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B.Pharm 3rd Semester

Sub: Pharmaceutical Microbiology

Unit V

From,

Pranab Kumar Bandyopadhyay

SIPS, Jharpokharia,

Teacher's ID : T080322602

Spoilage:

Spoilage is chemical and physicochemical degradation of pharmaceutical products rendering it unsuitable for use.

Spoilage is not desirable in pharmaceutical industry because deterioration of drugs and excipient occurs, as a result product may lose its quality and it may become ineffective. Entire batch may be required to be discarded. This may cost huge lose to the manufacturer. Moreover it may attract litigation from the consumers which may cause huge financial loss to the Company.

Damaged products may damage reputation of the manufacturing company which may attract further financial loss. Microbial spoilage may present potential health hazards to the consumers like toxicity , infection or even death. Toxic metabolites may be produced due to microbial growth which may cause health hazards. Microbes may deteriorate drugs and thereby reduce potency of the medicament. There could be change in the appearance of the product like decolourisation, phase separation, and odour formation ete. Following types of microbial spoilage occurs in pharmaceutical products.

1.Chemical spoilage. 2. Physicochemical spoilage 3. Biological spoilage.

Chemical Spoilage - Chemical spoilage means deterioration of chemical nature of drugs and excipients. Molecular structure of the ingredients may change. Thischange may affect physicochemical properties of the preparation. Potency of the drug may decrease. Further microbial growth may occur if chemical degradation of preservative occurs.

Similarly chemical degradation of surfactants, organic polymers ete may damage specialised micro-environment for which they are used. Along with microbial contamination some - chemicals may also cause chemical spoilage like pesticide, disinfectants, bleaching agent, sanitizer etc. may add to chemical spoilage.

Physicochemical spoilage - This type of spoilage may change physico-chemical properties of product. Following types of changes may occur.

i) Viable growth - Viable growth of microbes may occur inside the container. These growth may be visible in the form of floating layers, turbidity, hemps, ete. Contamination of products by fungus species like *Aspergillus sp.* may cause this type spoilage. Some bacterial species may also show viable growth.

ii) Gas production -Some microbes may produce gas inside the containers. These gases may be visible in the form of bubbles, floccules etc. Contamination of products with bacteria like *E. coli* may produce gas if it contains sugars.

iii) Colouration / Decolouration -Some microbes may decolourise formulation or it may produce unique colour which is different from the normal colour of the product.

iv) Odour formation-Microbial growth in the finished product may produce bad odour or characteristic odour.It may produce a characteristic rotten smell.

v) Taste change -Microbial spoilage may change the taste of the oral formulations. It may impart bitter or obnoxious taste to the oral formulation.

Bilogical Spoilage - Spoilage of pharmaceuticals may produce some undesirable and dangerous molecule which has undesirable biological effects. Some microbes may produce toxins, pyrogens or other harmful metabolites. These biomolecules may be present in the product from the very beginning. Spoilage may occur, although no microbial contamination was there.

Factors affecting the microbial Spoilage of the Pharmacultad 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 minimise the impact of spoilage. Following factors affect the microbial spoilage of Pharmaceutical products.

i) **Number of contaminating micro-organism** - There may not be sufficient spoilage if number of contaminants are less. This low level it contaminants may not able to multiply due to design of the formulation. However during long storage these microbes may cause damage. If initial contamination is high then it may present an undesirable challenge. Products will be damaged quickly due to presence of high numbers of microbes. High level of contamination occurs due to lapses in manufacturing process or due to high loads of contaminants in raw materials.

ii) **Type of migo-organisma** -Some microorganisms are more aggressive than others. They can quickly multiply and spoilage is much faster. However, aggressive microbes may not multiply initially due to presence of preservative and other substances. Contamination of product with natural communities of non-aggressive micro-organism can facilitate growth of other aggressive contaminants.

iii) **Presence of nutrients -** In product Micro-organism can utilise formulation components as nutrients and utilise these components for biosynthesis and growth. Many formulations may contain crude animal, vegetable and microbial products which creates a conducive environment for microbial growth and subsequent spoilage. Additives like sugar, amino acid, polyhydric alcohol may act as microbial nutrients. Primary contaminants may produce metabolites which could be used by aggressive microbes as nutrient. Demineralised water which is prepare by ion-exchange resins may contain nutrients.

iv) **Presence of water -** Presence of water in formulation may promote microbial growth and subsequent spoilage. Uncomplexed water or free excess water promotes this spoilage. Presence of free water may be measured by water activity. It is a ratio between 'vapour pressure of formulation and vapour pressure of water in similar condition'. If water activity is 1 then it is conducive for microbial growth. Chances of microbial growth decreases with decrease in value of water activity. A value less than 0.88 is considered safe to prevent

spoilage. However, some microbes can grow in extremely low water value condition. - *Aspergillus glaucus* can grow at a water activity value of 0.61.

v) **Oxidation-reduction potential**- Contaminating micro-organism may requires terminal electron accepter to facilitate functioning respiratory pathway. Presence of dissolved oxygen increases redox potential of the product thereby promote microbial growth and spoilage.

vi) **Temperature** -Storage temperature is a great controller of microbial growth and spoilage. However, spoilage may occur over a range -20^{o}C to 60^{o}C. Microbial growth and spoilage is less in low temperature and high temperature. Storing products at a cool place (8^{o}C to 12^{o}C) may cause negligible spoilage. High temperature can also prevent spoilage. Water for injection is stored above 80^{o}C before filling and sealing. So extreme temperature can minimise spoilage.

vii) **pH-** Microbes prefer neutral pH for their growth. However some microbes may prefer slightly acidic pH. Extremely acidic or alkaline pH of the formulation may prevent microbial growth and subsequent spoilage. Some communities of microbes can survive in extreme pH and change the pH, there by support growth of other micro-organism.

viii) **Containers and packaging** - Chances of contamination in single dose ampoules and vials are negligible. However multidose containers be contaminated by users themselves. Change in designs of these containers may minimize contamination and spoilage. Wide mouthed containers for creams and ointments are replaced by narrow mouthed tubes with screw cap closures. Multidose injections are stored in containers which has self sealing rubber wads. Containers are sealed such a way so that it can prevent entry of water and oxygen to minimise water activity and redox potential.

ix) **Presence of protection materials -** Some materials in formulation may protect microbes during sterilisation process. Polymers like gelatin, starch ete may increase microbial resistance to heat. Microbes may get adsorbed in particles and become more resistant to heat.

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.

Water which is purified by reverser osmosis process may also be contaminated if osmosis membrane is no properly disinfected. Even distilled water may also be contaminated if it is stored for few day at normal temperature.

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.

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 *Bacillius sp*. etc are also present. These spores and micro-organism may contaminate pharmaceutical products. This type of contamination is minimised 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 minimised by pooper health check-up, vaccination and hygiene of the personnel. Protective gear and pooper training of the personnel may also minimise the contamination.

Equipments -

Equipments of manufacturing may contain microbes if it is not sterilised properly. Grinder, blender, filter ete 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 micro-organism 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 of inhibit growth of micro-organism. These antimicrobial agents are included in formulation in order to minimise 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.

A good preservative should have following characteristics. It should be a broad spectrum antimicrobial agent, *i.e*. it should be effective against variety of microorganisms. However, most active preservatives are ineffective against some microbes.

A good preservative should have a fast killing and inhibition rate and it should selectively react with contaminants and it should not react with ingredients of the formulation. However, most preservatives interact with ingredients of the formulations. A good preservative should be non-irritant and non-toxic to the patients. However, many preservative have toxic effects and cause irritation of skin when used in formulation for topical application. Some preservatives even cause contact dermatitis. A good preservative should be stable and it should remain active throughout the shelf-life of the product. However effectiveness of the preservative may change depending upon so many factors.

Concentration of preservatives determine efficacy of its preservation power. Antimicrobial activity of preservative increases with the concentration. Activity of phenol decreases 64 times if concentration is halved.

Antimicrobial activity of preservative is also dependent on storage temperature. Decrease in storage temperature may reduce killing activity of preservative. It may be calculated by determining temperature co-efficient.

Preservative which is used in formulation may not be available for antimicrobial activity. An unstable equilibria is formed between preservative and available preservative which controls mass of micro-organism. Unavailable preservative helps to maintain equilibria by constantly supplying available molecule.

Availability of preservative may also decrease with increased solute concentration and decrease water activity. Ionic and weakly acidic preservatives exhibit their antimicrobial activity when they are not ionised. They produce maximum activity when ionisation is very low.

Preservative may distribute itself depending its affinity in a multiphase system. It may distribute itself in water phase and oil phase by an unstable equilibrium. Parameters like partition coefficient, polymer binding constant and oil-water ratio must be considered while using a preservative in a formulation.

Packaging material can interfere with preservation activity of preservative. Preservative may leach, permeate and interact with rubber, plastic, cork etc.

Different types of preservatives are used in pharmaceutical products. Acid, alkali and esters are used as preservative. Benzoic acid is used as preservative. Its sodium salt, sodium benzoate is also used as preservative. Its ester methyl hydroxybenzoate is also used as preservative.

Some alcohols like chlorobutol, benzyl alcohol etc are used as preservative. Some phenolic compounds like phenol, chlorocresol etc are also used as preservatives.

Some mercuric compounds, such as thiomersal etc are used as preservative. Many other compounds like benzalkonium chloride, cetrimide are used as preservative. Even compounds like chloroform and formaldehyde can be used as preservative.

Assessment of microbial contamination and Spoilage:

Assessment of microbial content of a pharmaceutical product is very essential. Sterile product should be perfectly free from micro-organism and that can be assessed by a test of sterility. However, non-sterile products may contain some micro-organisms. These micro-organisms could be pathogenic and non-pathogenic. All these micro-organisms can cause spoilage and may cause potential health hazards. Number of total micro-organisms present in a products must be low and should be within permissible limit. Types and nature of the microbes should also be assessed to determine the presence of specific microbes.

Total number of microbes and types of microbes is assessed by microbial limit test. Following tests are performed to assess the microbial contamination and subsequent spoilage.

1. Test of sterility-.

Indian pharmacopoeia, British pharmacopoeia and United States pharmacopoeia recommends test of sterility for some pharmaceutical products. Procedure are similar with little variations. Indian pharmacopoeia recommended tests are discussed briefly. Two methods are there i.e. direct innoculation and membrane filtration.

i) Direct inoculation- In this method, little amount of sample is directly added to the culture media which was specified in pharmacopeia. This inoculated media is then incubated for specified period of time. Presence of growth indicates the presence of micro organism which is sourced from sample. Hence it may be concluded that sample is not sterile. Sample may be termed sterile in absence of any growth.

ii) Membrane filtration- In this method sample is filtered through a membrane filter and washed with diluting fluid. Microbes, if present, will be there at the top of the filter paper. Now this filter paper is inoculated into specified culture media. If growth is observed then it indicates that product is not sterile.

A positive and negative control test must also be conducted in both the above mentioned method.

2. Microbial limit test-

The European pharmacopoeia recommends both qualitative and quantitative assessment of micro-organism. United States Pharmacopoeia recommends microbiological Limit test. It has two parts,(i) Total Aerobic microbial count and (ii) Test for specified microorganism.

(i) Total Aerobic microbial count-

In this method specified amount of test sample (10gm) is taken and mixed with specified amount (90ml) of peptone water.

This is sample dilution. There are specific procedure to make dilutions for water insoluble products and fatty products. Total microbial count is examined by following procedure-

First, examination of sample by membrane filtration method- 10 ml of prepared dilution is mixed with 90 ml of peptone water and filtered through membrane filter. It is then washed 3 times with sterile peptone water. One filter paper is placed in petridis containing Soyabean Caesin Digest Agar media and incubated for 5 days at 30-35^{o}C. Bacterial count is determined by counting colonies.

Another filter paper is placed in Sabouraud Dextrose Agar Media and incubated for 5 days at 20-25^{o}C. Fungal count is then determined.

Second, Examination of sample plate count method- In this method prepared dilution is directly transferred to 4 petridishes. Two for bacteria and two for fungus. In first two petridishes 15 ml of Soyabean Caesin Digest Agar media is added. Colonies are counter after incubation for 5 days at 30-35^{o}C. In remaining two petridishes 15 ml of Sabouraud Dextrose Agar Media was transfered and incubated at 20-25^{o}C. Colonies are counted.

(ii) Test for specified micro-organism-

Specific identification tests for *Escherichia coli, Salmonella, Pseudomonas aeruginea* and *Staphylococcus aureus* is performed.

E. coli is cultivated in Mac Conkes agar media. Colonies are identified by characteristics metalic shine.

Salmonella are cultivated on bismuth sulphite agar media and identified by black or green colonies.

Pseudomonas aeruginosa is identified by cultivating in cetrimide agar media. Colonies slow greenish colour.

Staphylococcus aureus is caltivated in Vogel Johnson agar media and identified by black colonies surround by yellow zones.

3. Periodic test-

Periodic test of the product for total microbial count must be done in order to determine continuing efficacy of the product.

Many alternative tests may be done for detection and determination of micro-organism *e.g* Luciferase test, Epifluorescence, electrical impedance etc.

Evaluation of microbial stability of formulation.

Microbial stability of a formulation is dependent on effectiveness of its preservative. Chemical assay and biological assay may assure effectiveness of preservative but it may lose its activity due to presence of other ingredients in the formulation. Some formulations do not require preservative because they act as self-preservative. Some formulation does not require preservative because it contains antimicrobial agents like antibiotics as ingredient. Some formulation may contain high sugar concentration, salt concentration and may act as self preservative. So, the ability of the formulation to protect itself from microbial growth must ascertain to determine microbial stability of the formulation. It is done by preservative efficacy test.

Basic principle of this test is to inoculate products with different types of specified micro-organism with specific quantity. Little amount of inoculated product is removed at a specific interval. Then viable count of this withdrawn sample is determined. United States Pharmacopoeia, European Pharmacopoeia etc recommends this type of tests.

The concentration of the test organism should be 10^5-10^6 cells per ml or gm. Total microbial count is performed in 0 hr, 6 hrs, 24 hrs, 48 hrs. 7 days, 14 days and 28 days. British pharmacopeia recommends test even after 28 days.

Different bacterial species are used for this purpose. They are *Staphylococcus aureus*, *Pseudomonus aeruginosa* and *E.coli.*

Different fungus species are also used. They are *Candida albicans*, *Aspergillus niger* etc.

This test allows to add designated micro-organisms if required.

After withdrawal of sample and before viable count, sample is mixed with chemicals which can deactivate preservative because presence of even very minute quantity of preservative may hamper microbial viable count.

There are two types of performance criteria. Criteria A is desired and recommended where as criteria B is satisfactory in justified cases.

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