→ Bacterial cells have -ve charge at its surface

Date / / Page No.

Staining Techniques

→ Stains are dye, organic compounds which have benzene ring & have chromoform group.

→ Stain classification
- → acidic (anionic nature (-vely charged) — - Eosin, - Picric acid, - acid fuchsin
- → basic (cationic nature (+tuly charged) — crystal violet, methylene blue, saffranin
- → neutral (no charge, it forms ppt when aq. & non aq. media combine
  - ex → eosinate.

→ Staining Techniques
- → Simple (single dye used)
  - → +ve staining (acid)
  - → -ve staing (basic)
- → Differential (two or more dyes used)
  - → Gram staining
  - → Acid fast staining

Procedure involved in staining

① Simple staining method

Prepare a smear
↓
dry it by air & then heat fixation is done
↓
crystal violet / methylene blue / carbol fuchsin (apply a drop)
↓
wash with water
↓
Observe in a microscope.

② Differential Staining method

two or more dyes are used at a time.

Scanned by CamScanner

(a) → Gram staining method
(b) → Acid fast staining

→ **Gram staining Method**

- The method is developed by Christian Gram (1884) in the country Denmark.
(Read from lab manual)

→ **Acid fast staining:-**

This method is developed by Paul Ehrlich (1882) & was modified by Ziehl-Neelsen & after this it was named as Ziehl-Neelsen staining method.

Method

Prepare a smear
↓
Air dry it & heating fixation is done
↓
* Carbol Fuchsin is applied & wait for few secs
↓
Dry is not allowing & staining is done for 2-3 times
↓
Now cool and wash the stain
↓
Drop of Alcohol is applied to decolourise it
↓
Wash it off with tap water
↓
The smear is treated with methylene blue for 2 mins.
↓
Again wash it with water
↓
Dry it & observe in microscope.

acid fast org. appear in Red colour on blue background & non-acid org will appear in blue colour.

"Death is the bird that feels the light when the dawn is still dark." – Rabindranath Tagore

Scanned by CamScanner

Measurement of bacterial growth

(A) Determination of cell mass
(B) Determination of cell activity
(C) Determination of cell no → Plate count technique
→ Membrane filter count
→ counting chamber method

(A) Determination of cell mass

(a) Direct method → dry wt. measurement
(b) Indirect method → measurement of cell Nitrogen
→ Turbidimetric method.

(a) Direct weight measurement

- This is a very common & simple method.
- In this method bacterial cells growing in liquid medium were collected by centrifugation.

↓

Now the centrifuged cells were washed to remove to remove extraneous matter

↓

Now the cells are dried and filled in a weighing bottle

↓

Now the cells are accurately weighed.

(b) Indirect method Measurement of cell Nitrogen.

- Nitrogen is a major chemical constituent of cell.
- Bacteria contains approximately 14 % N on a dry wt. basis
- Nitrogen is responsible to synthesize amino acids, purines, pyrimidines, etc.
- In this method to measure the growth cells should be centrifuged & washed

↓

After that quantitative analysis of Nitrogen is performed like determination of pre-amino acids, determination of nucleic acids i.e. DNA & RNA

Scanned by CamScanner

(b) Indirect method :- Turbidimetric Method

This is a rapid & simple method. This method involves the use of spectrophotometer

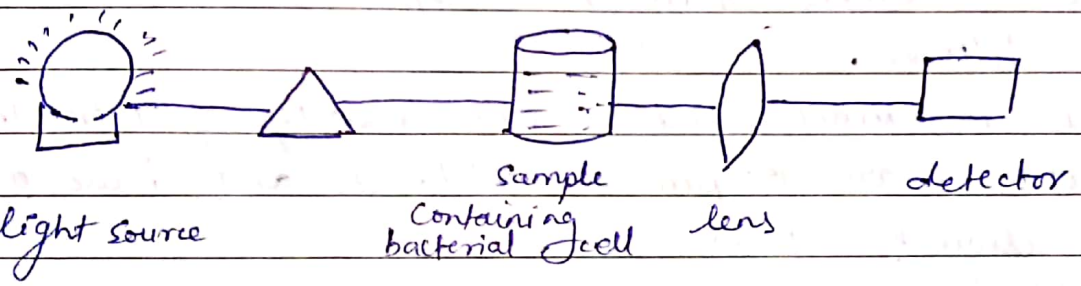
- Spectrophotometer can measure the extent of light scattering
- In this method a beam of light is passed through a suspension of bacteria

↓

The light is scattered in proportion to the no. of cells present

↓

The bacterial suspension appears turbid because each cell scatters light. If more cells are present more light is scattered & more turbidity is visible



(B) Determination of cell activity

This is a indirect method to measure the biochemical & metabolic activity.

ex → Bacterial species ferments glucose to produce acid, the amount of acid produced can be taken as a indirect method measure of bacterial growth

(C) Determination of cell no.

(1) Plate Count Technique

- This method is also c/d as viable cell count method.
- In this method a culture of medium containing bacterial cells & is made to series of dilutions

10 fold dilution is made (1ml of sample + 9ml of diluent)

↓

The dilution series is prepared in this way

↓

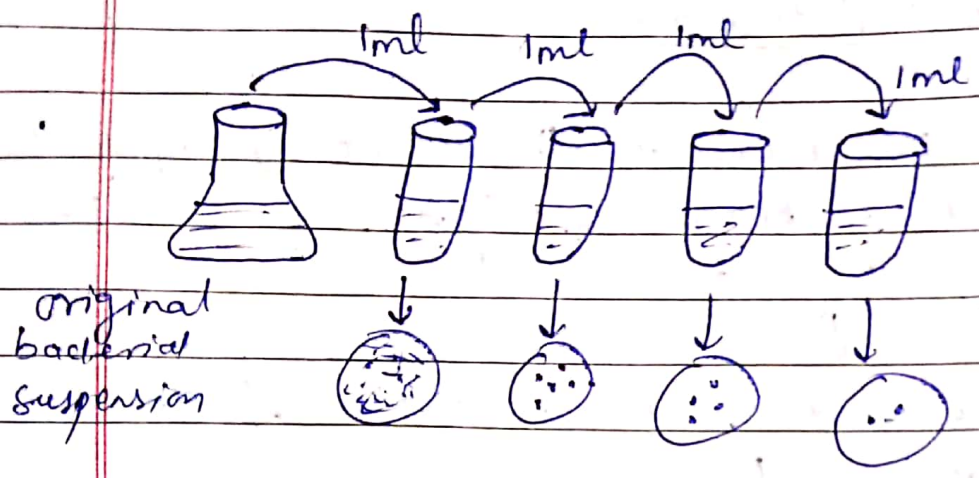
Each dilutn is poured into petri plate containing agar media & incubated

"Faith is the bird that feels the light when the dawn is still dark." –Rabindranath Tagore

Scanned by CamScanner

↓

Now the colonies were counted



(2) Membrane filter count (thickness = 150 μm, pore diam = 0.45 μm)

- In this method filtering a ~~non~~ known volume of bacterial media sample through a special membrane filter is filtered.
- This membrane filters are made up of nitrocellulose acetate & are 150 μm in thickness and have 0.45 μm in diameter pores.

Method

The sample is filtered by putting membrane filter

↓

The cells larger than 0.45 μm are trapped on the surface of the filter

↓

Now filter is carefully removed & placed into a petri plate containing agar media.

↓

Now incubate it for 24-48 hrs at 25°C - 37°C

↓

Each cell trapped on a filter will grow into a separate colony

Scanned by CamScanner

(3) Counting chamber method

In this method bacteria can be counted rapidly, simply & accurately with the help of petroff Hausser counting chamber or Haemocytometer

Method

a minute drop of culture is placed in a rectangular glass slide c/d neubars slide

↓

a suspension of unstained bacteria can be counted in the chamber using face contrast microscope

Scanned by CamScanner

Sterlization

Controlling of microbes – either by killing – Cidal effect (e.g → bactericidal)

or by inhibiting / preventing – Static effect e.g (bacteriostatic).

* Killing, removing microbes is c/d sterilizat^n.

Reasons for sterilization

- to prevent contamination, infection, from decaying, etc.

Methods (a) Physical methods –
- (1) dry heat.
- (2) Moist heat
- (3) Radiation
- (4) filteration.

dry heat →
- Red hot / heat
- flamming
- Hot air oven
- incineration
- Sunlight.

(b) Chemical methods.

"The butterfly counts not months but moments, and has time enough."–Rabindranath Tagor

Scanned by CamScanner

Hot air oven → 130-160 or 180°C for 1-2 hrs.
↳ can't sterilize plastic material
↳ oils are sterilized by hot air oven.

dry heat principle → It can denature microbial proteins but can't penetrate in it's cell.

Incineration - used materials (bandages, etc) converted into ashes.

Radiation → sunlight has UV rays which has germicidal ppt.

(2) Moist heat
- Most effective
↓
steam penetrates into bacterial cells breaks H- bonds & denature proteins. & Congalates them.

→ Pasteurization. (milk, wine, juices, etc)
→ Boiling
→ Autoclave (121°C used for 15 mins) → corrosion occurs.
→ Inspissation. (blood, serum are sterilized at 80°C - 85°C for 1 hr) (done for 3 days)

(3) filtrat^n → filter pore size (0.01 to 10 μm)
↳ membrane filter (nitrocellulose)
↳ asbestos filter (asbestos) or seitz filter

(4) Radiation → ionising (α, β ~~& cobalt~~)
→ non-ionising (UV rays)

→ Nucleic acid tnt in bacteria absorb light rays & denature chromatin material in bacteria.

Chemical methods → (a) Gas sterilisation (ethylene oxide, ozone)
(b) Surfactants (soaps, detergents, these remove charges from the bacterial cell)
(c) Disinfectants lysol, phenyl, dettol.

- High penetrating power & denature protein
- Generally used in hospitals.

Scanned by CamScanner

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

Scanned by CamScanner

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

The member of this family are:

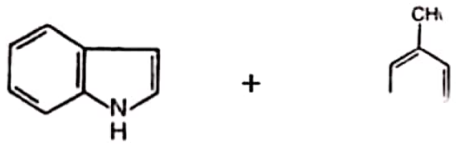
E.Coli, Enterobactor aerogenes, Klebsiella pneumoniae, Salmonella typhimurium, Shigella dysenteriae, Proteus vulgaris, Pseudomonas aeruginosa, Alcaligenes faecalis

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

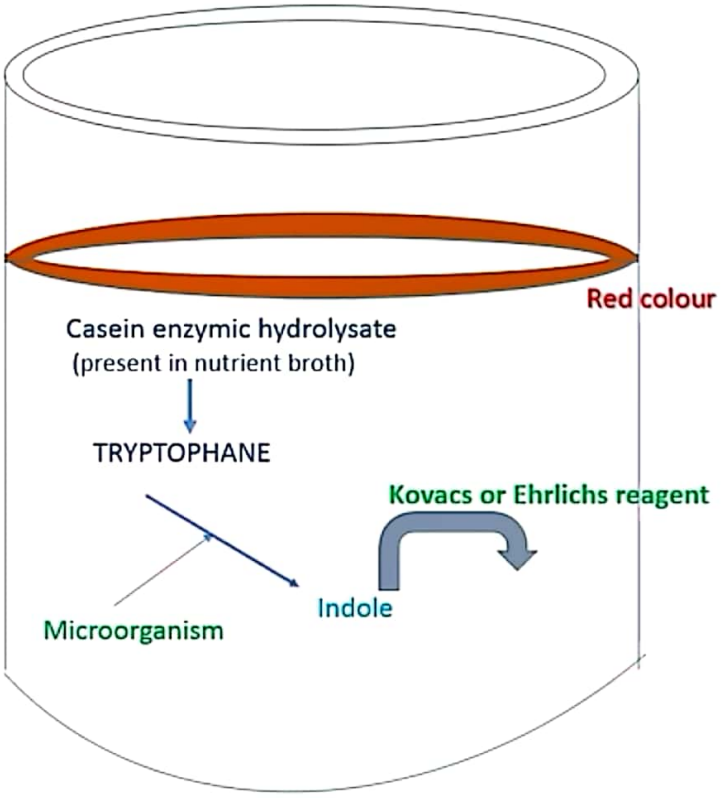
- These tests are also use to differentiate various members of Enterobacteriaceae family based on their biochemical properties (the properties to metabolize different compounds)

Scanned by CamScanner

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

Scanned by CamScanner

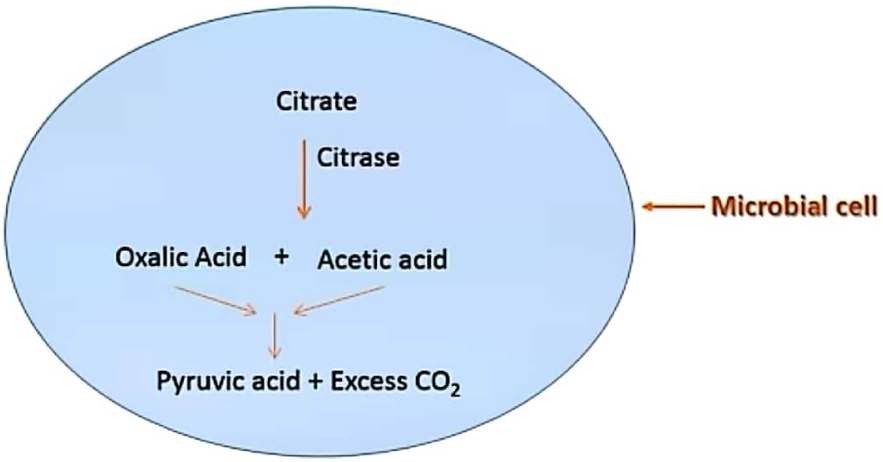


Scanned by CamScanner

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)

Scanned by CamScanner



CO_2 + Na ⟶ Na_2CO_3 + Alkaline pH

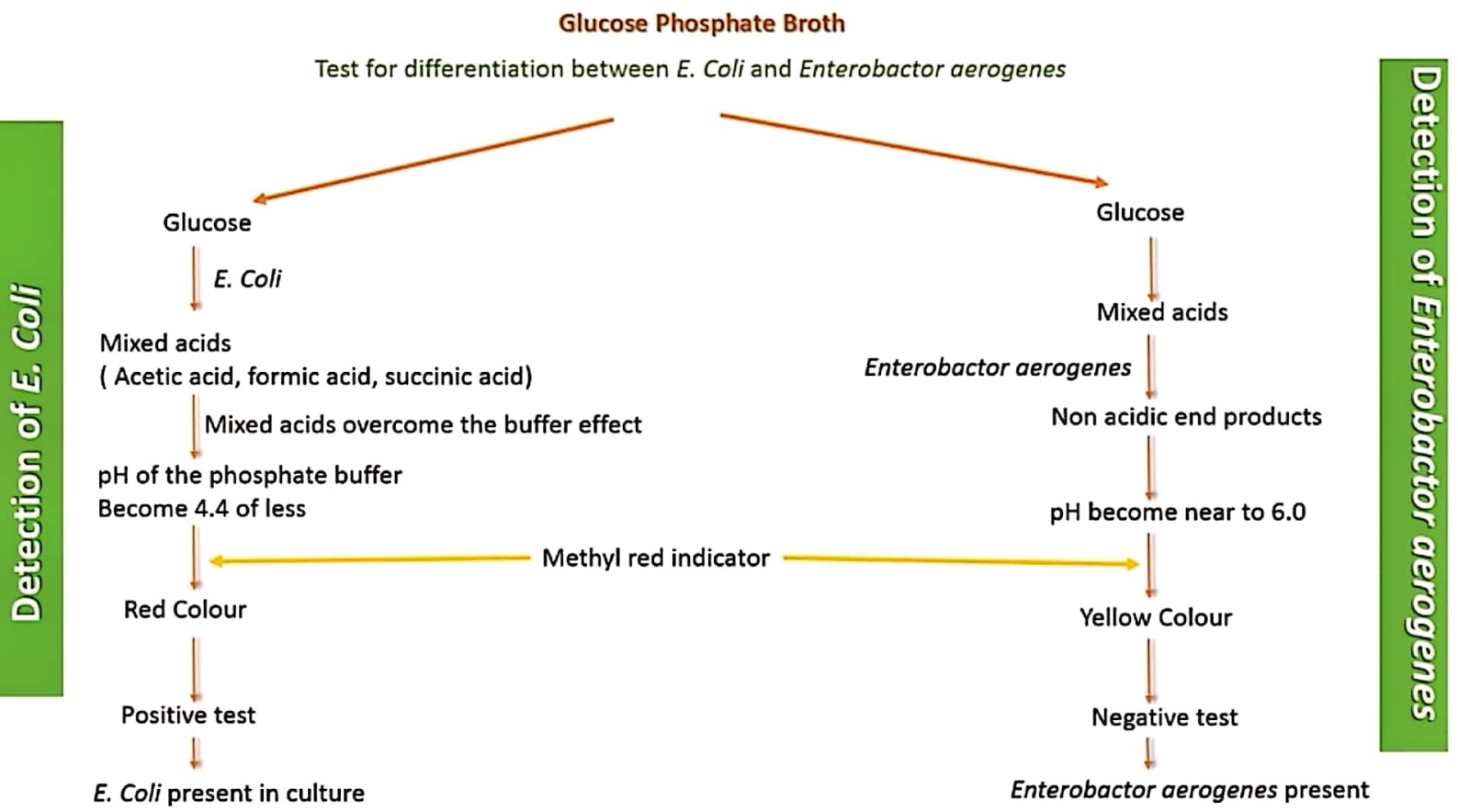
Bromothymol Blue (Green) —Due to alkaline pH⟶ Prussian Blue

Scanned by CamScanner

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 Yellow Colour indicate presence of Enterobactor aerogenes

Scanned by CamScanner



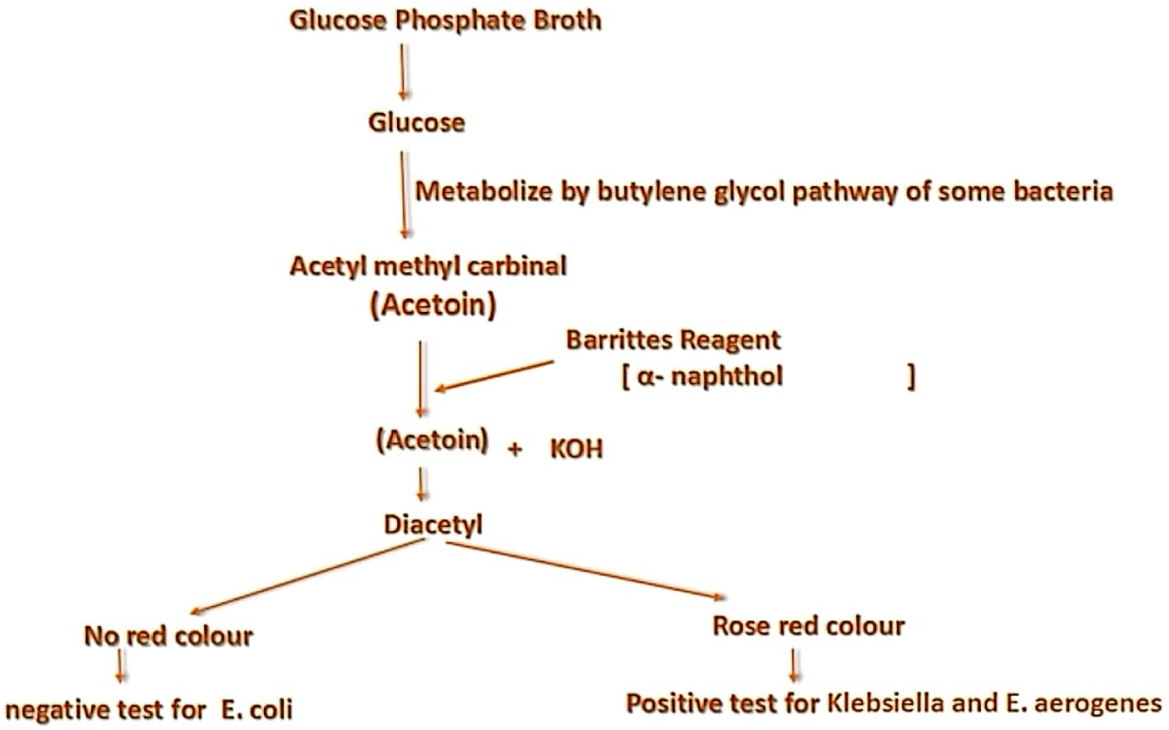
Scanned by CamScanner

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).

Scanned by CamScanner

Voges- Proskauer test



Scanned by CamScanner

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

Scanned by CamScanner

Sterility Indicators

Defination – The sterility indicator or sterilisation indicators are used to monitor visually to check the cleaning conditions are passing the test or not.

The controls are of two types for microbes :-

(a) Control on process sterilization
(b) Sterility testing of the products.

Dry heat sterilisation

Indicator	Sterilization methods	Principle	Devices	Parameters monitoned
Physical	Dry heat	Temp. record^n chart	Temp. record^n chart	Temperature
Chemical	Dry heat	Temp. sensitive coloured sol^n	Browne's tube	Temperature & time
Biological	Dry heat	Temp. sensitive microbes.	Bacillus subtilis	D - value

Physical Indicator

- To monitor process of dry heat sterilization, temp. record chart of every sterilization cycle is made & compared with the master temperature records.

Chemical Indicators

These are based on the ability to alter the physical and chemical properties of a given chemical substance.

- The chemical changes should take place only when it confirms the successful completion of sterilization cycle.

"The butterfly counts not months but moments, and has time enough." – Rabindranath Tagore

Scanned by CamScanner

- The chemical indicator usually shows a change in colour of indicator or undergo melting.

Biological indicator

- In this case, prep of stdardised bacterial spores are in the form of suspension or a culture media is dried on paper & kept in sterilizers as a biological indicator.
- for dry heat sterilization generally bacillus sublilis are used

② Moist Heat steri

Indicators.	Principle	Devices	Parameters Monitored
Physical	Temp. recordn charts.	Temp. recordn charts.	Temp
chemical	Temp. sensitive coloured soln	Browne's tube.	Temp & time
	Steam sensitive chemical.	Device infused with a carrier material.	saturated steam.
Biological	Temp. sensitive microbes	Bacillus stearothermophilus	D-value

Physical indicator.

To monitor the process of moist heat sterilization, a temp. record chart of each sterilization is made & compared against master temp. record.

Chemical indicator

- These indicators are based on the ability of heat to alter the physical & chemical property of a given substance.
- They usually show a change in colour or undergo melting

Scanned by CamScanner

Biological Indicator

- The spores of B. Stearothermophilus is sealed in ampoules, kept in sterilizers as a biological indicator.
- Once the process of sterilization is completed & it is incubated & examined for the growth.

(3) Gaseous Sterilization

(a) ~~Physical~~

Indicators	Steri. methods	Principle	Devices	Parameters ~~monitored~~
Physical	Gaseous	Temp record^n charts	Temp record^n charts	Temperature
Chemical	Gaseous	Reactive chemical	Indicator paper impregnated with reactive chemical	Gas conc, temp. and time
		Capillary principle	Based on same migration along wick principle	Gas conc, temp & time
		Temp sensitive chemical	A temp sensitive white wax concealing a black masked	Temp
Biological	Gaseous	Temp-sensitive microbes	Bacillus subtilis	D-value

Physical

- Temp are monitored for each sterilization cycle.
- Pressure & humidity measurements are recorded.

Chemical

- Royach Sachet is an indicator paper.
- A change in colour is seen & it indicates process completion.

Biological

- Bacillus subtilis is used & growth is checked after incubation.

Scanned by CamScanner

(4) Radiation

Indicator	Steril. method	Principle	Devices	Parameters monitored
Physical	Radiatn	Recordn charts	Recordn charts	Radiation dose.
Chemical	Radiatn	Radio chromic chemicals.	Plastic devices impregnated with radio sensitive materials which undergo colour change at relative low radiatn doses	Only indicate exposure to radiation.
		Dosimeter device	Acidified ferric $(NH_4)_2SO_4$ soln responds to irradiation dose & related changes in their optical density.	Accurately measures radiation doses.
Biological	Radiation	Radiatn-sensitive microbes.	Bacillus Pumilus	D-value.

(5) filteration Sterilisation

Indicators	Sterilisation methods	Principle	Devices	Parameters monitored
Physical	filteration sterilisation	forcibly passn of soln thru the membrane.	Bubble point pressure test	Pressure
Biological	filteration sterilisation	Retention of bacteria	P. diminuta	Size of microbes.

Scanned by CamScanner