10. Presence of inactivating agents – dirty condition changes of concentration
11. Great inoculum size
12. Efficiency of multiphase systems
13. Effect of container and packaging
14. Type and initial level of contamination

Methods of preservation of pharmaceutical products:

1. Physical protection
2. Preservative coating only
3. Waterproof protection
4. Water vapour proof protection
5. Water vapour proof protection with desiccant

Safety precautions:

1. **QA (quality assurance):** It encompasses a scheme of management which embraces all the procedures necessary to provide a high probability that pharmaceutical product will conform consistently to a specified description of quality.
2. **R & D (research and development):** In includes formulation design and development.
3. **GPMP:** It stands for good pharmaceutical manufacturing practice.
4. **QC:** QC stands for quality control. It means controlling the total manufacturing process in each step.
5. Post marketing surveillance
6. Contamination risk assessment.

3. Principles and Procedure of Sterilization of Pharmaceutical Products

Validation: Validation may be defined as establishing documented evidence which provides a high degree of assurance that specific process, method or system will consistently produce a result meeting predetermined acceptance criteria. Validation is that part of a quality assurance system that evaluates in advance the steps involved in operational procedures or product preparation to ensure quality, effectiveness and reliability.

Principles: Three principles should involve in validation process:

1. To build sterility into a product.
2. To demonstrate maximum level of probability that sterilization methods has established sterility to all batches of unit.
3. To provide greater assurance of result of end product sterility test.

Validation steps:

1. **Process design:** This is the research and development phase and involves defining a process for manufacturing the product. It usually includes the following:
   i. Creation of a Quality Target Product Profile (QTPP)
   ii. Identifying Critical Quality Attributes (CQAs)
   iii. Defining Critical Process Parameters (CPPs)
   iv. Conducting risk assessments
2. **Process qualification:** This stage evaluates or qualifies the process designed either to ensure that it can reproduce consistent and reliable levels of quality. It involves collecting and evaluating data on all aspects and stages of the manufacturing process. This includes:
   i. The building and facilities, i.e. ensuring they adhere to local regulations as well as pharmaceutical manufacturing regulations
   ii. The transportation of raw materials
   iii. Storage of raw materials
   iv. The knowledge, training and working practices of production line employees
   v. Every step of the process to turn raw materials into the finished product. This includes having pre-defined sampling points at various stages of the process.
   vi. Finished product packaging, storage, and distribution
3. **Continued process verification:** Continued process verification involves ongoing validation during production of the commercial product to ensure the process designed and qualified in the previous stages continues to deliver consistent quality. One of the main aims of this stage is to detect and resolve process drift. This stage involves product sampling, analysis and verification at various points in the manufacturing process and requires the involvement of employees with quality control training.

Sterilization: Sterilization is the killing or removal of all microorganisms including bacterial spores from a particular location or material.

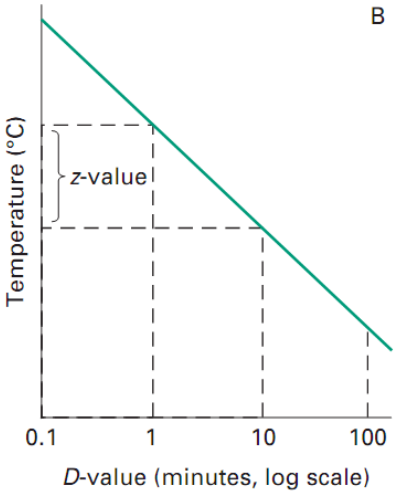
Process:

A. Physical
B. Chemical
C. Non-thermal

They are discussed below:

A. **Physical:** It includes:
   1. **Heat:** It may be –
      a. Dry heat
      - Red heat
      - Flaming

reduce the D value of an organism by 90% (i.e. 1 log cycle reduction). The Z value is not truly independent of temperature but may be considered essentially constant over the temperature ranges used in heat sterilization process.



F value: If the D value is measured at different temperature and pressure it can be seen that D value varies with the pressure, more powerful is considered the process. F value = a measure of the capacity to inactivate bacteria in function of the temperature. Mathematically the F value is expressed by the rate of mortality per minute in function of temperature for a given pressure. This concept applies de facto to steam sterilization only and is a measure of the power of a sterilizer.

Sterile pharmaceutical products: Sterile pharmaceutical products are products free of microorganisms. The presence of one single surviving microbial cell is sufficient to render the product non-sterile.

Types of sterile pharmaceutical products:

1. **Injections**
    - Aqueous intravenous solution
    - Oily solutions
    - Aqueous suspension
    - Oily suspension
    - Freeze dried powder
2. Contact lens solutions e.g. wetting agents, cleaning solutions, soaking solutions.
3. Surgical dressings
4. Implants
5. Absorbable haemostats e.g. oxidized cellulose, absorbable gelatin foam, human fibrin foam, calcium alginate.
6. Surgical ligatures and sutures e.g. catgut (non-absorbable types)
7. Sterile aerosol products
8. Purified cotton gauze

Quality control and quality assurance of sterile products

In a pharmaceutical organization, a quality control is a fundamental segment that refers to a process of striving to produce a product by a series of In Process Quality Control (IPQC) test in order to eliminate or prevent error at any stage of production.

Steps involved in In Process Quality Control (IPQC):

1. Identify types of formulations manufacturing or going to manufacture e.g. tablet, liquids, parenteral and ointments etc.
2. Identify which are the critical steps in the manufacturing of the product, where it will be necessary to check certain parameters to confirm that the process is in control.
3. Identify the specifications of the parameters which will confirm the parameters are within control.
4. Define the frequency of checking of parameter.
5. Create monitoring and control records for all IPQC process.
6. Keep a provision for modification of process if required.

The LAL is separated from the remaining cellular debris and its activity optimized using metallic cations, pH adjustment and additives and then freeze-dried.

↓

Equal volume of LAL reagent and test solution (usually 0.1 ml of each) are mixed in a depyrogenated test tube.

↓

Incubation at 37^0 C for 1 hour

↓

If endotoxins are present, a solid gel forms, indicating the presence of endotoxins.

↓

The British Pharmacopoeia describes six separate methodologies for the test for endotoxin. They are:

1. Gel clot limit test (most commonly used) → solid clot indicates positive result
2. Gel clot semi-quantitative
3. Turbidimetric kinetic method
4. Chromogenic kinetic method
5. Chromogenic endpoint method and
6. Turbidimetric endpoint method

Limitations:

i. Disturbed by endotoxin binding components like lipids, blood components etc.
ii. Difficult to correlate with rabbit test.
iii. False positive for cellulose and many herbal preparations.

7. **Sterility test:** The tests for sterility are intended for detecting the presence of viable microorganism in pharmaceutical preparation that is designed to be sterile. The test is based on the principle that if microorganisms are placed in a medium that provide optimum condition of nutrition, moisture, pH, aeration, temperature they can grow and their presence will be indicated by the presence of turbidity in clear medium. Test for sterility may be carried out by one of the following two methods:

a. **Membrane filtration method:** Membrane filters having a nominal pore size not greater than 0.4 µm are used to retain microorganisms. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions; and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products e.g. for antibiotics.

b. **Direct inoculation method:** In this method, the test sample is added directly into the required media, ensuring that the amount of sample is below 10%. An aliquot quantity of the material being tested is drawn aseptically from the container and transferred to a vessel containing a measured quantity of a suitable culture medium. The culture is incubated at appropriate temperature for not less than 14 days. The culture medium is observed at periodic intervals during the incubation period and at the end to detect presence of any microbial growth.

Monitoring of sterilization: There are three forms of monitoring required to ensure that sterilization is achieved. They are:

1. Physical or mechanical monitoring – record keeping
2. Chemical monitoring and
3. Biological monitoring

They are discussed below:

1. **Physical or mechanical monitoring (record keeping):** A log must be maintained on site for monitoring of each sterilization load. Records must be maintained on site for one year and on file for five years. It includes:
   a. Date and time of each load
   b. Temperature of each load
   c. Time of the sterilizer stayed in the recommended temperature range
   d. Maximum pressure reached in each load

Recommendations:

- Temperature, pressure and time must be within the manufacturer's recommended operating range.
- Record should be kept when repairs and spore tests are done along with the results of the spore tests and any other notable occurrences related to the sterilizer.

3. Selection of hybridomas
4. Screening the products
5. Cloning and propagation
6. Characterization of storage

They are discussed below:

1. **Immunization:** The very first step in hybridoma technology is to immunize an animal (usually a mouse), with appropriate antigen. The antigen, along with an adjuvant like Freud's complete or incomplete adjuvant is injected subcutaneously (adjuvants are non-specific potentiators of specific immune responses). The injections at multiple sites are repeated several times. This enables increased stimulation of B lymphocytes which are responding to the antigen. Three days prior to killing of the animal, a final dose of antigen is intravenously administered. The immune stimulated cells for synthesis of antibodies have grown maximally by this approach. The concentration of the desired antibodies is assayed in the serum of the animal at frequent intervals during the course of immunization.
   When the serum concentration of the antibodies is optimal, the animal is sacrificed. The spleen is aseptically removed and disrupted by mechanical or enzymatic methods to release the cells. The lymphocytes of the spleen are separated from the rest of the cells by density gradient centrifugation.
2. **Cell fusion:** The thoroughly washed lymphocytes are mixed with HGPRT defective myeloma cells. The mixture of cells is exposed to polyethylene glycol (PEG) for a short period (a few minutes), since it is toxic. PEG is removed by washing and the cells are kept in a fresh medium. These cells are composed of a mixture of hybridomas (fused cells), free myeloma cells and free lymphocytes.
3. **Selection of hybridomas:** When the cells are cultured in HAT medium, only the hybridoma cells grow, while the rest will slowly disappear. This happens in 7-10 days of culture. Selection of a single antibody producing hybrid cells is very important. This is possible if the hybridomas are isolated and grown individually. The suspension of the hybridoma cells is so diluted that the individual aliquots contain on an average one cell each. These cells, when grown in a regular culture medium, produce the desired antibody.
4. **Screening the products:** The hybridomas must be screened for the selection of the antibody of desired specificity. The culture medium from each hybridoma culture is periodically tested for the desired antibody specificity. The two techniques namely ELISA and RIA are commonly used for this purpose. In both the assays, the antibody binds to the specific antigen (usually coated to plastic plates) and the unbound antibody and other components of the medium can be washed off. Thus, the hybridoma cells producing the desired antibody can be identified by screening. The antibody secreted by the hybrid cells is referred to as monoclonal antibody.
5. **Cloning and propagation:** The single hybrid cells producing the desired antibody are isolated and cloned. Two techniques are commonly employed for cloning hybrid cells-limiting dilution method and soft agar method.
   - **Limiting dilution method:** In this procedure, the suspension of hybridoma cells is serially diluted and the aliquots of each dilution are put into microculture wells. The dilutions are so made that each aliquot in a well contains only a single hybrid cell. This ensures that the antibody produced is monoclonal.
   - **Soft agar method:** In this technique, the hybridoma cells are cultured in soft agar. It is possible to simultaneously grow many cells in the semisolid medium to form colonies. These colonies will be monoclonal in nature.
     In actual practice, both the above techniques are combined and used for maximal production of monoclonal antibodies.
6. **Characterization and storage:** The monoclonal antibody has to be subjected to biochemical and biophysical characterization for the desired specificity. It is also important to elucidate the monoclonal antibody for the immunoglobulin class or sub-class, the epitope for which it is specific and the number of binding sites it possesses.

Recombinant vaccines

Vaccination is the phenomenon of preventive immunization. In the modern concept, vaccination involves the administration (injection or oral) of an antigen to elicit an antibody response that will protect the organism against future infections.

Types: Vaccines are mainly of three types:

1. Dead bacteria or inactivated viruses
2. Live non-viral or weakened (attenuated) bacteria or viruses

29. Superoxide dismutase	*Mycobacterium spp. and Nocardia spp.*	Antioxidant, anti-inflammatory
30. Tyrosinase	*Streptomyces glausescens and Erwinia herbicola*	Antitumor, treatment of Parkinson's disease
31. Urease	*Lactobacillus spp. and Klebsiella aerogens*	Nitrogen metabolism of ruminants
32. Uricase	*Aspergillus flavus*	Gout
33. Urokinase	*Bacillus subtilis*	Blood clots
34. Vibrilase TM	*Vibrio proteolyticus*	Treatment of damaged tissue
35. α galactosidase	*Aspergillus spp. and Streptomyces griseoloalbus*	Fabry's disease, prevention of xenorejection, blood group transformation
36. β aminopeptidase	*Ochrobacterium anthropi*	Antioxidant
37. β galactosidase	*Aspergillus spp.*	Removal of lactose from milk
38. β lactamase	*Citrobacter freundii, Serratia marcescens*	Antibiotic resistance

Q. 29. Give a diagrammatic representation of a cloning strategy for industrial production of enzymes.