C. NO 9354222746

PHARMACEUTICAL MICROBIOLOGY

UNIT => II

PART => 1

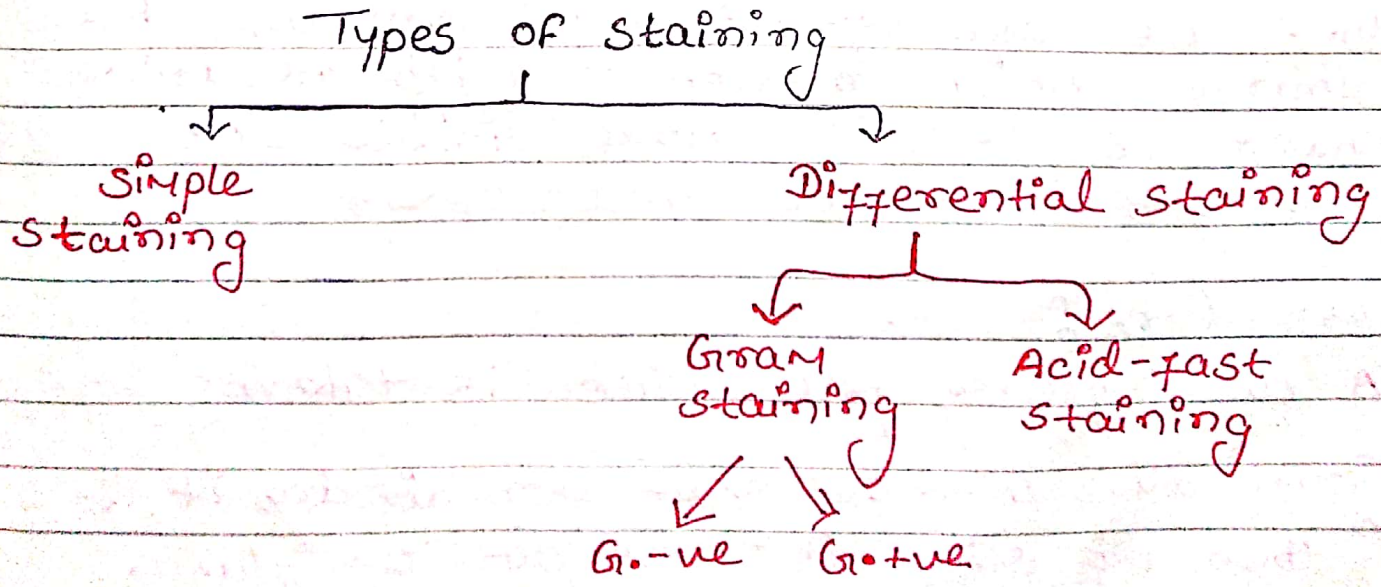
STAINING & ITS TYPES

Staining :-

-> Staining means identification of bacteria.

-> In our surrounding many types of bacteria is present (like) cocci, Bacilli, spirilli) and which cause different types of disease, The bacteria size are very small & we can't see by our naked eyes. so, with the help of Microscope & dyes solution (Methylene blue, crystal voilet, safranine) bacteria is identified.

-> staining is a technique by which we identify the structure, arrangements of bacteria by using different dye solution under Microscope.



* Simple staining :-

- Simple staining is a method of staining in which bacteria are stained by using a single stain / dye.
  - eg ⇒ Methylene blue, safranine, Malachite green.
- This staining is only use to examine cell shape & arrangement of bacteria.

→ Principle :

- In simple staining, we generally use +ve charge dye. So, first we develop -ve charge on the surface of bacteria by add^{n} of OH^- or removal of H^+ ion because many carboxyle group present on the surface of bacteria.
- Then we use dye & dye contains +ve charge which attached with negative charge & it will make visible of bacteria under microscope.

→ Procedure :

⇒ A clean grease free slide is taken [wash slide with detergent then air dry it & then the slide is pass out on flame 2 to 3 times rapidly].

culture media containing bacteria.

ii) on that slide make smear by using a sterile wire loop and cell suspension.

made up of nichrome wire

property → rapidly heat & cool.

iii) After making smear, the slide is allowed to air dry.

iv) After air drying these slide is rapidly passed through a flame for 3 or 4 times for heat fixation.

v) Now add few drops of different dye/ indicator into the surface of slide.

vi) Now allow the glass slide for drying few seconds.

vii) Then the slide is washed under tap/running water to wash the excess stain.

viii) Now wipe the ~~glass~~ below surface of slide with cloth or tissue paper.

ix) put the cover slip over the smear and place on the surface of microscope.

x) observe the bacteria in microscope.

↓

shape & arrangement.

* Differential staining

- Two or more dyes are used at a time.

→ Gram staining:

- The method is developed by Han's christian gram in 1884.
- The differential staining technique by which gram +ve & -ve bacteria is identified is called gram staining.

Gram +ve	Gram -ve
• After staining it gives purple/ Blue colour.	• After staining it gives Red/ pink colour.
• cell wall 20-30 nm thick & single layer.	• cell wall 8-12 nm thick & two layers
• outer membrane absent	• outer membrane present.
• Peptidoglycan layer is Multilayer.	• Peptidoglycan layer is single layer.
• outer membrane is Absent	• Outer membrane is present
• Lipid content (low 1-4%)	• Lipid content high (11-22%)
• eg ⇒ staphylococcus streptococcus Bacillus	• eg ⇒ vibrio E. coli.

Procedure

Requirements for Gram staining

- A clean grease free slide
- Bacteria cell suspension
- Nichrome wire loop.
- Primary stain - crystal voilet
- Mordant - Gram's iodine.
- Decolorizing agents - 95% alcohol (Ethanol 95%)
- counter stain -> Basic fuchsine or Safranine.

Gram staining procedure

Take a clean grease free slide

↓

Make a smear using Nichrome wire loop

↓

Air dry & Heat fix

↓

Flood smear with crystal voilet for 2 min.

↓

wash slide with tap water

↓

Flood smear with Gram iodine for 2 min.

↓

Treat the slide with decolorizing agent 95% Ethanol

↓

wash the slide with water

↓

Flood the smear with fuschin or safarin

↓

water wash the slide

↓

After observe it

[Note: gram staining if purple or blue colour is retain i.e, gram +ve bacteria & if shows red or pink i.e, gram -ve bacteria.

→ Acid fast staining

- In nature, there are variety of micro-organism each micro-organism have some special character.
- Most of the micro-organisms are easily stained by simple staining procedures.
- But their is some micro-organisms that are not easily stained by this technique because they have a waxy covering on its surface.
- Such organism require a special staining technique called Acid-fast staining.
- This staining technique was discovered by scientist Paul Ehrlich in 1883
- Acid-fast staining technique help us to differentiate the organism as acid-fast and non-acid fast organism.

Acid fast organism:

The organism that get stained by acid-fast staining technique but don't get decolorizes even by strong acid are called acid-fast organism.

Non-acid-fast organism:

- The organism that easily get stained & decolorizes easily by a strong acid are called non-acid fast organism.

Procedure

- Take a clean grease free slide and prepare a smear using nichrome wire loop.
  - ↓ Heat
- Air dry & fix the slide.
  - ↓
- The slide is flooded with ZN CF stain and placed on a boiling water bath for steaming for about 3-5 minutes
  - ↓
- During steaming the ZN CF stain is added repeatedly on the slide to avoid drying of smear.
  - ↓
- further the slide is treated to the decolorizing agent i.e., acid alcohol until the stain dissapear in washing.
  - ↓
- After decolorizatn do water wash.
  - ↓
- Further, the smear is flooded with counter-stain i.e, 1% Malachite green or 0.3% methylene blue for 2min.
  - ↓
- After 2min the slide is washed with water & air dried & observe it.

IMViC Test

I M V i C test is a group of test under which four tests are performed

- **I** Indole Test
- **M** Methyl Red Test
- **V** Voges Proskauer Test
- **C** Citrate Utilization test

- These tests are performed to observe the presence of the any bacteria that belongs to the family Enterobacteriaceae

The member of this family are:

E.Coli, Enterobactor aerogenes, Klebsiella pneumoniae, Salmonella typhimurium, Shigella dysenteriae, Proteus vulgaris, Pseudomonas aeruginosa, Alcaligenes faecalis

Cynobacterium xerosis, Micrococcus leuteus, lactococcus lactis, Staphylococcus aureus bacillus cereus

- These tests are also use to differentiate various members of Enterobacteriaceae family based on their biochemical properties (the properties to metabolize different compounds)

Indole Production Test

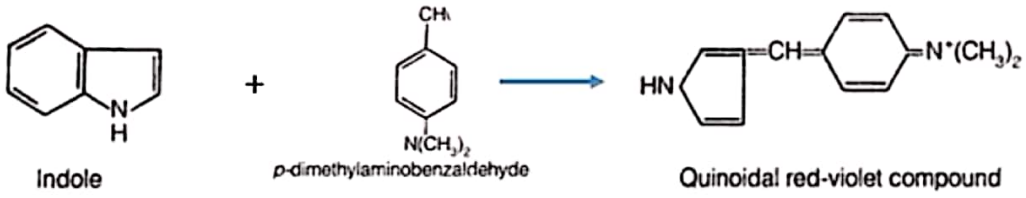
Objective

Identification of bacteria on their ability to degrade the amino acid Tryptophane.

Principle

Trypthophane is an essential amino acid, that can be metabolize by some bacterial cells to Produce indole. Not all bacteria have this property of conversion.

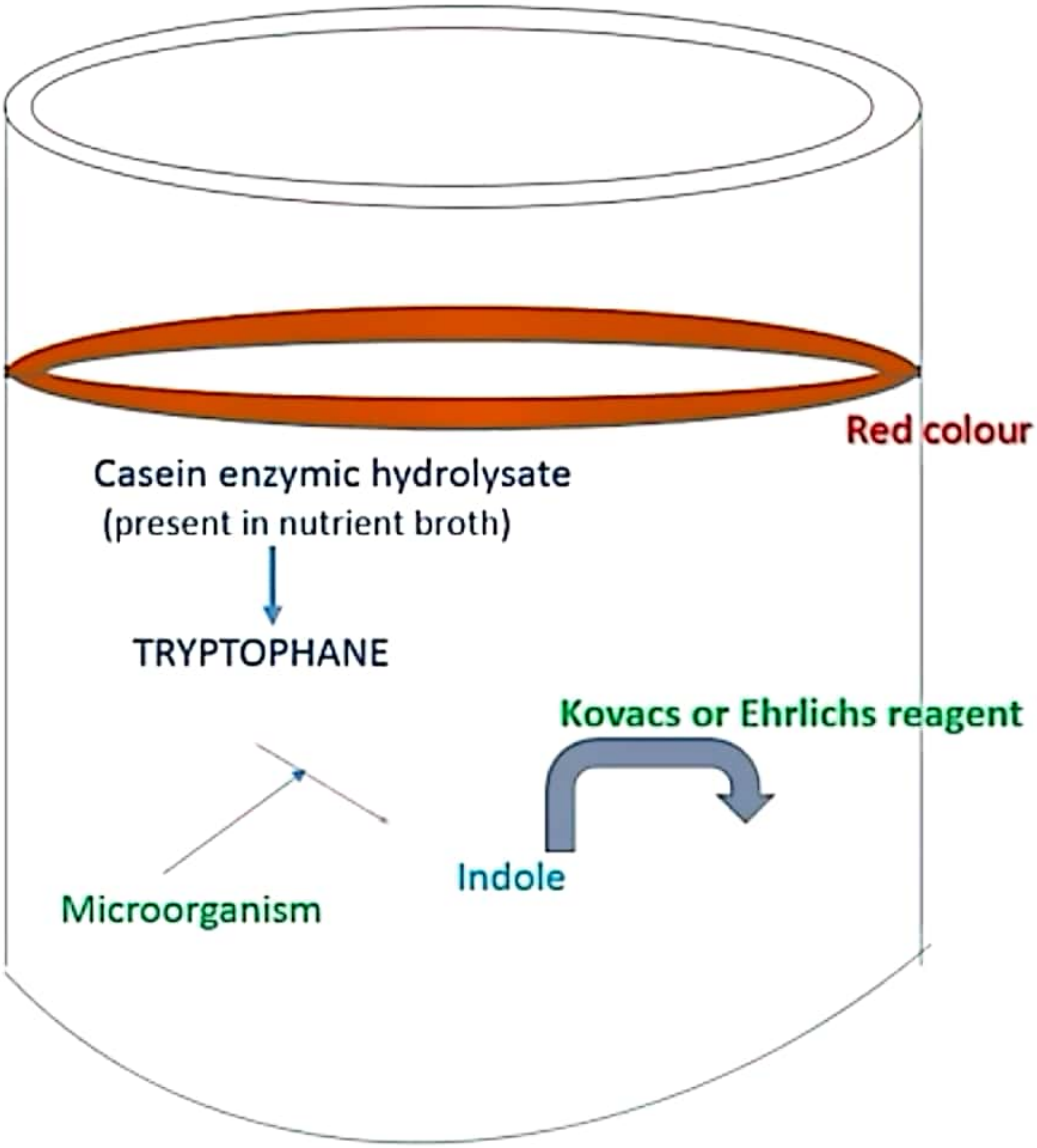
The indole is then reacted with p-dimethyl amino benzalgehyde portion of Kovacs reagent (p-dimethyl amino benzalgehyde + HCl + butanol) and form a red colour Complex, due to which media turns red at top layer of media.



Indole — *p*-dimethylaminobenzaldehyde — Quinoidal red-violet compound

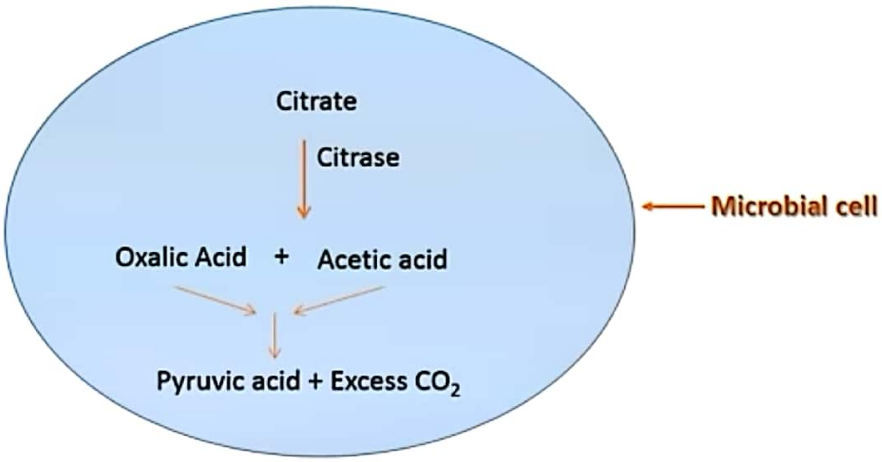
Result

The appearance of red colour on the top layer of the medium indicates presence of Microorganism.



Citrate Utilization test

Objective	Differentiate microorganism on the basis of their ability to metabolize citrate as sole / only source of carbon.
Principle	In the absence of glucose or lactose, some bacteria utilize citrate as carbon source for energy production. Citrate can be utilize by only those bacteria which has citrate permease enzyme in their cell. Citrate permease enzyme responsible for the intake of citrate inside the cell Inside the cell, another enzyme citrase convert citrate to oxalo acetic acid and acetate and then convert into pyruvic acid and CO_2. This generated CO_2 combines with sodium and water to produce Na_2CO_3 . Na_2CO_3 is an alkaline product. When an indicator Bromothymol Blue is added to this media, it turns from green to Prussian Blue (due to alkaline pH)
Result	Formation of Blue colour indicate positive test (presence of microorganism)



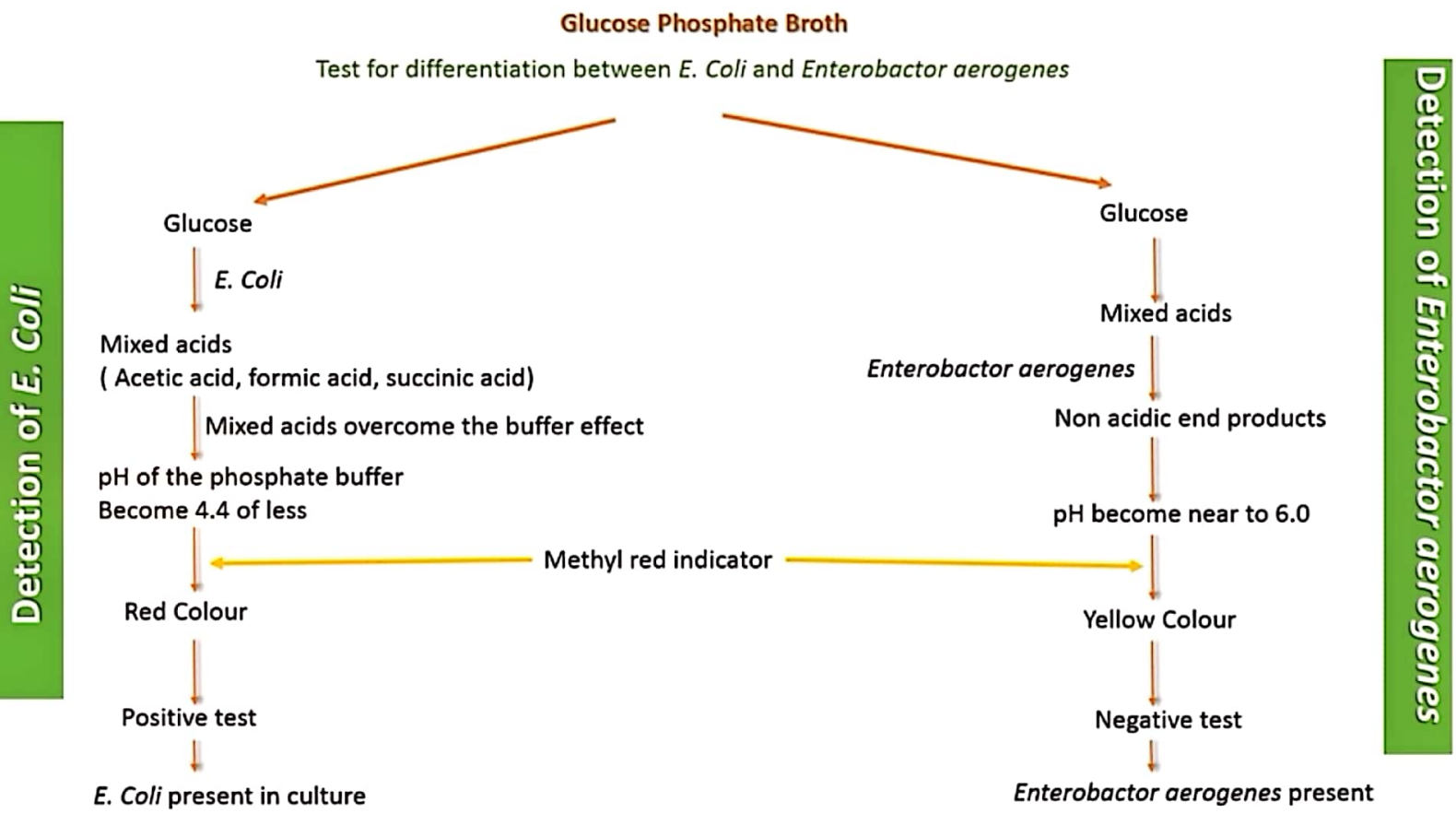
CO_2 + Na ⟶ Na_2CO_3 + Alkaline pH

Bromothymol Blue (Green) ⟶ Due to alkaline pH ⟶ Prussian Blue

Positive Test if Color appear

Methyl Red (MR) Test

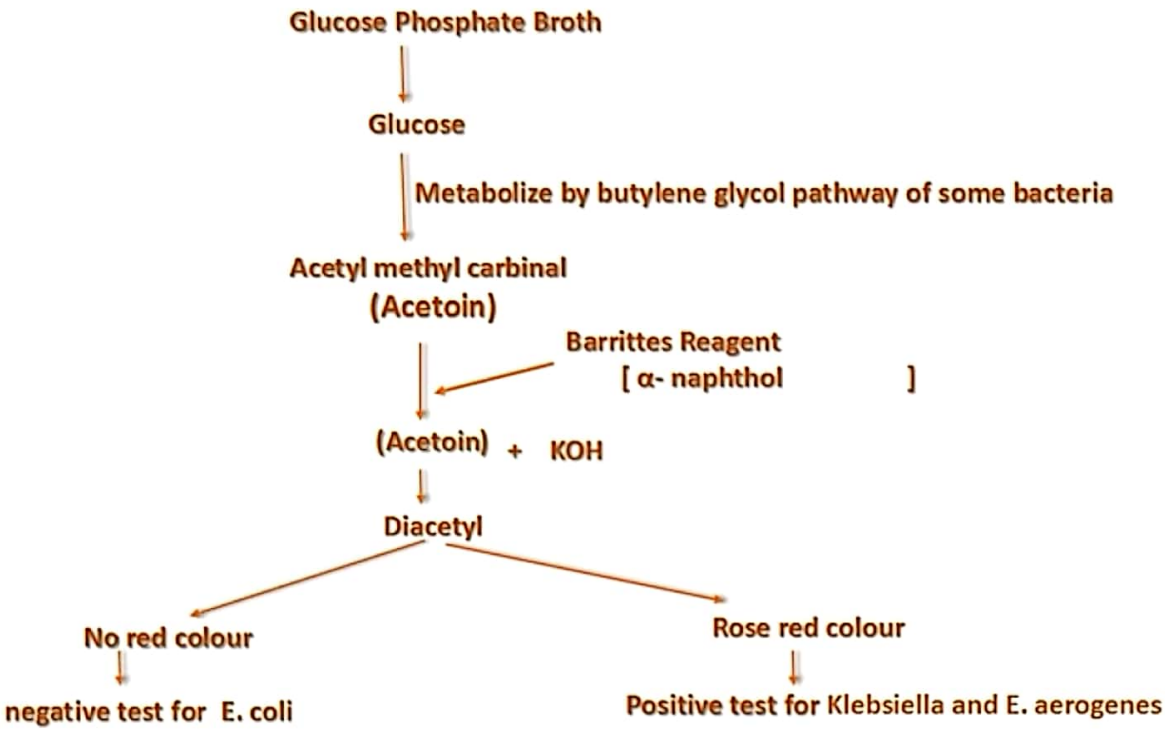
Objective	To determine the ability of organism to oxidize glucose and production of acid products To Differentiate E. Coli and Enterobactor aerogenes
Principle	Some specific organisms when grow on any glucose containing media, they use to produce mix acids (many acids) like acetic acid, formic acid or succinic acid. The amount of produced acid by these specific microorganisms reaches to that level at which It overcome the effect of phosphate buffer added in the glucose phosphate broth culture media. Due to the production of acid the pH of glucose phosphate broth reaches to near at pH 4. At this condition, after addition of an indicator methyl red, the colour of medium remains red, Because at pH 4.4 or lower the colour of methyl red is remains red and, remains stable for long time. If the color of methyl red changes from red to yellow it indicate the presence of bacteria E. aerogenes. Because E. aerogenes is present it utilizes the acids and produce non-acedic Neutral compounds and take pH towards neutral around pH 6. At the pH 6 the colour of methyl red is turns yellow
Result	**Red Colour indicate presence of E. Coli**



Voges- Proskauer test

Objective	To Differentiate E. Coli and Enterobactor aerogenes and Klebsiella
Principle	Some bacteria when inoculated in glucose phosphate broth, they utilize glucose by Butylene glycol pathway and produce acetyl methyl carbinol (acetoin). This acetoin reacts with the indicator Barritt's reagent (0.6 ml. of alcoholic solution of α- naphthol and 0.2 ml of 40% KOH) and form diacetyl and this diacetyl reacts with pepton (present in culture media) produce rose red colour in the media.
Result	Formation of rose red colour indicates presence of specific microorganism (Positive test).

Voges- Proskauer test



Microbes	Indole test	Methyle Red test	Voges-Proskauer test	Citrate utilization test
Escherichia Coli	+	+	-	-
Enterobactor aerogenes	-	-	+	+
Klebsiella	+ or -	-	+	+
Citrobactor freundii	-	+	-	+
Citrobactor diversus	+	+	-	+
Shigella species	Varies with different species	+	-	-

Table: Comparative table for test results and microbial strain

PHARMACEUTICAL MICROBIOLOGY

UNIT => 2

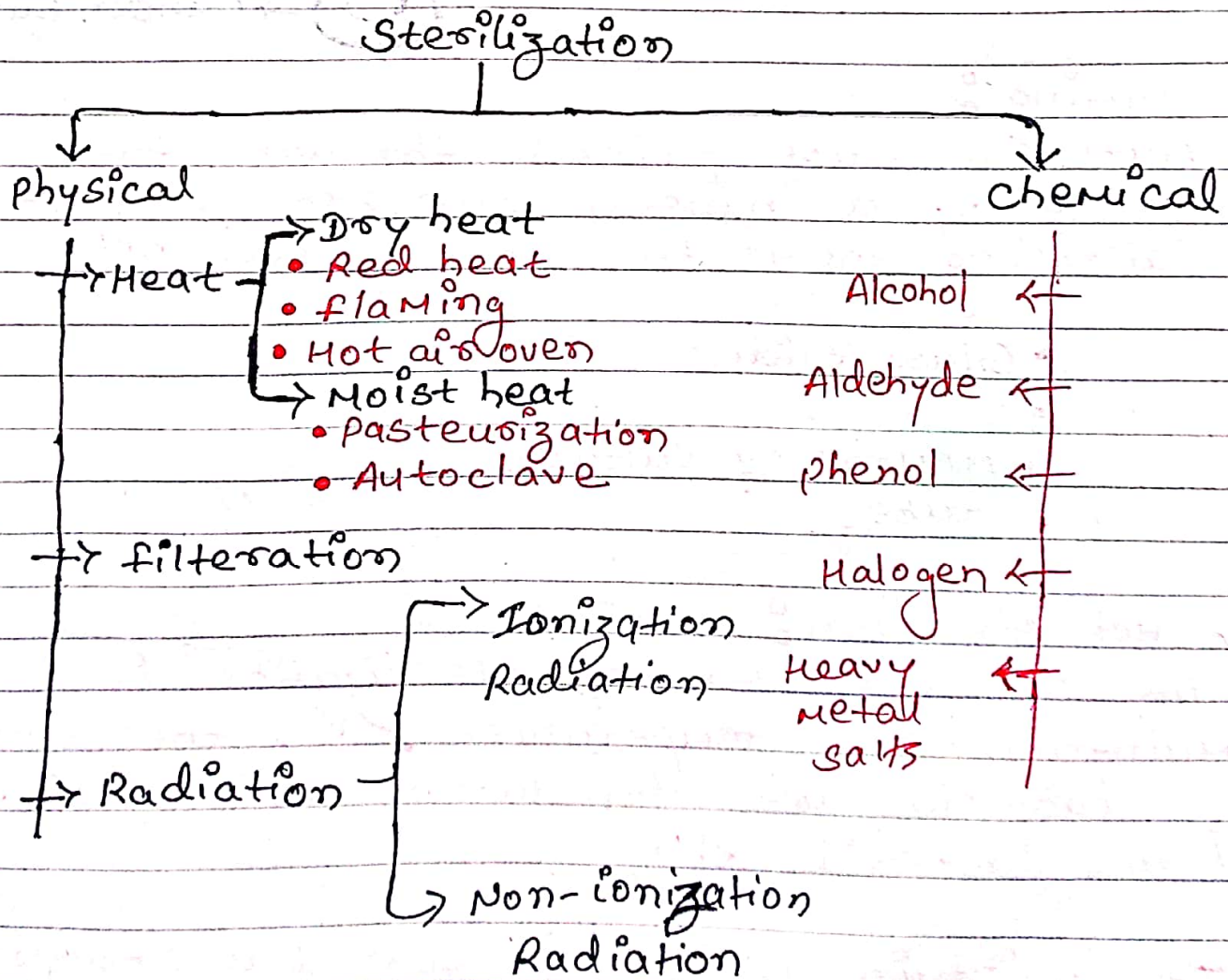
Part => 3

Sterilization :

- Sterilization is a process in which remove or kill all types of bacteria & viruses from living & non-living surfaces.

Q How can microorganism be killed ?

- Denaturation of proteins [e.g. wet heat, ethylene oxide]
- Oxidation [e.g. dry heat, hydrogen peroxide]
- filteration
- Interruption of DNA Synthesis [e.g. Radiation]



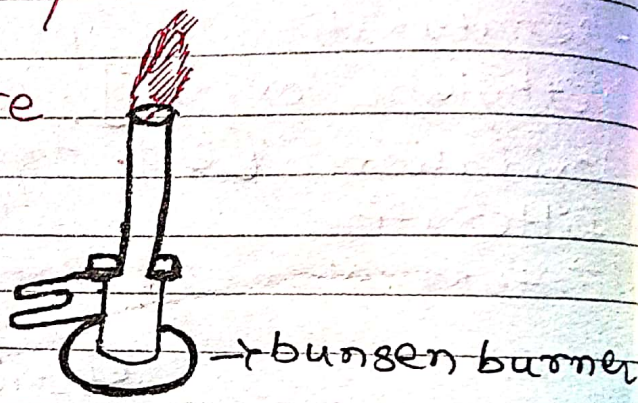
Physical sterilization :

* Heat :

→ Dry heat

i) Red heat :

- Materials are held in the flame of a bunsen burner till they become red hot.
  - innoculating wire
  - Tips of forceps
  - Needles



ii) Flaming :

- Materials are passed through the flame of a bunsen burner without allowing them to become red hot.
  - Glass slides
  - scalpels
  - Mouths of culture tubes.

iii) Hot Air Oven :

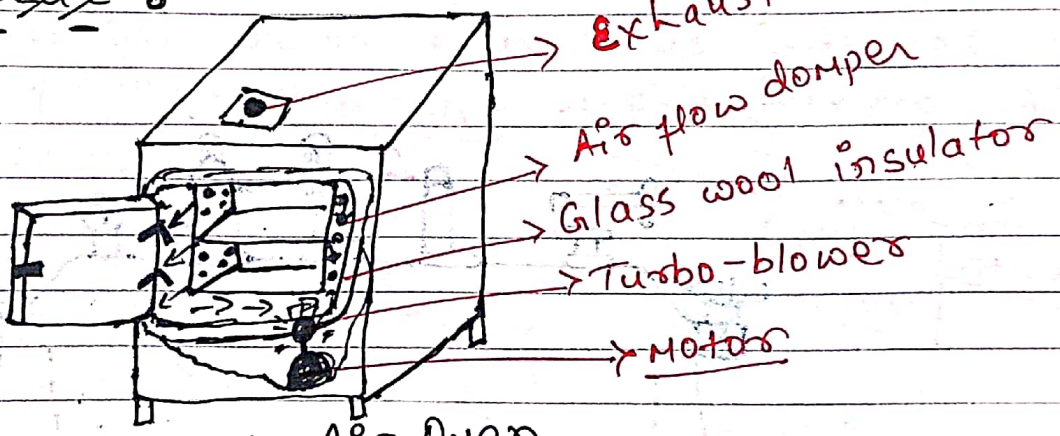
- The hot air oven of sterilization is to maintain high temperature. so, the protein of bacteria are denatured and oxidised. the bacteria kill

- It maintain 130°c - 180°c for 1 to 2 hours.
- It can't sterilize plastic material

Note: for 160°C → 2 hours
for 170°C → 1 hours
for 180°C → 30 Minutes.

- Hot Air oven is use for the sterilization of
- Glasswoare like glass syringes, petri dishes, pipettes and test tubes.
- surgical instruments like scalpels, scissors, forceps etc.
- chemicals like liquid paraffin, fats, etc.

→ ~~Moist heat~~ :



Hot Air Oven

→ Moist heat :

i) Pasteurization :

- It is a process of heating food (such as Milk, ice cream, fruit juices, etc) to a temperature that kill disease causing microorganism and substantially it reduce the level of spoilage organisms.
- we maintain less than 100°C temp.
- Eg ⇒ Pasteurization of Milk → takes 63°C for 30 Minutes.

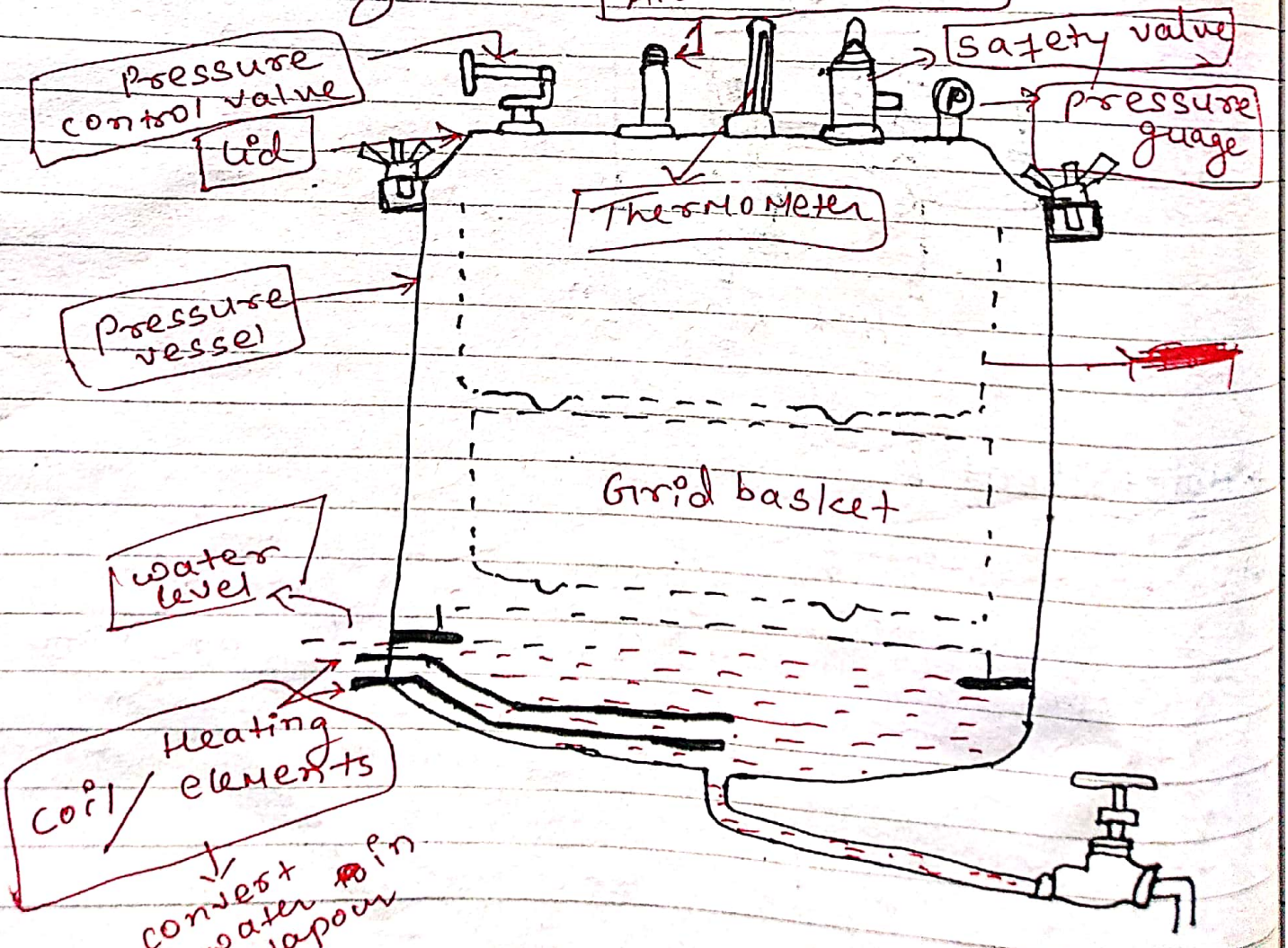
V.IMP

ii) Autoclave :

- Invented by charles chamberland in 1884.
- It is most Reliable Method of Sterilization.

→ Principle :

- It is based on the principle steam at high temperature.
- The high pressure is used to kill the microorganism
- It is the best and most widely method of sterilization.



Working :-

- The water is filled in autoclave and the materials is to be ~~st~~ sterilization is load on the basket inside autoclave.

- Now maintain the temperature and pressure for sterilization.

Temperature	Time	Pressure
121°c	15 min	15 lbs / inch²
126°c	10 min	20 lbs / inch²
133°c	3 min	30 lbs / inch².

- After cooling unload the materials.

→ filteration :-

- filteration is very useful for substances which get damaged by heat.

- In this method, we ~~steri~~ do sterilization of antibiotic solution, serum, carbohydrate soln etc.

- filteration is a process in which doesn't kill microorganisms but remove the microorganisms.

Scanned by CamScanner

- The filtration technique is based on three mechanism.

i) Sieving → Based on the size of microorganis

ii) Adsorption → Particles are adsorbed on the surface of filter medium.

iii) Trapping → Trapping of particle coithing gauze.

→ Radiation :

i) Ionizing Radiation :

- It includes x-ray, γ-rays, cosmic rays.
- Ionizing radiation causes mutation in DNA structure by changing their sequence of based pair.
- So, it damage the DNA and kill microorganism.

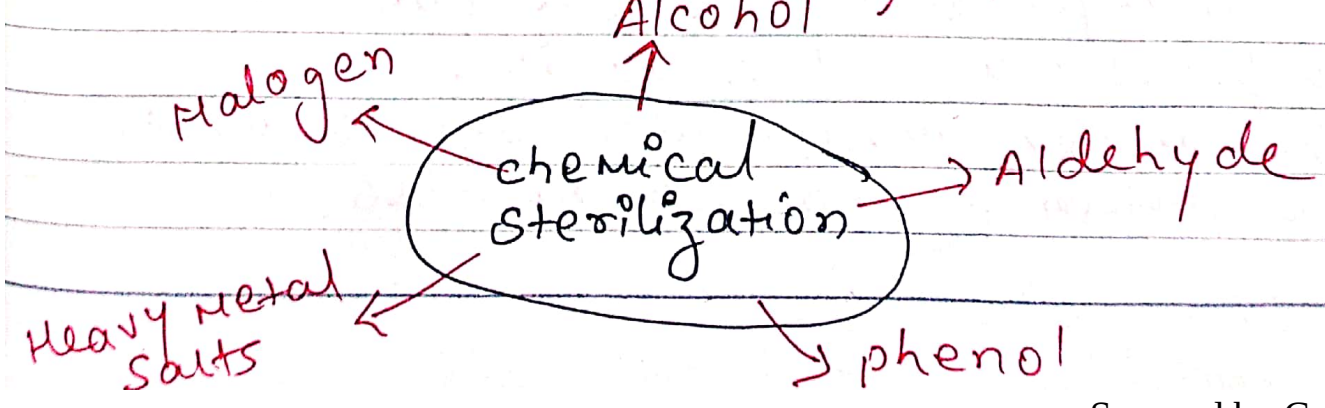
eg:) Gamma radiation are commercially used for sterilization of dispot sable items.

ii) Non-ionizing radiation :

- Non-ionizing radiation is of longer wavelength & it is used only for sterilizing surfaces.
- It includes uv rays & infrared-rays.
- Microorganisms absorb these rays and their purines & pyrimidines base unable to code the Adenine. so, the microorganisms are kill.

II chemical sterilization :

- The chemical substances are generally used on large scale in microbial / laboratories for purpose of sterilization of work areas, desk, glasswares, hand gloves etc.
- ideal properties of chemical sterilization —
  - → Effective in Acidic & basic medium.
  - → High penetrating power.
  - → Should be stable at high temp.
  - → Safe & easy use
  - → cheap & easily available
  - → wide spectrum / activity.



i) Alcohol :

- Alcohol is used for sterilization of microorganisms.
- It kill bacteria, fungi and some enveloped viruses.
- Alcohols dissolve the membrane lipid and denature protein of microorganism resulting in lysis of cells. So, the microorganism kill.

ii) Aldehydes :

- Aldehydes ~~are~~ acts on nucleic acid and protein present in the cell & inactivates the cell.
- Three types of aldehyde are used:
  - formaldehyde
  - Glutaraldehyde
  - ortho-phthaladehyde.

iii) phenols :

- phenols such as cresol, xylenol, lysol and ortho-phenyl phenol are used as disinfectant and sterilant in hospitals and laboratories.
- They destroy plasma membrane & denatured the protein present in microbes.

iv) Halogen:

- Halogen like Iodine and chlorine are used as skin antiseptics & disinfectants.

- Iodine acts as antiseptic (skin). It oxidises cell & kill microorganism.

v) Heavy metal salts:

- The heavy metal salts combine with the protein of the cells specially with sulphyhydryl group and carry out precipitation of cell proteins or lysis of microbial cell.

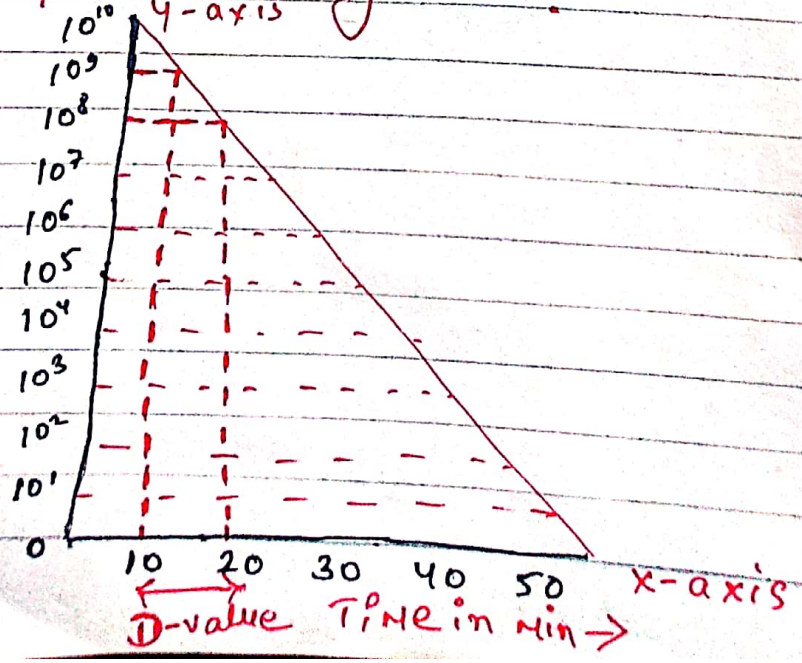
Evaluation of the efficiency of sterilization method :-

- Evaluation means to check the sterilization process (physical, chemical, radiation) are efficient to kill 100% microorganism.
- Evaluation can be determined by three values :

i) D-value ii) Z-value iii) F-value.

1) D-value :

- The D-value is also known as decimal reduction time.
- It is defined as time in minutes at any defined temperature to destroy microorganism (i.e., present in the sample/instrument which we need to sterilize) is called D-value.
- It is a set of condition to achieve 1 log reduction of microorganism.

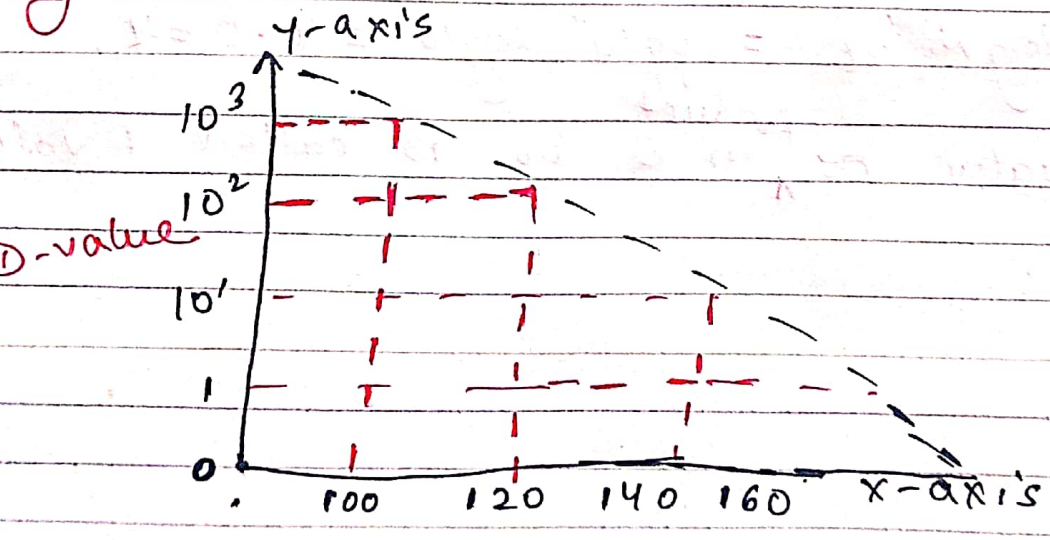


organisms	D-value min. at 121.1°C
. Bacillus stearothermophilus	4-5 min
. Desulfotomaculum nigrificanes	3-4 min.

- The time require for killing microorganism at low temp. is more but the time require for killing microorganism at high temp is less.
- It is specific value for each microorganisms
- If D-value is low then the sterilization would be great. & it is more efficient.

2) Z-value:

- when we plot the D-value on y-axis & temp. at x-axis then the temperature is required to reduce the D-value by one log is called Z-value



- If we increase the temperature more bacterias will kill then the D-value is decreases & z value is increases.

3) F-value:

- The time is required on heating method for sterilization to kill the population of bacteria spores in minute is called f-value.
- D-value for steamthermophilus to be 5 min & initial number (N_0) in the container to be 10,000, if it was required to reduce number to one (N_t) in the heat process, four decimal reduction would be needed.
  The time at 121.1°C would be $4 \times D_0 = 4 \times 5 = 20$ min
- The number of decimal reduct^n required is given by:
- $n = \log N_0/N_t = \log 10^4 - \log 10^1 = 4 - 0 = 4$.
- The value of product n & D_0 is called f value.

PHARMACEUTICAL MICROBIOLOGY

UNIT => 5

Sterility Indicator :

- sterility testing is defined as a test confirm that the products are completely free from micro-organism.
- It is very important for medical devices, pharmaceutical operations, etc.
- sterility indicator are that indicator use to check the quality of sterilization and monitoring of sterilization process.
- The sterility indicator are of following types :

i) Physical indicator
ii) Chemical indicator
iii) Biological indicator

* Physical indicator :

- The display on the sterilize or a recording device that print the parameters like time, temp, pressure associated with each sterilization cycle for each load, such indicator are consider as physical indicator.

Method		Device
1. Dry heat sterilization	→	Temp. recording chart
2. Moist heat sterilization	→	Temp. recording chart
3. filteration sterilization	→	Bubble point chart.
4. Gaseous sterilization	→	Temp. recording chart

* Chemical indicator :

- chemical indicator, these are chemical substance that are used in the process of sterilization in order to indicate that the process of sterilization is going as per requirements or it may indicate that the products are / sterile.

- The chemical indicator that are used in dry or moist heat sterilization, may melt / or change its colour only / when satisfactory condition that / the product is sterile.

* Biological indicator :

- The biological indicator are standardised bacterial spores used in the form of of suspension in water or culture media or of spores dried on the paper or plastic carriers that are kept / place in the sterilizer.
- According to the sterilization process we use that kind of bacterial spores like spores of bacillus subtilis is used as biological indicator in dry heat sterilization process to determine D-value ; while bacillus stereo-thermophilus spores are used in case of moist heat sterilization.

Method	Principle	Microorganism	Parameter
1 Dry heat	Temp. sensitive microbes	Bacillus subtilis	D-value
2. Moist heat	Temp. sensitive microbes	Bacillus stereo-thermophilus	D-value
3. Radiation	Radiation sensitive microbes	~~Bacillus~~ Bacillus pumilus	D-value