UNIT- V

(CHAPTER-14)

Introduction and Types of Spoilage

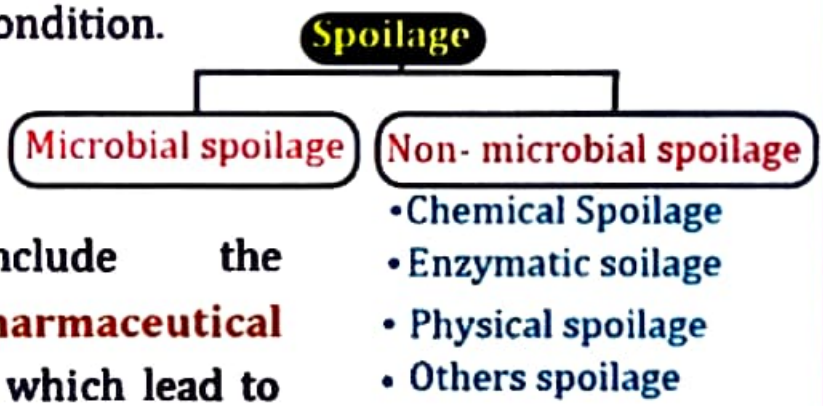
Points to be covered in this topic

1. INTRODUCTION
2. TYPES OF SPOILAGE
   - ✓ Enzymatic spoilage:
   - ✓ Physical spoilage:
   - ✓ Chemical spoilage:
3. SPOILAGE OF FRUITS AND VEGETABLES
4. FACTORS AFFECTING THE MICROBIAL SPOILAGE OF PHARMACEUTICAL PRODUCTS
5. SOURCES AND TYPES OF MICROBIAL CONTAMINANTS
6. PRESERVATION OF PHARMACEUTICAL PRODUCTS USING ANTIMICROBIAL AGENTS
7. ASSESSMENT OF MICROBIAL CONTAMINATION AND SPOILAGE

❑ INTRODUCTION OF PHARMACEUTICAL SPOILAGE

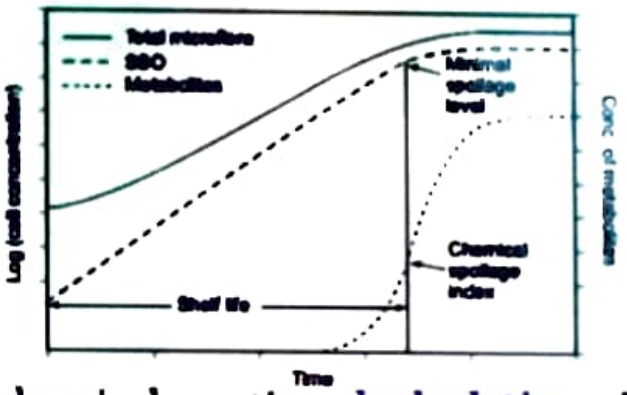
- **Spoilage** is **waste or scrap** arising from the production process. The term is most commonly applied to raw materials that have a **short life span**, such as **food used in the hospitality industry.**
- Normal spoilage is the standard amount of waste or scrap that is caused by **production**, and which is difficult to avoid. **Abnormal spoilage exceeds the normal or expected rate of spoilage.**
- **For example,** an **overcooked meal** cannot be served to a customer, and so is instead classified as abnormal spoilage.
- **Hence,** spoilage is a **complex reaction** in which a combination of microbial and biochemical activities are interact. Legally they are known as **spoilage**. These substandard **drugs when proceed further in order to be saleable as goods units are known as defectives.**
- **Examples:** Rancid meat, sour milk, moldy cheese etc.
- The spoilage is mainly determined based on microbial colonization form which is depends on the characteristics of products, method of processing and the storage condition.

❖ TYPES OF SPOILAGE



- Microbial Spoilage include the **contamination of Pharmaceutical products** with the microbes which lead to spoilage of the product affecting Drug safety and quality, and is not intended for use.
- Shortly Microbial Spoilage is defined as deterioration of pharmaceutical products by the contaminant microbe.

Enzymatic spoilage:



- It is based on **enzymatic reactions** occurred in foods and changes in chemical nature and resulted rancidity.
- This rancidity occurred by two types of chemical reactions **hydrolytic** and **oxidative rancidity**.
- **For example:** Triglyceride upon hydrolytic reaction in presence of lipase enzyme forms **glycerol and free fatty acid**.
- Further, **Linolenic acid** with various oxidative reactions forms 3-(E)-hexenal and 12-oxo-9 (Z) dodecenoic acid with the help of **lipoxygenase and hydroperoxide lyase**.

ENZYMES	FOODS	SPOILAGE ACTION
Lipase	Milk, oils	Hydrolytic rancidity
Thiaminase	Meat, fish	Thiamine destruction
Peroxidases	Fruits	Browning
Proteases	Egg, crab	Reduction of shelf life
Lipoxygenases	Vegetables	Destruction of vitamin A

Physical spoilage:

- It occurs due to **temperature, light, relative humidity** and results in **mechanical damage** of the food components.
- **For example:** Oxidation of food occurs due to light and changes colour, flavour and chemical nature, like greening of potatoes, sunlight flavour in milk, loss of vitamin D, E etc.

Chemical spoilage:

- It is based on **non-enzymatic chemical reaction** occurred within the foods and resulted change in flavor.
- **Chemical reactions** in food are responsible for changes in the **colour and flavour** of foods during processing and storage.

- **Others spoilage:** It occurs due to insects, rodents, birds, and other animals and resulting in changes in colour, odour, and chemical nature.
- Based on rate of spoilage, they are classified into three types like high perishable, semi-perishable and stable or non-perishable spoilage.
  - (i) High perishable: Meat, fish, poultry, eggs, milk, fruits and vegetables
  - (ii) Semi-perishable: Potatoes, some apple varieties, nutmeats.
  - (iii) Stable or non-perishable: Sugar, flour, dry beans.

❖ Spoilage of fruits and vegetables

- Fruits and vegetables are rich source of energy, body-building nutrients, vitamins and minerals. Protected mechanically by the pectins which constitute a protective gum between the cells and gives firmness.
- Spoilage in fruits and vegetable starts with the hydrolysis of the pectin.
- Once the pectinases have damage the structure of the fruit/vegetable, other organisms start to contribute to the soft rot.

FOODS	TYPE OF SPOILAGE	SPOILAGE MICROORGANISMS
Fresh Meat	Putrefaction	*Clostridium, Pseudomonas, Proteus*
	Souring	*Chromobacterium, Lactobacillus*
Cured Meat	Mouldy	*Penicillium, Aspergillus, Rhizopus*
	Souring	*Pseudomonas, Micrococcus, Bacillus*
	Slimy	*Leuconostoc*
Fish	Discolouration	*Pseudomonas*
	Putrefaction	*Chromobacterium, Halobacterium*
Fresh vegetables and fruits	Bacterial soft rot	*Pseudomonas spp.*
	Gray mould rot	*Botryitis cinerea*
Milk	Bitterness	Pseudomonas spp.
	Souring	Lactobacillus thermophilus
Canned food	Thermophilic acid	Clostridium thermosaccrolyticum
	Sliminess	Yeast, molds

❖ FACTORS AFFECTING THE MICROBIAL SPOILAGE OF PHARMACEUTICAL PRODUCTS

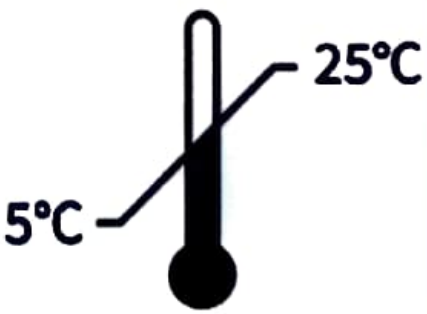
- There are so many factors which affects Microbial spoilage. Some of these factors **reduce rate of spoilage** where as some **factors increases the rate of spoilage.**
- These factors are related to **nutritional requirement of micro-organisms**, environment and nature of micro-organism.
- There factors must be studied to minimize the **impact of spoilage**. Following factors affect the microbial spoilage of Pharmaceutical products.

➢ pH

- **Extremes** of **pH prevent microbial attack**. They grow at neutral pH, therefore acidic or alkaline formulations are less susceptible to spoilage.
- Around neutrality bacterial spoilage is more likely, with reports of **pseudomonas and related Gram-negative** bacteria growing in antacid mixtures, flavoured mouth washes and in **distilled or demineralized water**. Above **pH-8**, the spoilage is rare for **soap-based emulsions.**
- Products with low pH levels such as the **fruit juice-flavoured syrups are attacked by mould or yeast.**
- Yeasts are metabolizes of organic acids and raise the pH to levels where secondary bacterial growth occurs. In food industry, low pH adjustment is made to preserve foodstuffs only.

➢ Storage Temperature

- The **actual storage temperature** determines the spoilage by **particular types of microorganisms**. Spoilage of pharmaceuticals occurs potentially over the range of about **20°C to 60°C.**
- Storage in a deep freeze at **−20°C** or lower is used for **long-term storage** of foodstuffs and some pharmaceutical raw materials, and dispensed total parenteral nutrition feeds are stored in hospitals for **short periods at −20°C** to even **further minimize the risk of spoilage.**



Nutritional Factors

- Many spoilage microorganisms have simple nutritional requirements and metabolic adaptability which enables them to utilize many formulation components as substrates for biosynthesis, growth and also trace materials contained in them.
- The use of animal products and crude vegetable materials in a formulation provides an additionally nutritious environment.
- Demineralized water prepared by ion-exchange methods, contains sufficient nutrients to allow significant growth of many water-borne Gram-negative bacteria like *Pseudomonas spp.*

Water

- It is the most important cause of the survival and growth of micro-organisms.
- Some solute-rich medicines such as syrups appear to be 'wet', microbial growth in them may be difficult since the microbes have to compete for water molecules with the large numbers of sugar and other molecules of the formulation which also interact with water via hydrogen bonding.
- An estimate of the proportion of the non-complexed water in a formulation available to equilibrate with any microbial contaminants and facilitate growth can be obtained by measuring its water activity (Aw).

$$A_w = \frac{\text{Vapour pressure of formulation}}{\text{Vapour pressure of water under similar condition}}$$

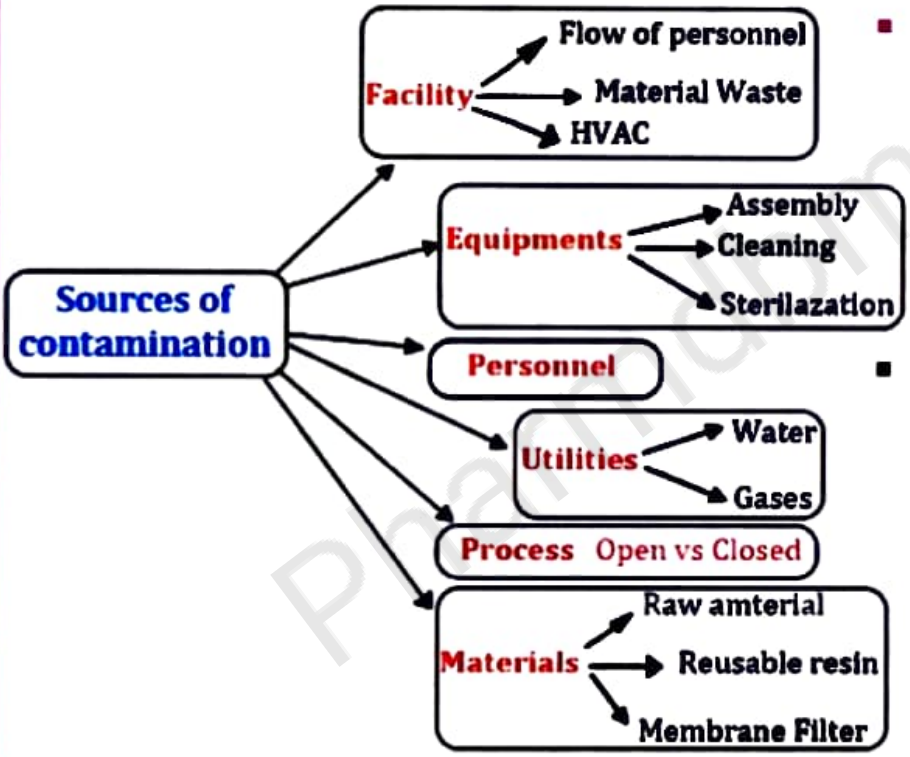
Redox Potential

- The ability of microbes to grow in an environment is influenced by its oxidation-reduction balance since they require compatible terminal electron acceptors to permit function of their respiratory pathways.
- The redox potential in viscous emulsion is high due to the high solubility of oxygen in most fats and oils.
- It has a major influence on microbial stability of some formulations in controlling the access of contaminants during both storage and use.
- The most important dosage form such as parenteral drugs is protected because of the high risks of infection by this route.

- **Self-sealing rubber closures** are used to **prevent microbial entry into multi-dose injection containers** following withdrawals with a hypodermic needle.
- Wide-mouthed cream jars are replaced with **narrow nozzle** and **flexible screw capped tubes** to remove the likelihood of operator-introduced contamination during use.
- Other factors affecting microbial spoilage of pharmaceutical products include:

• Relative Humidity • Oxygen Availability
• Osmotic Pressure • Surface Tension

❑ SOURCES AND TYPES OF MICROBIAL CONTAMINANTS



- **Source and types** of microbial contaminants Microbes are very important part of our environment.
- They are present almost **anywhere and everywhere.**

- So, contamination may occur to **pharmaceutical products** in large scale manufacturing, in small scale hospital manufacturing or during use by the patient.
- Following are the different source of microbial contamination in pharmaceutical products.
- **In large scale manufacturing:** - In large scale manufacturing as well as medium and small scale manufacturing contamination may occur from following sources.

➢ Water

- Water is a **major source of contamination**. Common water borne micro-organism like ***Pseudomonas, Achromo bacteria*** and other low demand gram negative groups are present in portable water as well as in purified water.

- Ion-exchange column may be contaminated by water source and micro-organism may **multiply there to contaminate purified water**.

➢ Raw materials

- Pharmaceutical products are **prepared from varieties of raw materials**. Clays and earth materials like **Bentonite, kaolin** ete may contain **anaerobia spores** like Clostridium sp. Starch may contain **coliform batteria** like E. Coli. Gums may contain **actinomycetes**.
- Animal Products may contain a variety of bacterial like ***E. coli, Salmonella sp*** ete.

➢ Equipments

- Equipments of manufacturing may contain microbes **if it is not sterilized properly. Grinder, blender, filter** etc may contain non-specific and local communities of micro-organism.

➢ Containers

- Containers may cause contamination if it is not sterile. In hospital manufacturing -**In hospital manufacturing water and environment are the major source of contaminants.**
- In hospitals, water is stored in storage tank which may develop **fungus, bacteria and algi type of microbes.**
- Hospital air may be contaminated with **pathogenic microorganism** due to the presences of infected patients and numerous visitors.

❖ Herman source

- Pharmaceutical products **may be contaminated during use.** Patient may self contaminate his medicine.
- Contaminants may travel to other **patients through doctors nurses** ete.

❑ PRESERVATION OF PHARMACEUTICAL PRODUCTS USING ANTIMICROBIAL AGENTS

- Antimicrobial agents are those substances which **can kill or inhibit growth of micro-organism.** These antimicrobial agents are included in formulation in order to **minimize levels of contaminated micro-organism**. These antimicrobial agents are called **preservatives**. These preservatives can generally prevent or kill low levels of **contamination**.
- Preservatives are not used in those formulations which has **low risk of contamination and subsequent microbial growth.**
- Formulations which contains **high level of acid, alkali, sugar** etc may **not require preservative.**
- Formulations of antibiotics and **other anti-microbial substances may not require preservative.**

❖ Air of the manufacturing area

- Air is filled with billions of suspending particles and microbes. **Fungus spores, like Penicillium, mucor, Aspergillus ete.**
- Bacterial spores like ***Bacillus sp.*** etc are also present. These spores and **micro-organism may contaminate pharmaceutical products.**
- This type of contamination is **minimized by practice of manufacturing in clean room** and in aseptic room under continuous flow of sterile air through HEPA filter.

❖ Personnel

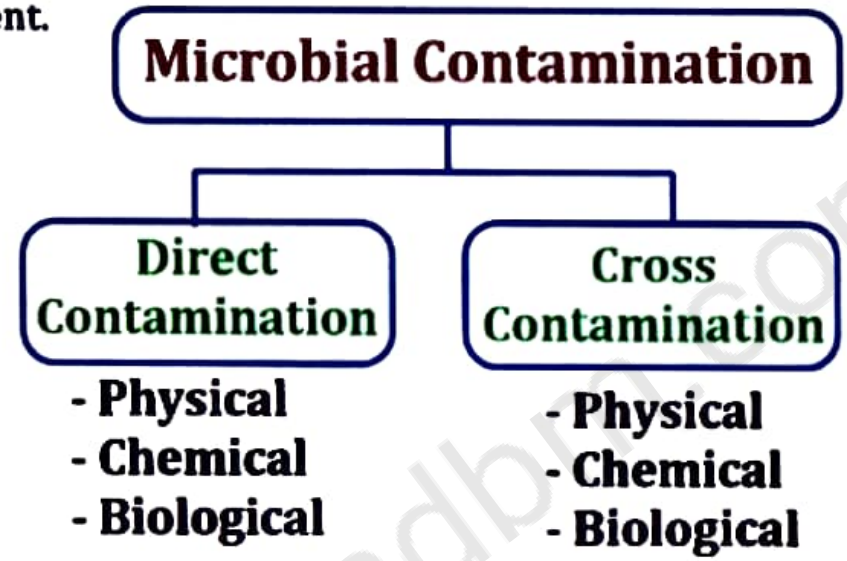
- Manufacturing **staff** may also contaminate **pharmaceutical products.**
- Personnel may be infected with various types of infections like ***coliform bacteria, staphylococci, strepto-cocci, Actino bacteria, Candida.***
- This types of contamination may be minimized by proper health check-up, **vaccination and hygiene of the personnel**. Protective gear and pooper training of the personnel may also minimize the contamination.

❖ TYPES OF MICROBIAL CONTAMINANT

- Microbial contamination is broadly classified in to direct contamination and cross contamination **(Flowchart 2).**

(a) Direct contamination: Contamination occurred by microbial components and poorly maintained heating, ventilation and air conditioning system.

(b) Cross contamination: It is the process by which microbes are spread indirectly from one to another through improper and unsterilized equipment.



❖ DIRECT CONTAMINATION IS CLASSIFIED AS FOLLOWS

(a) Direct Physical contamination: **Examples:** Particles, fibres, metal parts etc.

(b) Direct chemical contamination: **Examples:** Moisture, Gases, Vapors etc.

(c) Direct biological contamination: **Examples:** Microorganisms like bacteria, viruses, molds, fungi etc.

❖ CROSS CONTAMINATION IS CLASSIFIED AS FOLLOWS:

(a) Physical cross contamination: **Example:** Leakage of oil seal from the reactor.

(b) Chemical cross contamination: **Examples:** Moisture content is increased when a product exposed to high relative humidity.

(c) Biological cross contamination: **Example:** Improper cleaning of equipment, unclean equipments used for manufacturing process.

PHARMACEUTICAL PRODUCTS	CONTAMINANTS
Plague vaccine	*Clostridium tetani*
Serum vaccine	*Staphylococcus aureus*
Thyroid tablets	*Salmonella muenchen*
Antibiotic eye ointments	*Pseudomonas aeruginosa*
Talcum powder	*Clostridium tetani*
Saline solution	*Serratia marcescens*
Antiseptic mouth wash	*Coliforms*
Surgical dressings	*Clostridium species*
Hand cream	*Klebsiella pneumoniae*

❖ ASSESSMENT OF MICROBIAL CONTAMINATION AND SPOILAGE

- The evaluation of a medicinal product's microbiological composition is critical. A sterile product should be completely devoid of microorganisms, as determined by a sterility test.
- Non-sterile items, on the other hand, may include microorganisms. These microorganisms have the potential to be both pathogenic and non-pathogenic.
- Microorganisms like these can cause deterioration, which can pose health risks. The overall number of microorganisms present in a product must be minimal and below the allowable limit.
- To detect the existence of certain bacteria, the types and characteristics of the microbes should also be examined.
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- **Non-sterile items,** on the other hand, may include microorganisms. These microorganisms **have the potential to be both pathogenic and non-pathogenic.**
- Tests to determine the total amount of microorganisms and types of bacteria are called **microbial limit tests.**
- The following tests are carried out to evaluate **microbial contamination** and **consequent deterioration**.
- **Test of sterility** - Some pharmaceutical items should be tested for sterility, according to the **Indian pharmacopoeia**, the British pharmacopoeia, and the **United States pharmacopoeia**. The procedures are comparable with minor differences.
- The tests prescribed by the Indian Pharmacopoeia are briefly reviewed. There are two methods: **direct inoculation and membrane filtering.**

1. **Direct inoculation –**

- In this procedure, a **little amount of material is immediately introduced to the pharmacopeia-specified culture medium.** This inoculation medium is then incubated for a certain amount of time.
- **The existence of growth implies the presence of a microorganism** derived from the sample. As a result, it is possible to determine that the sample is not sterile. In the lack of any growth, a **sample is said to be sterile.**

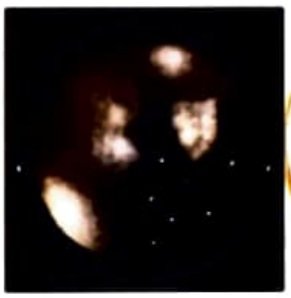
2. Membrane filtration –

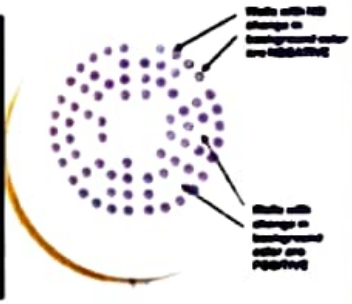
- **The material is filtered through a membrane filter and rinsed with diluting solution in this procedure.**
- **If microbes are present, they will be near the top of the filter paper.** This filter paper **is now injected into the appropriate culture medium.**
- **If growth is detected, it shows that the product is not sterile.**

- In both the above-mentioned methods, a positive and negative control test must be performed.
- **Microbial limit test** - The European Pharmacopoeia suggests assessing microorganisms both qualitatively and quantitatively. The microbiological
- Limit test is recommended by the United States Pharmacopoeia. It is divided into two sections:
  - (i) Total Aerobic Microbial Count
  - (ii) Test for Specific Microorganisms.

i) Total Aerobic Microbial Count

- In this approach, a defined amount of test sample (10gm) is combined with a specific amount of peptone water (90ml).
- This is called sample dilution. Dilutions of water-insoluble and fatty compounds must follow precise procedures. The total microbial count is determined using the following procedure:
- To begin with, the sample is filtered through a membrane filter by mixing 10 ml of distilled water with 90 ml of peptone water.
- After that, it is rinsed three times with sterile peptone water. One filter paper is incubated for 5 days at 30-35oC in Petri dish with Soyabean Caesin Digest Agar medium. Colonies are counted to ascertain bacterial count.

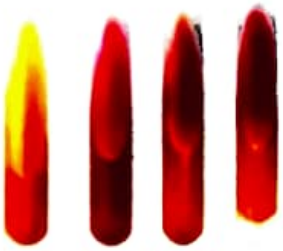




- Another filter paper is immersed in Sabouraud Dextrose Agar Media and incubated at 20-25°C for 5 days. The fungal count is then calculated.
- Second, the sample plate count technique is examined. In this approach, created dilution is directly transferred to four Petri dishes.
- There are two for bacteria and two for fungus. 15 ml of Soyabean Caesin Digest Agar medium is added to the first two Petri dishes. After 5 days of incubation at 30-35°C, colonies are counted.
- 15 ml of Sabouraud Dextrose Agar Media was transferred to the remaining two Petri dishes and incubated at 20-25°C. Colonies are tallied.

ii) Test for Specific Microorganisms

- *Escherichia coli, Salmonella, Pseudomonas aeruginea,* and *Staphylococcus aureus* are identified using specific assays.



- Mac-Conkey agar medium is used to culture *E. coli.* Colonies are distinguished by their metallic gleam.
- Salmonella colonies are detected as black or green on bismuth sulphite agar medium.
- ***Pseudomonas aeruginosa*** is recognized by growing it on cetrimide agar medium. Colonies gradually turn a greenish colour.

➢ Good Pharmaceutical Manufacturing Practice (GPMP):

- Quality Control (QC) is that part of GPMP dealing with specification, documentation and assessing conformance to specification.
- A high assurance of overall product quality is raised only from a detailed specification, control and monitoring of all the stages that contribute to the manufacturing process.
- Parametric release is accepted as an operational alternative to routine sterility testing for batch release of some finished sterile products where the manufacturer can provide assurance that the product is of the stipulated quality, based on the evidence of successful validation of the manufacturing process and review of the documentation on process monitoring carried out during manufacturing.

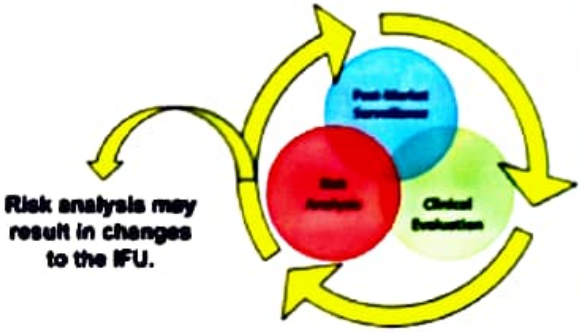
➢ Quality Assurance (QA):

- It is a combined scheme of management which embraces all the procedures necessary to provide a high probability that a medicine will conform consistently to a specified description of quality.
- It includes formulation design and development (R&D), good pharmaceutical manufacturing practice (GPMP), quality control (QC) and post-marketing surveillance.

- The risk of microbial infection and spoilage are raised from microbial contamination during manufacture and storage and hence preservatives are recommended further protection against environmental microbial contaminants but it is relatively non-specific in their reactivity.
- Laboratory tests are devised to challenge the product by used 'preservative challenge tests' where relatively large inocula of various laboratory cultures are added to aliquots of the product and determine their rate of inactivation by viable counting methods (single challenge tests).

➢ Post-market Surveillance

- It is most important stage to follow up a medicine that is smooth floating in the market without any complain by the customers.



- A proper quality assurance system is included for monitoring in-use performance and for responding to customer complaints.
- These are constantly followed up in great detail in order to decide carefully constructed and implemented schemes for product safety.

UNIT- V

(CHAPTER- 15)

PRESERVATION OF PHARMACEUTICAL PRODUCTS USING ANTIMICROBIAL AGENTS

Points to be covered in this topic

1. INTRODUCTION
2. IDEAL PROPERTIES OF PRESERVATIVES
3. CLASSIFICATION OF PRESERVATIVES
   - CLASSIFICATION OF PRESERVATIVES BASED ON MECHANISM OF ACTION
   - CLASSIFICATION BASED ON SOURCE
4. METHODS OF PRESERVATION
5. MICROBIAL STABILITY TEST
6. GROWTH OF ANIMAL CELLS IN CULTURE
7. TYPES OF ANIMAL CELL CULTURE
8. GENERAL PROCEDURE FOR CELL CULTURE
9. APPLICATION OF CELL CULTURES IN PHARMACEUTICAL INDUSTRY AND RESEARCH

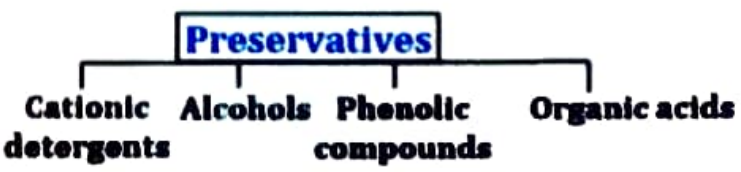
❑ INTRODUCTION

- Preservatives are the chemical substances used to improve or amplify shelf life of drugs by decreasing or lowering the oxidation of active ingredients and Excipients by reducing microbial production.
- Preservatives are substances added to various pharmaceutical dosage forms and cosmetic preparations to prevent or inhibit microbial growth.
- An ideal preservative would be effective at low concentrations against all possible micro-organism, be nontoxic and compatible with other constituent of the preparation and be stable for the shelf-life of the preparation.

❖ IDEAL PROPERTIES OF PRESERVATIVES

- ✓ It should **not be irritant.**
- ✓ It should **not be toxic**.
- ✓ It should be physically and chemically **stable**.
- ✓ It should be **compatible with other ingredients used in formulation.**
- ✓ It should be act as good antimicrobial agent and **should exert wide spectrum of activity.**
- ✓ It should act in small concentration i.e. **it must be potent.**
- ✓ It should **maintain activity** throughout product manufacturing, shelf life and usage.
- ✓ It must decrease the percentage of the microbes and prevent any re-growth **They can be:**
- ✓ Microbiostatic & Microbiocidal in nature.
- ✓ **Some preservatives are ineffective with some microbial strains and should be combined with others to be effective. Such as**
- ✓ Benzalkonium chloride, Organo-mercurial, Cetrimide, chlorhexidine and 3- cresol are combined

❖ CLASSIFICATION OF PRESERVATIVES



I. **Cationic detergents: Examples:** Benzalkolium chloride, alkyl trimethyl ammonium chloride.

II. **Alcohols: Examples:** Chlorbutanol, bronopol, phenyl and phenoxy ethanol.

III. **Phenolic compounds: Examples:** chlorinated and isopropyl derivatives of meta cresol.

IV. **Organic acids: Examples:** Salicylic acid, benzoic acid, acetic acid, lactic acid, hydroxyl benzoic acid.

❖ CLASSIFICATION OF PRESERVATIVES BASED ON MECHANISM OF ACTION

1. Antioxidants: The agents which prevent oxidation of active pharmaceutical ingredient which otherwise undergo degradation due to oxidation as they are sensitive to oxygen.

For example: Vitamin E, Vitamin C, Butylated-hydroxyanisole (BHA), Butylated-hydroxytoluene (BHT).

2. Antimicrobial agents: They are the agents that are active against gram positive and gram negative micro-organism which causes degradation of pharmaceutical preparation, active in small inclusion level.

For example: Benzoates, Sodium benzoate, Sorbates etc.

3. Chelating agents: They are the agents which form the complex with pharmaceutical ingredient and prevent the degradation of pharmaceutical formulation.

- **For example:** Disodium ethylenediaminetetraacetic acid (EDTA), Polyphosphates, Citric acid.

❖ CLASSIFICATION BASED ON SOURCE

i. Natural Preservatives: These preservatives are obtained by natural sources that are plant, mineral and animal sources etc.

Example- Neem Oil, Salt (sodium chloride), Lemon, Honey.

ii. Artificial Preservatives: These preservative are man made by chemical synthesis and active against various microorganisms in small concentration.

Example- Benzoates, Sodium benzoate, Sorbates, Propionets, nitrites.

❖ Factor affecting the efficacy and availability of preservatives:

- Temperature.
- Chemical structure of preservatives
- Capacity of preservatives.
- Inoculum size.
- Effect of pH.
- Effect of containers and packaging.
- Changes of concentration.

❖ METHODS OF PRESERVATION

➢ Physical protection:

- It is used for proper packaging of the pharmaceutical products under aseptic condition or else there is a chance for microbial growth.
- Operating persons are also an important factor for proper processing of the products under aseptic environment.

➢ Preservative coating:

- Aqueous raw materials used in the formulation of paints and coatings create the perfect environment for the growth of bacteria, fungi and yeast. They can destroy valuable pharmaceutical formulations.
- Controlling these microorganisms helps increase efficiency, helps deliver a better end-use product and helps employees and consumers avoid contact with spoilage microorganisms.
- Biocides are necessary for protecting the integrity and functionality of water-based paints and coatings from destruction by microbial contamination as a result lengthening of product's shelf life and protect dry film from algae, mold and mildew.

➢ Water proof protection:

- Packaging of the pharmaceutical products should be under water proof protection because water favours the growth of microorganisms.

Water vapour proof protection:

- This method is applicable for certain pharmaceutical products when they are packing under proper care for minimizing microbial activity.
- For 'dry' dosage forms with very low water activity (Aw) provides protection against microbial attack. The moisture vapour properties of packaging materials require careful examination.

Water vapour proof protection with desiccant:

- This method is also used for dry products that absorbed moisture from the environment and is spoiled due to growth of microorganisms.
- Packing should be proper with this method to minimize the microbial growth and spoilage of the products.

Mode of Action: Preservatives interfere with the growth, multiplication and metabolism of the microorganisms by one or more of the following mechanisms.

(i) Modifying the membrane permeability,

(ii) Denaturation of enzymes and other cellular proteins

(iii) Oxidation of cellular constituents

(iv) Hydrolysis.

- For use in pharmaceutical products the antimicrobial preservatives should be selected from those recommended in pharmacopoeias and in minimum effective concentration.

Benzalkonium chloride	Benzoic acid	Benzyl alcohol
Butyl paraben	Cetrimonium bromide	Cetylpyridinium chloride
Chlorobutanol	Chlorocresol	Cresol
Ethyl paraben	Methyl paraben	Phenol
Phenoxyethanol	Phenyl ethyl alcohol	Phenylmercuric acetate
Phenylmercuric nitrate	Potassium benzoate	Potassium Sorbate
Propyl paraben	Sodium Benzoate	Sodium propionate
Sorbic acid	Thimerosal	Thymol

- **Examples of antimicrobial preservatives commonly employed in manufacturing of pharmaceutical products include:** Methyl, ethyl, propyl and butyl parabens; Sorbic acid, Na, K & Ca Sorbate; Benzoic acid, Na, K and Ca benzoate; sodium metabisulfite, Propylene glycol, **BHT** (butylhydroxy toluene), **BHA** (Butylhydroxyanisol), benzaldehyde, essential oils, phenol and mercury compounds. Parabens are among the most commonly used antimicrobial preservatives.

❖ MICROBIAL STABILITY TEST:

- Microbial contaminants usually originate from two different sources during production and filling and during the use of the cosmetics by the consumers.
- It is necessary to carry **out routine microbiological analysis** of each batch of the finished product. The main potential pathogens in cosmetics are *Pseudomonas aeruginosa, Candida albicans, and Staphylococcus aureus.*
- These pathogens must not be detectable more than **0.1 g or 0.1 ml** in a cosmetic product.

SOME TESTS ARE AS FOLLOWS:

1. Screening test:

- It is also known as plate count or dip slide method. This method is **used to detect aerobic bacteria** in aqueous sample. Dip slide is coated on both sides with a solid agar gel medium.
- A small quantity of **TTC (2,3,5-triphenyl tetrazolium chloride)** is added to **detect aerobic bacteria in the sample.**
- The slide is dipped in to the aqueous solution for **10** seconds and excess liquid is drained off from the slide. Then it is incubated at **35-37°C for 18-48 hours.**
- The colour appeared is compared with **calibration chart.** Aerobic bacteria species grow on this medium and is detected by their ability to reduce **TTC dye to a red coloured Formosan dye.** Developing bacterial colonies are alter the **TTC dye** and are appeared as **red spots**

❖ Quantitative test:

- Quantitative tests determine the **actual count level of bacteria, molds and yeasts in cosmetic products**. This method is used for isolation of microorganisms from cosmetic products include **direct colony counts and enrichment culturing.**
- Microbial stability studies are also carried out in Pharmaceutical products. Raw materials play vital role in the **product formulation.** Pharmaceutical raw material is defined as a substance that is used in the manufacturing of pharmaceutical products.
- During the manufacture of pharmaceuticals and cosmetics, untreated raw materials are **contaminated with microorganisms beyond acceptable limits.**

- Hence it is **necessary to control microbiological contamination** from **raw materials under GMP** regulations in the pharmaceutical industry.
- These microorganisms from plants **(e.g. species of Erwinia, Pseudomonas, Lactobacillus, Bacillus or Streptococcus)** such as gum acacia, Tragacanth, agar, powdered rhubarb, and starches contains bacteria, which causes disease.
- Water plays major role in **product formulations because water is the good media for microbial growth.**
- **The water activity (Aw)** of pharmaceutical and cosmetic products is the measure of free water in the formulation. The amount of water that is in **free form is available to microorganisms for survival.**
- The measurement of Aw in pharmaceutical and cosmetic products predicts the type and number of microorganisms responsible for product degradation and maintains chemical stability.
- Water activity is determined by **dew point method and using electric hygrometer (measure relative humidity).**

❖ Antimicrobial Effectiveness Test

- This test is used for estimating of **preservative in a product**. This test is mainly used during development of formulation and in stability studies.
- In this test, a product is inoculated with a controlled quantity of specific microorganisms.
- The test then compares the level of microorganisms found on a control sample versus the test sample over a period of 28 days.
- Test organisms are used for the purpose of challenging the preservative system in a product. The suitable media are Soyabean Casein digest or **Sabouraud Dextrose Agar** used for the test. The bacteria, yeast and mold are used for this test.
- A product is inoculated or contaminated with a number of organisms between **1 × 105 (100,000) to 1 × 106 (1,000,000)** colony forming units **(CFU)** per ml of product. At various intervals, the product is tested to determine its ability to control reproduction or destroy the microorganisms.
- Test and standard are incubated at **32.5 ± 2.5°C and 22.5 ± 2.5°C** for bacteria and yeast respectively and the concentration of the microorganisms in each of the **standardized inoculum is determined by plate count method.**

➢ **Pharmaceutical Products:** For testing purposes, the USP has divided test articles into four separate categories:

Category 1 – Injections, other parenterals including emulsions, sterile nasal products made with aqueous bases or vehicles.

Category 2 – Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes.

Category 3 – Oral products other than antacids made with aqueous bases or vehicles.

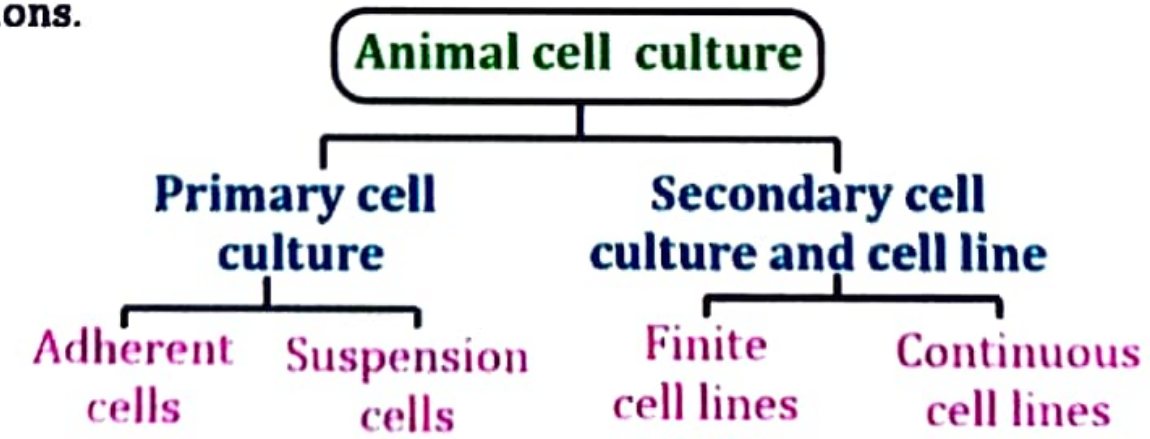
Category 4 – Antacids made with an aqueous base.

❑ GROWTH OF ANIMAL CELLS IN CULTURE

- Cell culture is a technique which involves isolation of cells from animal/plant body i.e. from their natural environment **(in vivo)** and practicing to grow isolated cells in cell specific media in plastic flask or petri dish in a controlled environmental artificial condition **(in vitro).**
- Cell culture means to **keep cells alive and grow in an in vitro condition in a nutritive media** which are widely used for research and diagnosis of different pathogens and to understand the function and mechanism of **operation of many cells.**
- Animal cell cultures are initiated by the **dispersion of a piece of tissue** into a suspension of its **component cells**, which is then added to a culture dish **containing nutrient media.**
- In animal cells **fibroblasts and epithelial cells** are selected because these cells grow very fast.
- The plastic surface of dishes is used for cell culture. In 1907, **Harrison cultivated frog nerve cells** in a lymph clot and observed the growth of nerve fibres in-vitro for several weeks and hence he is recognized as the **father of cell culture.**

❖ TYPES OF ANIMAL CELL CULTURE:

- Based on the number of cell divisions, cell culture is classified as primary cell culture and cell lines. Cell lines can undergo finite or infinite cell divisions.



Primary Cell Culture:

- This cell culture is obtained from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture.
- The culture is comprised of heterogeneous cells and most of the cells divide only for a limited time. Based on their origin, primary cells grow either as an adherent monolayer or in a suspension.

Adherent Cells :

- These cells are propagated as a monolayer and are anchorage dependent. Monolayer cultures are defined as when the bottom of the culture vessel is covered with a continuous layer of cells of one need to be attached to a solid or semi-solid substrate for proliferation.
- They adhere to the culture vessel with the use of an extracellular matrix which is derived from tissues of organs that are immobile and embedded as connective tissue.
- For example: Fibroblasts and epithelial cells.
- Majority of continuous cell lines grow as monolayers and such type of cells are transferred directly to a cover slip for examination under microscope.

Suspension Cells:

- These types of cells do not attach to the surface of the culture vessels. Hence, they are also known as anchorage independent or non-adherent cells which are grown in liquid culture medium.
- Hematopoietic stem cells and tumor cells are grown in suspension much faster which do not require frequent replacement of the medium. These cultures have short lag period.

Secondary Cell Culture and Cell Line

- Secondary culture is the culture when a primary culture is sub-cultured. It is also known as cell line or sub-clone.
- The process involves removing the growth media and disassociating the adhered cells by enzymatic treatment.

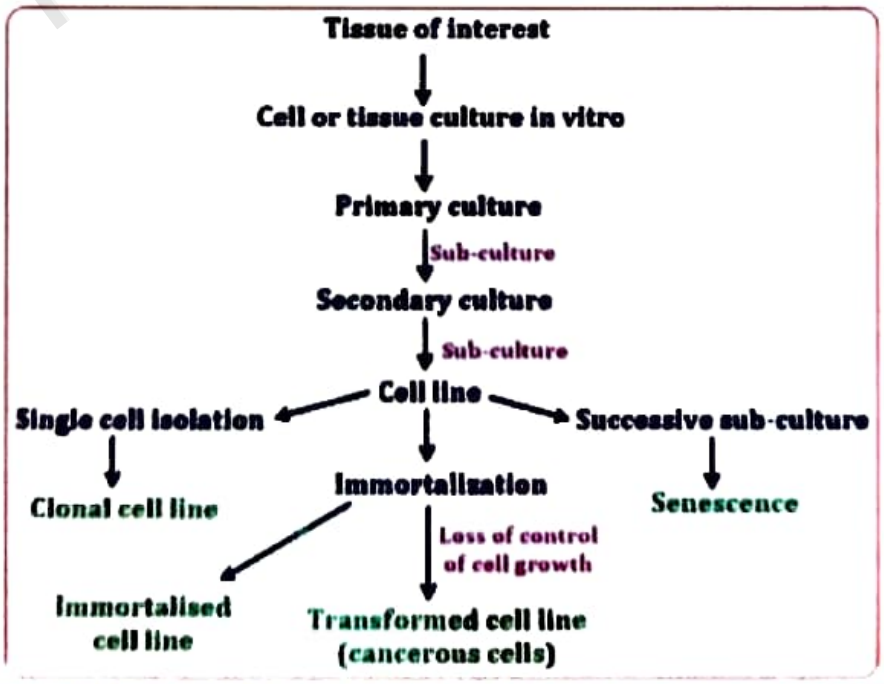
- Sub-culturing of primary cells to different divisions leads to the **generation of cell lines.** During growth period, cells with the highest **growth capacity predominate** and result in genotypic and phenotypic uniformity in the population.
- Based on the life span of the culture cell lines are two types **viz. Finite cell lines and Continuous cell lines.**

➢ Finite cell lines

- It is defined as the cell line that undergoes limited number of cell division with a limited life span. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as senescence. Cell lines derived from primary cultures of normal cells are finite cell lines.

➢ Continuous cell lines

- It is defined as a finite cell line which undergoes transformation and acquires the ability to divide indefinitely. This transformation occurs spontaneously or chemically or virally induced or from the establishment of cell cultures from malignant tissue.
- Prepared cell cultures are then sub-cultured and grown indefinitely as permanent cell lines. These cells are less adherent and fast growing as a result the cell density becomes higher and different in phenotypes from the original tissue. They grow more in suspension medium.



❑ GENERAL PROCEDURE FOR CELL CULTURE

(a) Requirements:

- Vertical Laminar air flow, Incubator, Refrigerator, Microscope, Tissue culture ware.

(b) Temperature:

- The temperature sets at as the same as body temperature of the host from which cells are procured. Most animal cells required 36-37°C.

(c) Substrate:

- Good compatible substrate is required for attachment and optimum growth. Glass and specially treated plastics are commonly used as substrate.
- Thereafter attachment factor such as collagen, gelatin, laminin etc. are used as substrate coating to improve growth and function of normal cells derived from brain, blood vessels, kidney, liver, skin etc.

(d) Culture medium:

- It is an important and complex factor for the cell growth. The culture medium is supplemented with various growth factors, pH and osmolality regulator and provides essential gases like oxygen and carbon dioxide.
- The medium is also supplemented with various nutrients like amino acids, vitamins, minerals and carbohydrates which are essential for growth of cells and provided energy for metabolism.

- Choice of media is used based on the cells being cultured. Generally, media like Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM), Glasgow Minimum Essential Medium (GMEM) are used for cell culture. Prepared media is filtered and incubated at 4°C.

(e) Media and growth requirement:

- Temperature should maintain at 37°C and optimum pH is 7.2 to 7.5. The humidity is required to be maintained properly in the media with proper gas phase ratio (Bicarbonate concentration and carbon dioxide in equilibrium).

- For growth of cultured cells light intensity also plays a vital role. Inside environment cells are cultured in dark because light induced production of toxic compound.
- Commonly used antibiotics are penicillin, streptomycin, Kanamycin etc. Trace elements like iron, zinc, selenium, sugar, amino acids, vitamins, choline, inositol etc.

(f) Selection of organ:

- Different type of cells are grown in cultures including connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin, and kidney) and many different types of tumor cells.
- On the basis of morphology (shape and appearance) or on the functional characteristics of cells, they are divided into three types.
- **Epithelial like** - attached to a substrate and appears flattened and polygonal in shape.
- **Lymphoblast like** - cells do not attach remain in suspension with a spherical shape.
- **Fibroblast like** - cells attached to an substrate appears elongated and bipolar.

(g) Culturing of cell:

- Cells are cultured as anchorage dependent or independent. Cell lines derived from normal tissues are considered as anchorage-dependent which grows only on a suitable substrate **e.g. tissue cells.**
- Suspension cells are anchorage independent e.g. blood cells whereas Transformed cell lines either grows as monolayer or as suspension.

Steps:

1. **Use sterile technique:** Tissue part is harvested and processed using sterile equipment, reagents and techniques. Personal protective equipments are used to avoid contamination. All enzymes and reagents are filtered sterile condition using a **0.22 micron membrane**.

2. Mince/cut tissue: Mince the tissue specimen into small pieces **(usually 2 × 4 mm)** with sterile scissors or scalpel, and then placed the small pieces into selected buffer, media or salt solution.

3. Wash and add enzyme: Wash tissue two to three times to eliminate excess blood proteins and then add enzyme(s) of choice, likely, collagenase, protease, papain or trypsin.

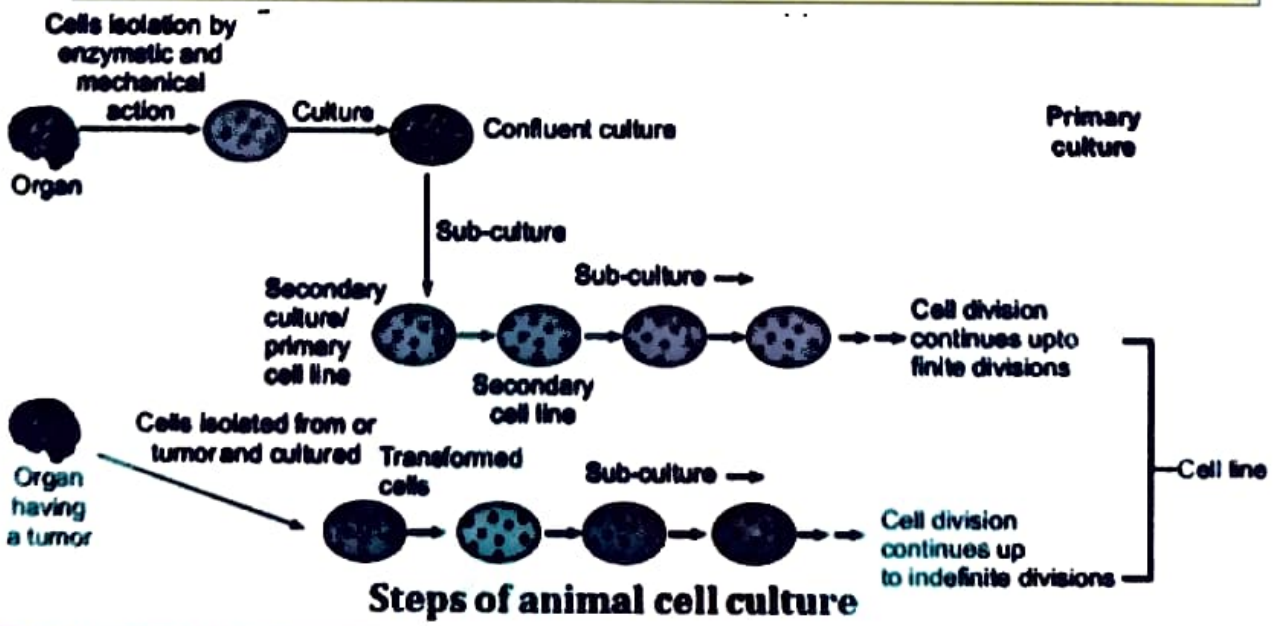
Usually about **0.5 to 1.5 mg/ml of selected enzyme is sufficient.**

4. Incubation: Further tissue specimen is incubated in optimum temperature at 37°C for 30 to 90 minutes with periodical mixed the rock specimen.

5. Disperse and wash cells: Cells are dispersed by gently pipetted them and then cell suspension is filtered using a fine mesh. The cells became settled and decanted excess liquid containing enzymes. Further cells are washed two to three times with **Fetal Bovine Serum (FBS),** Bovine Serum Albumin (BSA) or other inhibitors can also be used to halt enzyme digestion.

6. Resuspension and measure cells: Cells are resuspended in the correct medium or buffer and then quantitatively determined the cell yield and viability. This is an important step in the cell isolation process to evaluate the result by dissociation technique. Most researchers are used a haemocytometer for determining cell yield and trypan blue diazo dye to measure cell viability.

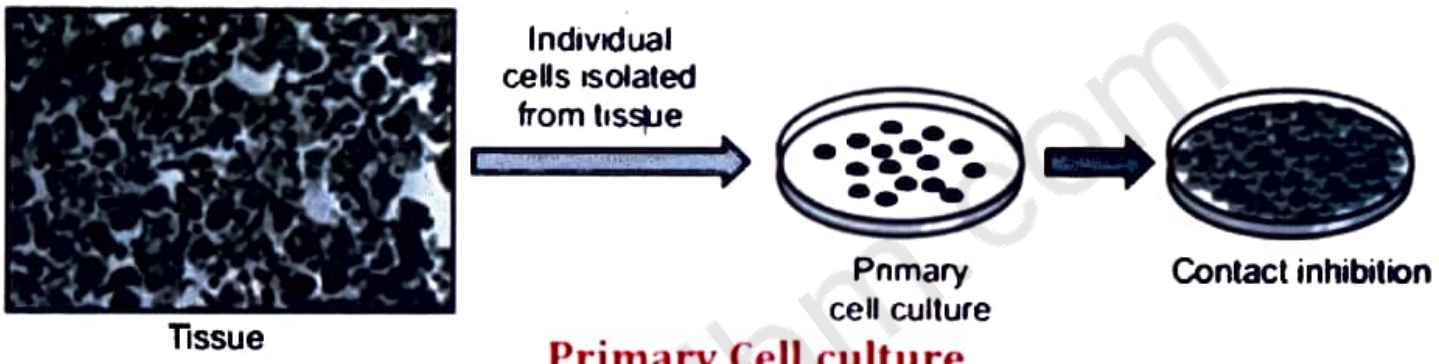
$$\%\,\text{OF VIABLE CELLS} = \frac{\text{NUMBER OF UNSTAINED CELLS X 100}}{\text{TOTAL NUMBER OF CELLS}}$$



Steps of animal cell culture

➢ PRIMARY CULTURE

- Cells when **surgically or enzymatically** are removed from the organism and are **placed in suitable culture environment and grown are called primary culture.**
- They have finite life span and contain heterogeneous population of cells. These cells upon **subculture lead to generate cell lines which have limited life span.**
- Lineage of cells are originated from the primary culture is **known as cell strain.** Primary cultures are **morphologically similar to the parent tissues.**



Primary Cell culture

ESTABLISHED CELL CULTURE

- Primary cell culture when **first subcultured** is known as secondary cell culture. Established or immortalized cell line is the ability to proliferate indefinitely by random mutation and artificial modification such as artificial expression of the telomerase gene.

➢ **Advantages:**

✓ Many kinds of cell lines.
✓ Generally easy to grow and manipulate.
✓ Proliferate indefinitely.
✓ Contact inhibition.

Example: HeLa, Sf-9, Cervical cancer.

➢ Transformed Cell Culture

- Transformation is a process of **conversion of normal cell into a cell having some or many of the attributes of different cell.** When cell is transformed, cells lose contact inhibition and become immortal.

- **For example: NIH 3T3 mouse cells are partially transformed and became immortal but contact inhibited and grows in a mono layer.**
- **Transformed cells lack contact inhibition of movement due to change of cell surface property and loss of many receptors.**
- **They continue to grow and pile up on top of one another as they proliferate.**
- **Transformed cells are also cultured through suspension medium where cells do not attach to the surface of the culture vessels and are grown liquid culture medium.**
- **Hematopoietic stem cells and tumor cells are grown in suspension much faster which do not require the frequent replacement of the medium.**

❑ APPLICATION OF CELL CULTURES IN PHARMACEUTICAL INDUSTRY AND RESEARCH

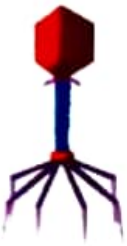
- **Cell culture is used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (for example: metabolic studies, aging), the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis.**

✓ **Model System:** **Cell culture is used as model system to study basic cell biology and biochemistry. It is also used to study the interaction between cell and disease causing agents like bacteria, virus. It helps to study the effect of drugs and to study triggers for ageing.**

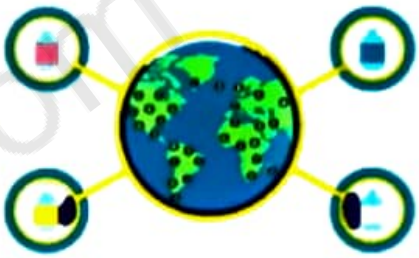
✓ **Genetic Counseling:** **Fetal cell culture extracted from pregnant women is used to study or examine the abnormalities of chromosomes, genes using karyotyping and these findings are used in early detection of fetal disorders.**

✓ **Toxicity Testing:** **Animal cell culture is used to study the effects of new drugs, cosmetics and chemicals and growth of multiple cells, especially liver and kidney cells. This technique is also used to determine the maximum permissible dosage of new drugs.**

- ✓ **Cancer Research:** Cell culture is one of the most important tools in cancer research. The basic difference between normal cell and cancer cell can be studied using animal cell culture technique. Normal cells are induced cancer cells by using radiation, chemicals, viruses and then cause of cancer is studied.



- ✓ **Virology:** Animal cell cultures are used to replicate the viruses instead of animals for the production of vaccine. Cell culture can also be used to detect and isolate viruses, and also to study growth and development cycle of viruses.



- ✓ **Vaccine Production:** Cultured animal cells are used for virus production and these viruses are used to produce vaccines. For example: Vaccines like polio, rabies, chicken pox, measles and hepatitis B are produced using animal cell culture.

- ✓ **Gene Therapy:** Cultured animal cells are genetically altered and are used in gene therapy technique. First cells are removed from the patient lacking a functional gene or missing a functional gene. These genes are replaced by functional genes and altered cells are culture and grown in laboratory condition and are introduced into the patient.

- ✓ **Drug Screening and Development:** Animal cell cultures are used to study the cytotoxicity of new drug. This is also used to find out the effective and safe dosage of new drugs. **Cell-based assay plays an important role in pharmaceutical industry.**

- ✓ **Genetically Engineered Protein:** Animal cell cultures are used to produce commercially important genetically engineered proteins such as monoclonal antibodies, insulin, hormones etc. Proteins extracted from biological sources are important for substitution therapy. Interferon is discovered in 1957 by cell cultured method by viral infection.
- ✓ **Replacement Tissue or Organ:** Animal cell culture is used as replacement tissue or organs. For example: artificial skin is produced to treat patients with burns and ulcers. Recently artificial organ culture such as liver, kidney and pancreas are successfully carried out for transplantation.