that can exist in two morphological forms:
  - As yeast form (unicellular) under parasitic/tissue conditions (usually at 37°C).
  - As mould form (filamentous) in environmental/saprophytic conditions (usually at 25°C).
- Example: *Histoplasma capsulatum*, *Blastomyces dermatitidis*.

Reproduction in Fungi

- Fungi reproduce by both asexual and sexual methods.
- The type of reproduction depends on the species and environmental conditions.
- Reproduction in fungi usually involves the formation of spores, which function somewhat like seeds in plants, helping in multiplication, survival, and dispersal.

1. Asexual Reproduction

- Most common form of reproduction in fungi.
- Occurs without the fusion of gametes.
- Produces genetically identical offspring (clones).

Methods of Asexual Reproduction

1. Budding
    - A small outgrowth (bud) develops on the parent cell, grows, and finally detaches to form a new individual.
    - Common in yeasts.
    - Example: *Saccharomyces cerevisiae*.
2. Fragmentation
    - The mycelium (hyphae) of the parent fungus breaks into small fragments.
    - Each fragment develops into a new mycelium under suitable conditions.
    - Example: *Rhizopus*.
3. Fission
    - The parent cell divides into two daughter cells by binary fission.
    - Seen in some yeasts.
    - Example: *Schizosaccharomyces*.
4. Spore Formation

- Fungi produce a variety of asexual spores, each with specific structures:
  - Sporangiospores: Produced inside sporangia (e.g., *Rhizopus*).
  - Conidiospores (Conidia): Produced externally on conidiophores (e.g., *Aspergillus*, *Penicillium*).
  - Chlamydospores: Thick-walled resting spores formed within hyphae (e.g., *Candida*).
  - Arthrospores: Formed by fragmentation of hyphae (e.g., *Coccidioides*).
  - Blastoconidia (Blastospores): Formed by budding (e.g., *Candida albicans*).

2. Sexual Reproduction

- Involves the fusion of two compatible haploid nuclei from different mating types (+ and – strains).
- Results in genetically diverse offspring, which increases adaptability.

Main Stages of Sexual Reproduction

1. Plasmogamy
   - Fusion of cytoplasm from two different mating types.
   - Produces a dikaryotic stage, where two haploid nuclei coexist in the same cell without fusing.
2. Karyogamy
   - Fusion of the two haploid nuclei.
   - Forms a diploid nucleus (2n).
   - In some fungi, this stage may be delayed, leading to a prolonged dikaryotic phase (especially in Basidiomycota).
3. Meiosis
   - The diploid nucleus undergoes meiotic division.
   - Produces haploid spores, which germinate to form new fungal organisms.

3. Sexual Spores in Fungi

1. Zygospores
    - Thick-walled resting spores.
    - Formed by fusion of two gametangia in Zygomycetes.
    - Example: *Rhizopus*.
2. Ascospores
    - Produced inside a sac-like structure called ascus.
    - Characteristic of Ascomycetes.
    - Example: *Saccharomyces cerevisiae*.
3. Basidiospores
    - Produced externally on basidia.
    - Characteristic of Basidiomycetes.
    - Example: *Agaricus* (mushroom).
4. Oospores
    - Thick-walled spores formed by fusion of oogonium (female) and antheridium (male).
    - Seen in Oomycetes.
    - Example: *Phytophthora infestans*.

Cultivation of Fungi

- Cultivation of fungi refers to the **artificial growth of fungi under controlled laboratory, greenhouse, or industrial conditions** for various applications such as food, medicine, and research.
- **Purpose of Cultivation:**
    1. **Food Production** – Production of edible mushrooms (*Agaricus bisporus*), fermentation products (bread, beer, wine, cheese).
    2. **Medicinal Applications** – Production of antibiotics (*Penicillium* → penicillin), immunosuppressants (*Tolypocladium* → cyclosporin), vitamins, enzymes.
    3. **Industrial Applications** – Citric acid (*Aspergillus niger*), ethanol (yeast fermentation), organic acids, enzymes (amylase, cellulase, protease).
    4. **Research and Teaching** – Study of fungal genetics, pathogenicity, physiology, and drug testing.

Culture Media for Fungi

The most widely used medium is Sabouraud Dextrose Agar (SDA).
It selectively supports fungal growth and inhibits most bacteria due to its slightly acidic pH (5.6).

Composition of Sabouraud Dextrose Agar (per 100 ml):

- Dextrose – 4 g → Source of carbohydrate/energy.
- Peptone – 1 g → Source of nitrogen and amino acids.
- Agar – 2 g → Solidifying agent.
- Distilled Water – 100 ml → Solvent.
- pH – Adjusted to 5.6 → Enhances fungal growth and suppresses bacterial contaminants.

 Sometimes antibiotics like chloramphenicol or gentamicin are added to inhibit bacterial contamination.

Process of Cultivation of Fungi

1. **Isolation**
    - Obtaining a pure fungal isolate from natural sources (soil, air, water, plant material, clinical specimen).
    - Techniques: *Streak plate, pour plate, dilution plate*.
2. **Inoculation**
    - Transfer of fungal spores or mycelial fragments onto sterile growth medium (SDA or liquid broth).
    - Done aseptically to avoid contamination.
3. **Incubation**
    - Plates/tubes are incubated under optimal growth conditions:
        - **Temperature:** 25–30°C for most fungi; 37°C for pathogenic fungi.
        - **pH:** 4.5–6.0 (slightly acidic).
        - **Aeration:** Most fungi are aerobic; require oxygen supply.
    - Duration: 3–7 days for yeasts, 1–3 weeks for moulds.
4. **Observation & Identification**
    - Colony morphology (color, texture, growth rate).
    - Microscopic examination (Lactophenol cotton blue staining).
    - Biochemical tests or molecular methods for confirmation.

Applications of Cultivated Fungi

- **Pharmaceuticals:** Antibiotics (penicillin, griseofulvin), statins, immunosuppressants.
- **Industrial Enzymes:** Amylases, cellulases, proteases for food and textile industries.
- **Food Industry:** Fermented foods, alcoholic beverages, citric acid, edible mushrooms.
- **Medical Research:** Studying pathogenic fungi (*Candida, Aspergillus*).

Viruses

- Viruses are **microscopic infectious agents** that can only replicate inside the living cells of an organism.
- They are **not true living organisms**; instead, they are considered **obligate intracellular parasites**, meaning they completely depend on the host cell's machinery for reproduction.
- They contain **either DNA or RNA** as genetic material (never both).
- Size: typically **20 nm to 300 nm**, much smaller than bacteria.
- They can infect **all forms of life**: animals, plants, and even microorganisms (bacteria, protozoa, fungi).

Structure of a Virus

A fully assembled, infectious virus is called a virion. It consists of:

1. Core – the genetic material, which can be DNA or RNA.
2. Capsid – the protein coat that surrounds and protects the genetic material.
    - Shapes: icosahedral, helical, or complex.
3. Envelope (in some viruses) – a lipid membrane derived from the host cell.
    - Embedded with viral glycoproteins that help in attachment and entry.

Classification of Viruses

Viruses can be classified on the basis of:

1. **Based on Host**

- Animal Viruses – infect animals (e.g., Rabies virus, Influenza virus).
- Plant Viruses – infect plants (e.g., Tobacco mosaic virus).
- Bacteriophages – infect bacteria (e.g., T4 bacteriophage).

2. **Based on Morphology**

- **Icosahedral Viruses** – spherical, highly symmetrical shape.
  Examples: Adenovirus, Poliovirus.
- **Helical Viruses** – rod-like or filamentous shape.
  Examples: Rabies virus, Tobacco mosaic virus.
- **Complex Viruses** – complicated structures with additional features such as tail fibers.
  Example: T4 bacteriophage.
- **Enveloped Viruses** – have an outer lipid membrane (envelope) derived from host.
  Example: Influenza virus, HIV.
- **Non-enveloped (Naked) Viruses** – lack an envelope.
  Example: Norovirus.

Viral Replication (Life Cycle of a Virus)

Viral replication is a **stepwise process** consisting of:

1. **Attachment (Adsorption)**
   - Virus binds to specific receptors on the host cell surface using viral proteins/glycoproteins.
2. **Entry (Penetration)**
   - Viral particle or its genetic material enters the host cell.
   - Mechanisms: direct fusion, endocytosis, or injection (in bacteriophages).
3. **Uncoating**
   - Viral capsid is removed, releasing the viral genome into the host cell cytoplasm/nucleus.
4. **Replication (Genome Synthesis)**
   - Viral genome is replicated using host cell machinery (DNA polymerase or RNA-dependent enzymes).
5. **Biosynthesis (Protein Synthesis)**
   - Viral proteins are synthesized by host ribosomes according to viral genetic instructions.

6. **Assembly (Maturation)**
    - Newly formed viral genomes and proteins assemble into complete virions.
7. **Release**
    - Viruses exit the host cell to infect new cells:
        - **Budding (Exocytosis):** Enveloped viruses acquire lipid envelope while leaving.
        - **Cell Lysis:** Non-enveloped viruses rupture host cell.

Cultivation of Viruses

- Viruses are obligate intracellular parasites, meaning they can only grow and replicate inside living host cells.
- Unlike bacteria and fungi, they cannot be grown on artificial nutrient media.
- For cultivation, living systems are required such as whole animals, embryonated eggs, or cell cultures.
- Viral cultivation is essential for:
    - Studying viral properties
    - Producing vaccines
    - Research on pathogenesis
    - Laboratory diagnosis of viral infections

Methods of Viral Cultivation

1. Cultivation in Animals
    - Viruses are inoculated into living animals such as mice, rabbits, guinea pigs, etc.
    - Used mainly for:
        - Pathogenicity studies (how a virus causes disease)
        - Vaccine development
    - *Example:* Rabies virus grown in mice.

2. **Cultivation in Embryonated Eggs**
    - One of the most widely used methods, especially for vaccine production.
    - A fertilized chicken egg (7–10 days old) is used.
    - Virus is injected into different sites depending on type:
        1. Chorioallantoic Membrane (CAM): for Poxviruses, Herpesviruses.
        2. Amniotic Sac: for Influenza virus.
        3. Allantoic Cavity: for Newcastle disease virus, Influenza virus.
        4. Yolk Sac: for Yellow fever virus, some Arboviruses.

    Advantages:
    - Cost-effective and easily available.
    - Provides a sterile, nutrient-rich environment.
    - Suitable for large-scale vaccine production (e.g., Influenza vaccines).
3. **Cultivation in Cell Cultures**
    - Involves growing viruses in laboratory-maintained cell lines derived from animal or human tissues.
    - *Example:* Monkey kidney cells for Poliovirus.
    - Types of cell cultures:
        - **Primary Cell Culture:** Directly derived from animal tissue; short-lived but closest to natural cells.
        - **Diploid (Semi-continuous) Cell Lines:** Limited lifespan, retain normal chromosome number.
        - **Continuous Cell Lines:** Immortalized cells, can be maintained indefinitely (e.g., HeLa cells).

    Advantages:
    - Useful for virus isolation and diagnostic work.
    - Allows detailed study of viral replication cycle.

    Disadvantages:
    - Technically demanding.
    - Cells are short-lived (except continuous lines).
    - Risk of contamination.

Disinfection

- Disinfection is the process of eliminating or reducing harmful microorganisms (such as bacteria, viruses, fungi) on inanimate objects and surfaces.
- It does not necessarily kill all microorganisms, but reduces their number to a level considered safe by public health standards.
- Disinfection is mainly carried out using chemical agents, which are divided into:
    1. Disinfectants
    2. Antiseptics

Disinfectants

- Chemicals used to kill or inhibit harmful microorganisms on inanimate (non-living) objects or surfaces.
- Generally stronger than antiseptics and may be too harsh for living tissues.
- Examples: Bleach (sodium hypochlorite), Hydrogen peroxide, Alcohol-based cleaners, Formaldehyde.

Antiseptics

- Chemicals used to prevent or stop the growth of microorganisms on living tissues such as skin or mucous membranes.
- They are less harsh than disinfectants and safe for living cells.
- Examples: Hand sanitizers (alcohol-based), Iodine solution, Chlorhexidine.

Ideal Characteristics of a Disinfectant

An ideal disinfectant should:

- ✓ Have a broad spectrum of activity (effective against bacteria, viruses, fungi).
- ✓ Be fast-acting.
- ✓ Be non-toxic and safe for humans/animals.
- ✓ Be effective at all pH levels.
- ✓ Be stable and retain activity over time.
- ✓ Have a long shelf life.
- ✓ Have high penetration power into surfaces.
- ✓ Be inexpensive and easily available.
- ✓ Have a pleasant odor and appearance.

Classification and Mode of Action of Disinfectants

1. **Phenols & Phenolic Compounds**
    - *Mode of Action:* Disrupt cell membranes and denature proteins.
    - *Example:* Phenol, Lysol (cresol).
    - **Uses:**
        1. Disinfecting floors, surfaces, hospital equipment.
        2. Used in soaps and antiseptic solutions.
    -
2. **Alcohols**
    - *Mode of Action:* Denature proteins and dissolve lipids → damage cell membranes.
    - *Example:* Ethanol, Isopropyl alcohol.
    - **Uses:**
        1. Skin antiseptic before injections.
        2. Disinfection of thermometers, medical instruments.

3. Hand sanitizers.

3. **Aldehydes**
   - *Mode of Action:* Inactivate proteins and nucleic acids by alkylation.
   - *Example:* Formaldehyde, Glutaraldehyde.

   Uses:
   4. Disinfection of hospital instruments and dialysis equipment.
   5. Preservation of vaccines and biological specimens.
   6. Formaldehyde fumigation of operation theatres.
   -

7. **Halogens**
   - *Mode of Action:* Oxidize and denature proteins.
   - *Example:* Chlorine compounds (bleach), Iodine.

   Uses:
   8. Water disinfection (chlorine).
   9. Wound cleaning (iodine solution).
   10. Surface disinfection in hospitals.
   -

11. **Heavy Metals**
   - *Mode of Action:* Bind to proteins and inactivate enzymes.
   - *Example:* Silver nitrate, Mercuric chloride.

   Uses:
   - 1% Silver nitrate for prevention of ophthalmia neonatorum (eye infection in newborns).
   - Copper sulfate for controlling algae in reservoirs.
   - Mercury salts (historical use, now limited due to toxicity).
   -

12. **Oxidizing Agents**
   - *Mode of Action:* Release oxygen radicals that damage cell structures.
   - *Example:* Hydrogen peroxide, Potassium permanganate.

Uses:
- Wound cleansing (H_2O_2).
- Gargles and mouthwash ($KMnO_4$).

- Water treatment (ozone).

13. **Dyes**
    - *Mode of Action:* Interfere with microbial cell wall and protein synthesis.
    - *Example:* Crystal violet, Malachite green.

    Uses:

    14. Treatment of skin infections (fungal).
    15. Used in selective culture media (inhibit bacteria, allow fungi).

16. **Detergents (Surface-active agents)**
    - *Mode of Action:* Disrupt cell membranes and cause leakage of cell contents.
    - *Example:* Quaternary ammonium compounds (Cetrimide, Benzalkonium chloride)

    Uses:

    - Disinfection of floors, surfaces, surgical instruments.
    - Used in mouthwashes and skin antiseptics.

Factors Influencing Disinfection

The effectiveness of a disinfectant depends on several factors:

1. **Concentration of Disinfectant**
   - Higher concentration generally increases microbial killing.
   - However, excessively high concentration may reduce activity (due to coagulation of proteins preventing penetration).
2. **Temperature**
   - Most disinfectants are more effective at higher temperatures.
   - Extremely high temperatures may cause evaporation or degradation of disinfectants.
3. **Contact Time**
   - Adequate exposure time is essential for killing/inhibiting microorganisms.
   - Shorter times may result in incomplete disinfection.
4. **pH of the Environment**
   - Some disinfectants work better in acidic conditions (e.g., phenols).
   - Others are more effective in alkaline conditions (e.g., quaternary ammonium compounds).
5. **Surface Condition**
   - Smooth, clean surfaces allow better action.
   - Rough/damaged surfaces may protect microorganisms and reduce disinfectant effectiveness.
6. **Characteristics of Microorganisms**
   - Different microorganisms have different resistance.
   - Bacterial spores, Mycobacteria, and non-enveloped viruses are more resistant**.**
   - Vegetative bacteria and enveloped viruses are more susceptible.
7. **Water Hardness**
   - Hard water (containing Ca^{2+}, Mg^{2+}) reduces the effectiveness of some disinfectants, especially quaternary ammonium compounds**.**

Evaluation of Disinfectants

Evaluation is carried out to measure the efficacy of disinfectants. Major methods include:

1. Tube Dilution & Agar Plate Method

- Principle: Determines the minimum concentration of disinfectant that inhibits microbial growth.
- Steps:
    1. Prepare serial dilutions of disinfectant.
    2. Inoculate each tube with a standardized microbial suspension.
    3. Incubate at suitable temperature for 24–48 hours.
    4. Observe growth (turbidity) or no growth.
- Use: To determine the MIC (minimum inhibitory concentration) of disinfectants.

2. Cup Plate (Cylinder Plate) Method

- Principle: Zone of inhibition around disinfectant shows antimicrobial activity.
- Steps:
    1. Inoculate agar plates with microbial suspension.
    2. Punch wells (cups) in agar and add disinfectant solution of different concentrations.
    3. Incubate plates and measure zone of inhibition.
- Use: To compare different disinfectants and concentrations.

3. Ditch Plate Method

- Principle: Growth inhibition around a ditch containing disinfectant indicates activity.
- Steps:
    1. Streak microorganisms on agar plate.
    2. Cut a ditch in the agar and fill with disinfectant.

3. Incubate and observe inhibition zone.

- Use: Simple method for screening disinfectants.

4. Phenol Coefficient Method

- Principle: Compares the killing power of a disinfectant with standard phenol against a test organism (e.g., *Salmonella typhi*).
- Formula:

$$\text{Phenol Coefficient} = \frac{\text{Dilution of test disinfectant killing in 7.5 min but not in 5 min}}{\text{Dilution of phenol killing in 7.5 min but not in 5 min}}$$

Interpretation:

- Coefficient = 1 → Equal to phenol.
- Coefficient > 1 → More effective than phenol.
- Coefficient < 1 → Less effective than phenol.

Types:

- Rideal-Walker Test
- Chick-Martin Test (modification for organic matter).

5. Kelsey-Sykes Test

- Principle: Measures bactericidal activity of disinfectants under practical conditions.
- Steps:
  1. Mix bacterial suspension with disinfectant.
  2. At 0, 8, 18, and 28 minutes, transfer samples into recovery medium.
  3. Incubate to check for surviving organisms.
- Use: Mimics real-life situations with repeated contamination.

Bacteriostatic vs Bactericidal Actions

- **Bacteriostatic Action**
  - Inhibits growth and multiplication of bacteria but does not kill them.
  - Microorganisms resume growth once the agent is removed.
  - Examples: Chloramphenicol, Tetracyclines.
- **Bactericidal Action**
  - Kills microorganisms directly and irreversibly.
  - Preferred in severe infections or when immune response is weak.
  - Examples: Penicillins, Aminoglycosides.

Sterility Testing of Products

- Sterility testing is a microbiological test performed to demonstrate that a product is free from viable contaminating microorganisms (bacteria, fungi, spores).
- Required for parenterals, ophthalmic preparations, implants, and other sterile dosage forms.
- Performed according to pharmacopeial standards: IP (Indian Pharmacopoeia), BP (British Pharmacopoeia), USP (United States Pharmacopeia).

Products Requiring Sterility Testing

1. **Solids** – e.g., powders for injection, implants.
2. **Liquids** – e.g., injections, infusions.
3. **Ophthalmic Preparations** – eye drops, ointments.
4. **Other Sterile Products** – surgical dressings, sutures, medical devices.

Pharmacopeial Guidelines (IP, BP, USP)

All three pharmacopeias describe **two main methods** for sterility testing:

1. Membrane Filtration Method

- **Principle:** Product is filtered through a 0.45 µm or finer membrane filter, which retains microorganisms. The filter is then placed in culture media.
- **Suitable for:** Large volumes, filterable aqueous/oily solutions, and products containing preservatives.

Procedure:

- Pass the test solution through sterile membrane filter.
- Wash filter with sterile fluid to remove inhibitory substances.

- Divide the filter into two parts and place in:
  - **Fluid Thioglycollate Medium (FTM):** For detection of anaerobic and aerobic bacteria.
  - **Soybean–Casein Digest Medium (SCDM / TSB):** For detection of fungi and aerobic bacteria.
- Incubate for **14 days**:
  - FTM at **30–35°C**.
  - SCDM at **20–25°C**.
- Observe for turbidity (microbial growth).

2. Direct Inoculation (Direct Transfer) Method

- **Principle:** A portion of the product is directly inoculated into culture media.
- **Suitable for:** Small-volume products, solids, non-filterable preparations.

Procedure:

- Transfer specified quantity of test sample directly into:
  - FTM (30–35°C, 14 days).
  - SCDM (20–25°C, 14 days).
- Observe for visible microbial growth.

Culture Media Used

1. **Fluid Thioglycollate Medium (FTM):**
   - Detects **aerobic and anaerobic bacteria**.
   - Incubation: 30–35°C.
2. **Soybean–Casein Digest Medium (SCDM / TSB):**
   - Detects **aerobic bacteria and fungi (molds & yeasts)**.
   - Incubation: 20–25°C.

Precautions (as per IP, BP, USP)

- Test must be performed under aseptic conditions **in a** cleanroom/laminar airflow hood**.**
- Proper environmental monitoring should be done.
- Negative controls (media without product) must be incubated to ensure media sterility.
- Growth promotion test (inoculating media with standard microorganisms) must be performed to confirm media suitability.

Interpretation of Results

- **Sterile:** No turbidity or growth in media after 14 days incubation.
- **Non-Sterile:** Visible growth (turbidity, sediment, pellicle, or surface colonies).
- If growth occurs, investigation must confirm whether it is due to product contamination or procedural error.

Comparison – IP, BP, USP

- All three pharmacopeias prescribe Membrane Filtration as the preferred method (for filterable products) and Direct Inoculation as an alternative (for non-filterable products).
- Incubation period: 14 days in all three.
- Media: Fluid Thioglycollate Medium + Soybean–Casein Digest Medium.
- Temperature: FTM (30–35°C), SCDM (20–25°C).