UNIT => 4

Designing of aseptic area :

- Aseptic area is the well defined area where the production of sterile preparation should be carried out and the entry at these area is strictly through the airlock for personnel and/or for equipment and material.

- The various operations of sterile preparation, filteration, filling, sterilization is carried out under controlled condition designed to control the presence of any microbial cell or any air particles.

- During designing of aseptic area focus on control of temp., humidity, air quality control to maintain & to make aseptic area environment acceptably clean.

- The aseptic area is divided in main 4 Grades as per the operations performed inside the area.

→ Grade A : This zone performed high risk operations, eg => aseptic filling & transfer.

→ Grade B : Background area of Grade A processes (aseptic preparation & filling).

→ Grade C : Preparation of sterile soln to be filter

→ Grade D : Handling of components after washing.

* following factors are considered in designing of any aseptic area.

- All aseptic area should as far as possible to avoid the entry of unrelated personnel's.

- In all aseptic areas, all exposed surfaces, walls, roofs should be smooth, unbroken, free from any cracks to minimize the shedding or deposition of microbial cells.

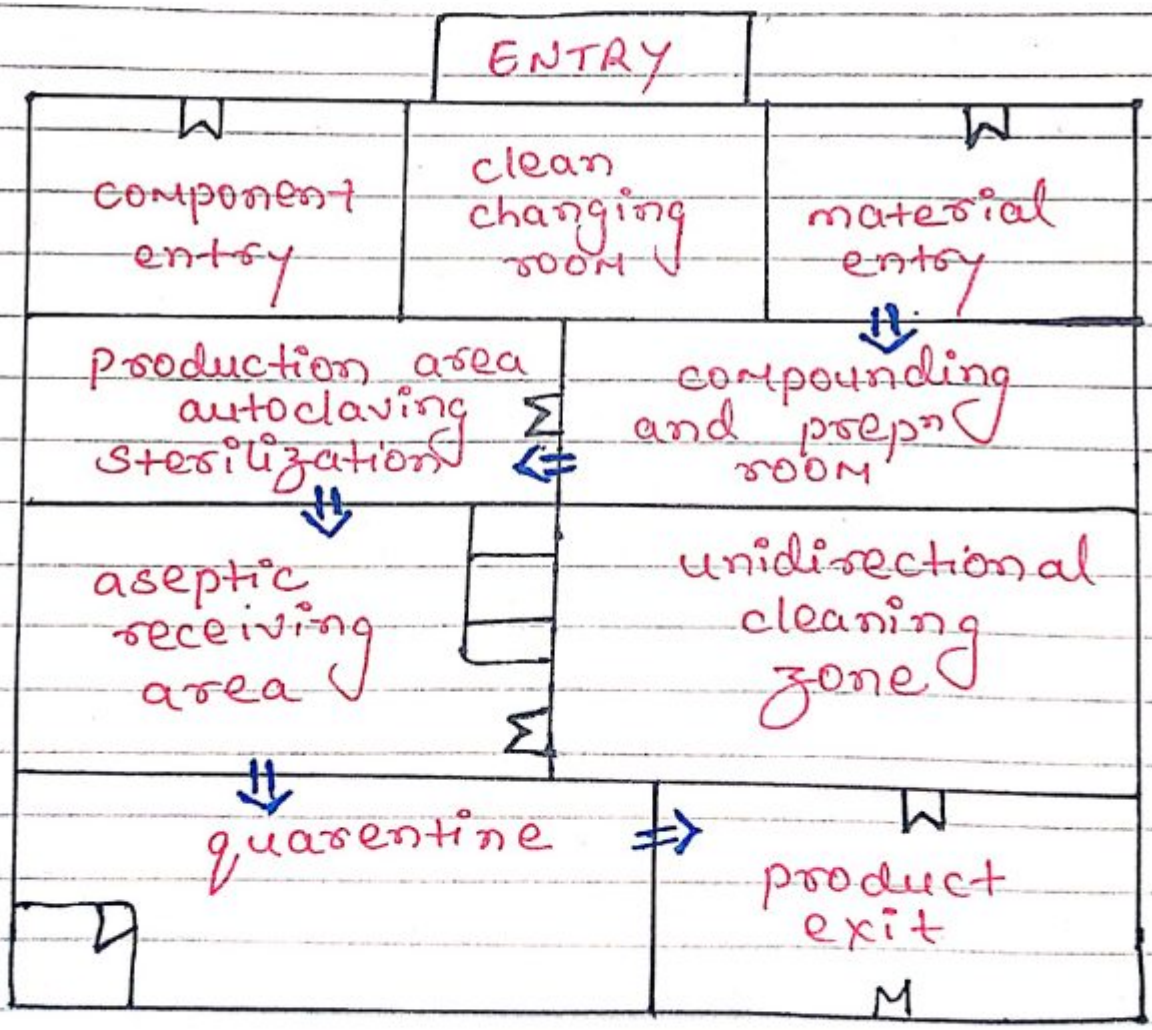
- Doors should be carefully designed to avoid any air contamination. sliding doors are not allowed for aseptic area. swing doors should be open in the flow direction of air or from positive air pressure side to negative air pressure side.

- Pipes and ducts or other things should be installed in such a way that they do not create unsealed opening & surfaces that are difficult to clean.

- changing rooms should be designed as air locked.

- Air locked doors should not be open at same time in double door. when 1st door is open, the second door should be close.

An warning alarm system should be there to warn in the condition of both doors are open.



LAYOUT OF ASEPTIC AREA

MICROBIOLOGY

UNIT => 4

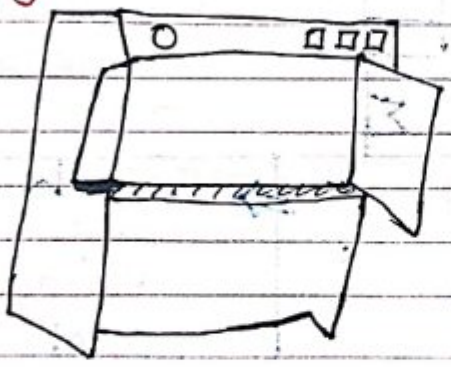
PART => 2

Laminar flow Equipments

- Laminar flow cabinet or laminar airflow hood is an enclosed bench
  ↓
  designed to
  ↓
  prevent contaminat^n/particles at time of
  - Biochemical testing
  - Performing rkn
  - inoculating Microbes (for pure culture)
  - for obtaining clean/aseptic area.

or

- A laminar flow clean bench provides a space to work with a product or specimen where it will be protected from contamination by particulates such as Microorganisms.



Laminar flow chamber
or
Laminar flow cabinet
or
Tissue culture hood

→ construction :

- Laminar flow cabinet consist of :
  - filter pad or pre-filter assembly
  - A fan (Blower) - HEPA filter
  - switches for [UV light, visible light & motor]

→ working :

- Before starting work on laminar flow cabinet
  ↓
  UV germicidal lamp switched on for about 15-20 min to kill germs
  ↓
  Then switched off as can cause skin burn or cancer
  ↓
  After this surface is wiped with ethanol before & after use.

→ principle :

- fan suck air → Pre filter assembly [dust trapped] → Prefiltered air passes by
  ↓
  HEPA filter → contaminating microbes were trapped → sterile air flows in cabinet

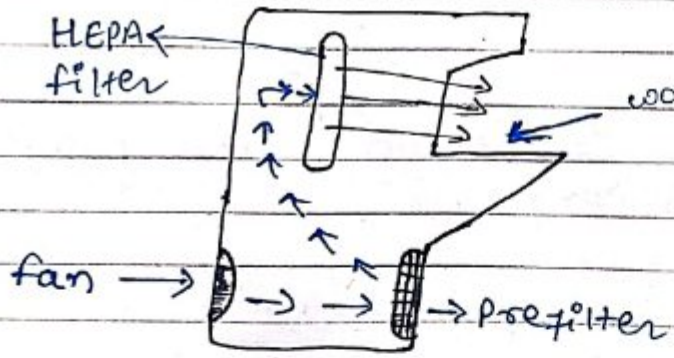
→ HEPA (High Efficiency Particulate Air) filter

- In laminar Air flow System, clean air is obtained through HEPA filter.
- The air flow at a speed of 100ft/min & sweeps dust particles making entire room free of particulate matter.

→ Types of laminar flow cabinet :

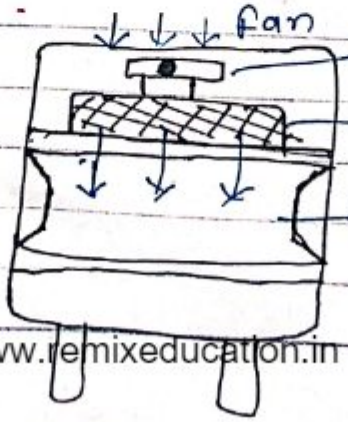
① Horizontal laminar hood

- These hoods filter air from back to front of the hood.



② vertical laminar hood.

- These hoods filter air from top to downward on working area.



→ Different sources of contamination in an aseptic area:

1. Personnel / operator
2. Building
3. Equipments & utensils
4. Raw materials
5. Manufacturing process
6. HVAC [Heating, ventilation & Air-conditioning] system.

→ Personnel:

- Person = supervising, performing & controlling drug manufacturing is reason for microbial contamination due to following reason:
  - Inadequate training
  - Eating / drinking / Smooking.

2. → Building

- Rough floor, walls, ceilings — presence of moisture
- Absence of air filteration system
- Improper washing, cleaning — toilet & personal cleanliness.

→ Equipments & utensils

- Improper cleaning & sanitation of Equipments.
- using defective equipments

→ Raw Materials

- degradation of raw material due to extreme environmental condition
- wrong labelling
- Improper storage & handling
- Incorrect sampling & testing.

→ Manufacturing process :

- Improper sterilization.
- lack of labelling, cleaning.

→ HVAC :

- Improper Air filteration system.
- Non-Maintenance of pressure in the area.

* Method of Prevention of contamination

(1) Personnel

- Personal hygine maintained
- unauthorised personnel restricted.
- Trained persons allowed.
- should wear protective clothing → masks.

(2) facility design

- Pressure & temp. should be maintained in aseptic rooms.
- Air filteration system must be provided.
- Disinfectants are used to clean area.

(3) Building design

- smooth, crack free & easily cleanable floors.
- windows & doors = closed properly.

(4) cleaning & Disinfection :

- time to time cleaning.
- good quality cleaning agents must be used.

* Clean area classification :

- Clean room → constructed → where conc. of airborne particle controlled by use of HEPA filter
  ↓
  Aseptic compounding is done.

- Acc. to Iso [International organisation of standardisation] → clean room → refers to → level of particulate cleaniness
  ↓ based on
  no of airborne particles of a certain size percubic meter.

- A clean room should include :
  - HEPA filter
  - Air lock entry system
  - Maintain of air pressure.

Microbiological assay

- A microbiological assay may be defined as quantitative or qualitative determination of any chemical compound from a simple or even complex material with use of microorganisms.

- It is necessary to assay antimicrobial agents for determination of potency ,for determining the pharmacokinetics of a drug in animals or man and for monitoring and controlling antimicrobial chemotherapy.

- Many therapeutic agents which either inhibit the growth of microorganisms (antibiotics) or are essential for their growth (vitamins and amino acids) can be standardized by microbiological assay.

- Microbiological assay are simple, inexpensive and convenient methods which are similar in accuracy as that of chemical method

Microbiological assay of antibiotics

➢ Microbiological assay of antibiotics include following general procedures

1. Preparation of media
2. Selection of microorganism
3. Preparation of inoculum

➢ Microorganisms used in microbiological assay of antibiotics :

Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis

➢ The microbiological assay of antibiotics may be carried out by the following two methods:

i) Method A: Cup-plate or cylinder-plate method.

ii) Method B: Turbidimetric or tube assay method.

1)Method A: Cup-plate or cylinder-plate method

➢ This method depends on the diffusion of an antibiotic from a vertical cavity or a cylinder, through the solidified agar layer in a Petri plate.

➢The growth of test microorganisms is inhibited entirely in a circular area or zone around the cavity or cylinder containing a solution of the antibiotic.

➢A liquified assay medium (43 to 45°C) is inoculated by suspension of test microorganisms and the inoculated medium is poured immediately into sterile Petri plate by using an assay medium and then spread the test culture or microorganisms on the surface of plates (spread plate technique).

➢Solutions of known concentrations of the standard preparation and the test antibiotic are prepared in appropriate solutions.

➢These solutions are added in sterile cavities or cylinders prepared in a solid medium.

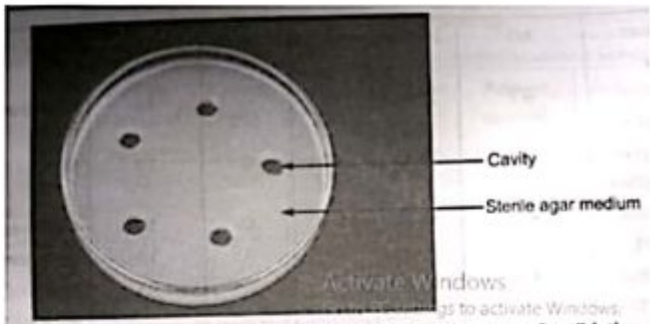


Fig. 23.1: Cavities prepared in sterile agar medium for assay of antibiotics

- The plates are left standing for 1 to 2 hours at room temperature 4°C.
- All plates are then incubated for about 18 to 24 hours.
- The diameters or areas of the circular inhibition zones produced.
- The graph which relates zone diameter to the logarithm of the concentration of antibiotics is plotted and the unknown concentration of test antibiotics is calculated.

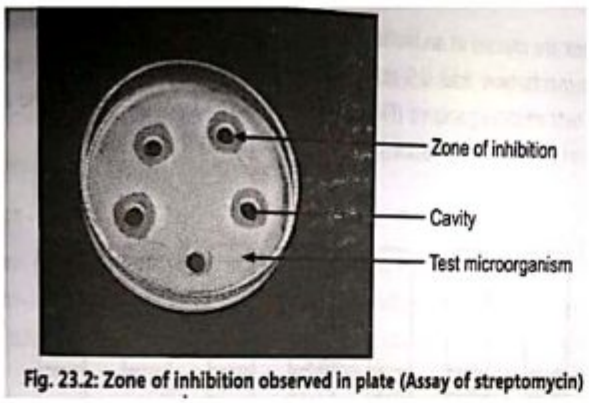
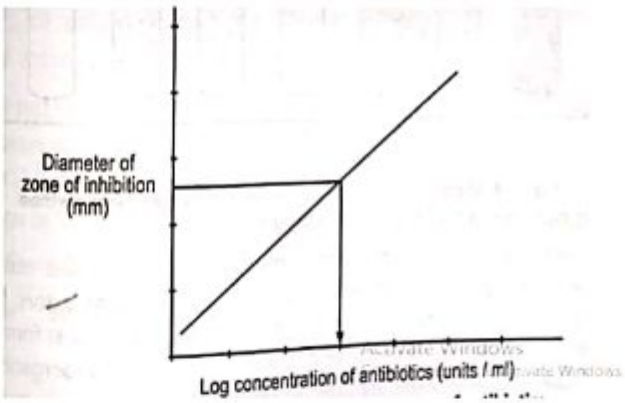


Fig. 23.2: Zone of inhibition observed in plate (Assay of streptomycin)



2) Method B: Turbidimetric or tube assay method:

- This method depends upon the growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favourable to its rapid growth in the absence of the antibiotic.
- Advantage: has a shorter incubation period for the growth of the test microorganisms.
- This method is not recommended for cloudy or turbid preparations.
- Five different concentrations of the standard solution are prepared by diluting the stock solution.
- A median concentration is selected and the test sample of the antibiotic solution is adjusted by dilution to obtain approximately this concentration.
- One ml of each concentration of the standard solution and of the sample solution are placed in each of the tubes in duplicate.
- To each-tube, 9 ml of nutrient medium is added.

- It is the ability of these test microorganisms to synthesize the factor being assayed that forms the basis of the microbiological assay of vitamins and amino acids.

- Microbiological assay of cyanocobalamin (Vitamin B12) may be performed by the following methods:

  i) Titrimetric method.

  ii) Turbidimetric method.

- At the same time, three control tubes, one containing the inoculated culture medium, another identical with it but treated immediately with 0.5 ml of dilute formaldehyde solution (blank) and a third containing un-inoculated culture medium are prepared.
- All the tubes are placed in an incubator for 4 to 5 hours.
- After incubation add 0.5 ml of dilute formaldehyde solution to each tube.
- The growth of the test microorganisms is measured by determining the absorbance at about 530 nm using turbidometer.

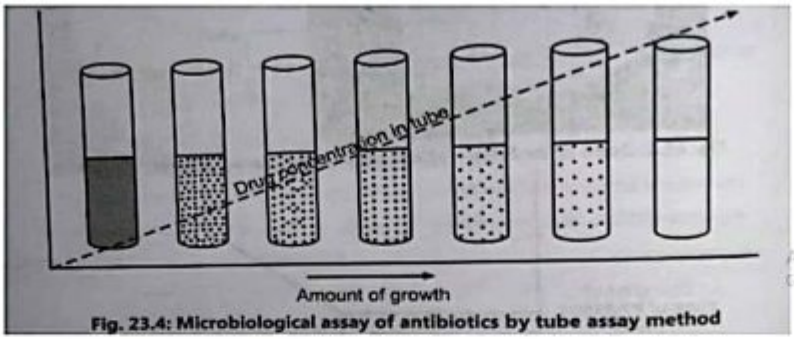


Fig. 23.4: Microbiological assay of antibiotics by tube assay method

Microbiological assay of Vitamins

- Vitamins are important growth factors needed for growth and multiplication of microbes.
- They are very sensitive to small amounts of growth factors.
- It is the ability of these microbes to synthesize the factor being assayed that forms the basis of the microbiological assay of vitamins and amino acids.
- Examples of microbes used in microbiological assay of vitamins are *Tetrahymena thermophila* , *Lactobacillus plantarum.*
- Vitamins are important growth factors needed for growth and multiplication of microorganisms.
- They are very sensitive to small amounts of growth factors.

1) Titrimetric method:

Procedure:

- Clean ten test tubes and add 0.0 ml, 0.5 ml, 1.0 ml, 1.5 ml, 2 ml, 2.5 ml, 3 ml, 4 ml, 4.5 ml and 5 ml respectively of standard **cyanocobalamin** solution (0.01 – 0.04 ug/ml).
- To each test tube add 5 ml **Basal medium stock solution** and adjust the final volume (10 ml) by using water.
- In the other four test tubes add 1.0 ml. 2.0 ml. 3.0 ml, 4.0 ml respectively of the **test solution to be assayed.**
- To each test tube add 5 ml **Basal medium stock solution** and adjust the final volume (10 ml) by using water.
- Sterilize all test tubes in autoclave at 121°C for 5 minutes.

- After sterilization, cool all test tubes upto room temperature and inoculate with one drop of inoculum (*Lactobacillus leichmanii ATCC 7830*).
- Incubate the tubes for 64 to 72 hours at any chosen temperature within the range of 30 to 37°C.
- Titrate the contents of each tube with 0.05 N NaOH, using bromothymol blue as an indicator (converts to green colour).
- Plot the graph and determine the concentration as activity per ml of test solution by interpolation of vitamin B12 activity.

Table 23.8: Procedure for microbiological assay of Vitamin B_{12} (Titrimetric method)

Tube number	Std. Cyanocobalamin solution (0.01 to 0.04 µg/ml) (ml)	Basal medium stock solution (ml)	Volume of sterile water (ml)
1	0.0	5	5.0
2	0.5	5	4.5
3	1.0	5	4.0
4	1.5	5	3.5
5	2.0	5	3.0
6	2.5	5	2.5
7	3.0	5	2.0
8	4.0	5	1.0
9	4.5	5	0.5
10	5.0	5	0.0
1'	1.0 (Test solution)	5	4.0
2'	2.0 (Test solution)	5	3.0
3'	3.0 (Test solution)	5	2.0
4'	4.0 (Test solution)	5	1.0

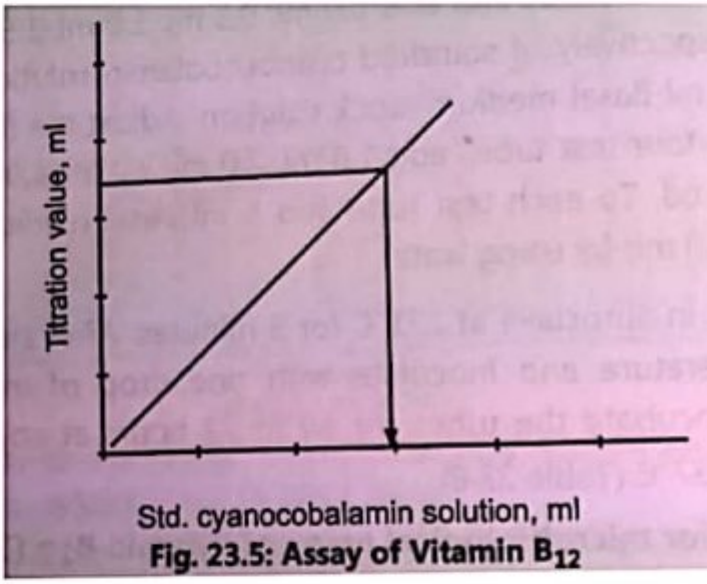


Fig. 23.5: Assay of Vitamin B_{12}

2) Turbidimetric method:

Procedure:

- Apparatus, reagents and procedures are same as titrimetric method but this test includes two more test tubes to which neither standard cyanocobalmin solution nor test solution, nor inoculum is added.
- Incubate all test tubes at 30 to 37°C for 16 to 24 hours.
- By using an 'un-inoculated blank tube' adjust the transmittance at 640 mu to100% in the photoelectric colorimeter.
- Thoroughly mix the contents of each tube and record the transmittance reading.
- Plot the graph and calculate the concentration of the test solution of cyanocobalmin.

Assessment of new antibiotics and testing of anti-microbial activity of new substance:

- Assessment of new antibiotics is done by figuring out their Minimum Inhibitory Concentration (MIC).
- MIC is the lowest concentration of antimicrobial compound found to inhibit the growth of particular test microorganisms.
- MIC values are usually expressed in terms of µg/ml or units/ml.
- MIC of different anti-microbial compounds may be determined by
- 1) Liquid dilution method
- 2) Solid dilution method

1) Liquid dilution method:

- It is also know as test tube method.
- The method involves use of a series of test tubes which contain a double-strength medium and are labeled as shown in adjacent table.
- The first test tube (un-inoculated) is used for checking the sterility of the medium.
- In all other eleven test tubes Inoculum (m/o) is added to achieve a desired concentration (10^5-10^6 cells/ml) of microbes .

Tube number	Volume of double strength medium (ml)	Volume of test chemical (ml)	Volume of sterile water (ml)
0 (uninoculated)	5	0.0	5
0' (control)	5	0.0	5
1	5	0.5	4.5
2	5	1.0	4.0
3	5	1.5	3.5
4	5	2.0	3.0
5	5	2.5	2.5
6	5	3.0	2.0
7	5	3.5	1.5
8	5	4.0	1.0
9	5	4.5	0.5
10	5	5.0	0.0

- In all eleven test tubes, test chemical is added ranging from 0.5 to 5 ml.
- The second test tube (control) is used to check the suitability of the test microbes and viability of the inoculum.
- The final volume (10 ml) in all test tubes is adjusted by using sterile water.
- The contents of all test tubes are properly mixed and incubated at 37°C for 2 to 3 days.
- After incubation,all test tubes are examined for growth in the form of turbidity and results are recorded and minimum inhibitory concentration is calculated
- It also necessary to conduct a preliminary experiment to determine the approximate range (test solution) which would be suitable for the test.

2) Solid dilution method:

- In this method ,test chemical is fixed into molten agar and then poured into Petri plate .
- After solidification, the inoculum is spread on the surface of agar medium.
- All plates are incubated at 37°C for 2-3 days.
- After incubation, all plates are observed for growth of inoculum and the minimum inhibitory concentration of the test chemicals is calculated
- Certain advantages of this method are :
- 1.Several microorganisms can be tested at the same time by use of multipoint inoculator.
- 2.Contaminations are easily detected, because colony features on solid media are more distinctive than turbidity differences in fluid media