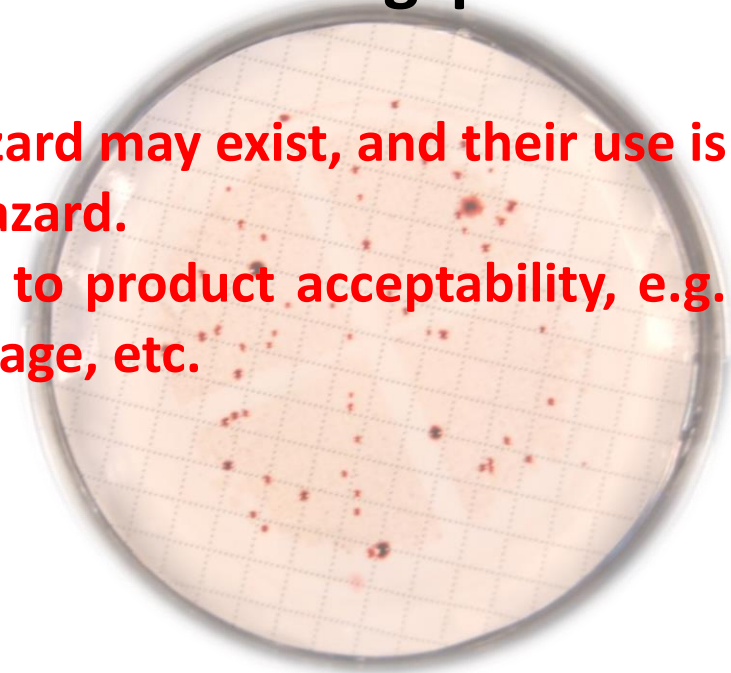




Enumeration of Hygiene Indicator Organisms

- ✓ **Indicator organisms** are bacteria that are used as sign of quality or hygienic status in food, water or environment.
- ✓ Conditions have been related to in-sanitation and public health concerns.
- ✓ Indicator organisms are important components of microbiological testing programs conducted both by regulatory agencies and food industry.
- ✓ Signify potential presence of pathogens, lapse in sanitation as required in good manufacturing practices (GMPs), or a process failure.
- ✓ Safety indicators suggest that microbial hazard may exist, and their use is intended to minimize risk of exposure to hazard.
- ✓ Quality indicators assess issues important to product acceptability, e.g. shelf life, organoleptic characteristics, spoilage, etc.



Selection Criteria for Indicator Organisms

Presence of indicator should suggest a faulty process or potential for spoilage

Survival/stability of indicator should be similar to or greater than hazard or spoilage organism

Growth characteristics of indicator should be similar or faster than hazard or spoilage organism

Identifiable characteristics should be stable

Method for detection should be easy, rapid, inexpensive, reliable, sensitive, and validated and suitable for in-plant use

Quantitative results should show correlation between concentration of indicator and level of hazard

Hygiene Indicators

- Types and number of microorganisms present influence taste, aroma, texture, color and shelf life of product.
- Among these microbial populations, a particular one may be useful as an indicator to reflect quality changes in product.
- Such quality indicators are used to ensure that product is microbiologically stable and aesthetically acceptable.
- Primary attribute of quality indicator is that its growth and numbers should be inversely related to acceptable product quality.
- Indicator should be present in all products whose quality is to be assessed, its growth is unaffected by other microbial populations present and simple methods available for detection, differentiation, and quantization.
- **Example: Yeast and mold determination, which serve as a quality indicator for cereal grains.**

Commonly Used Indicator Organisms

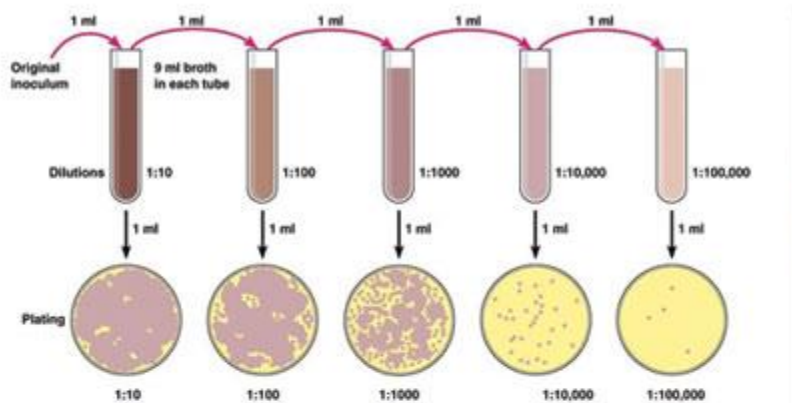
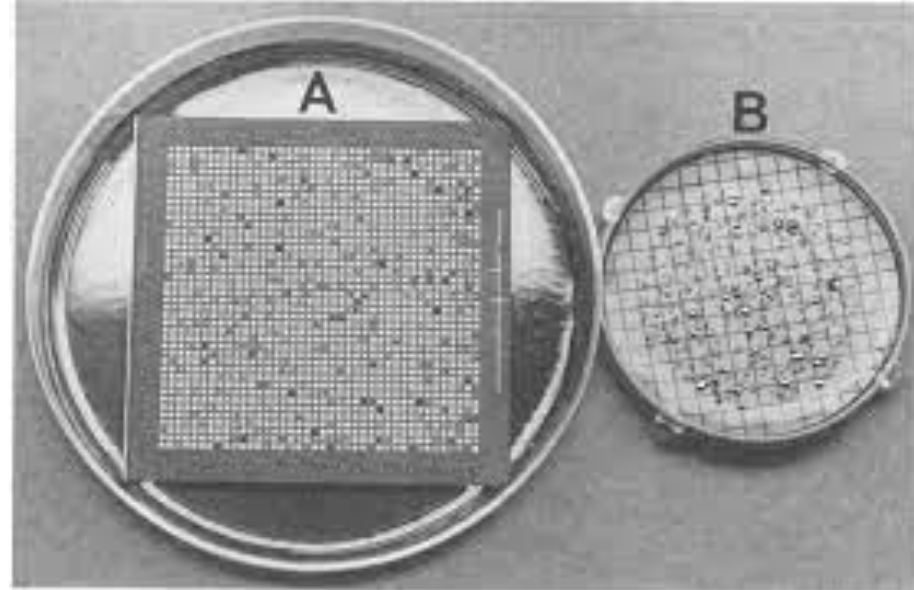
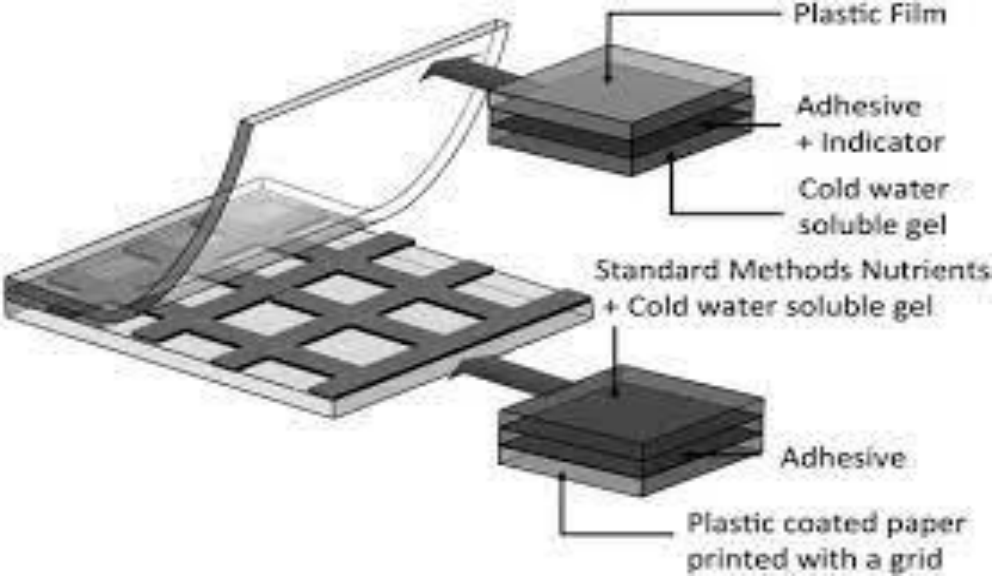


Aerobic plate count (APC):

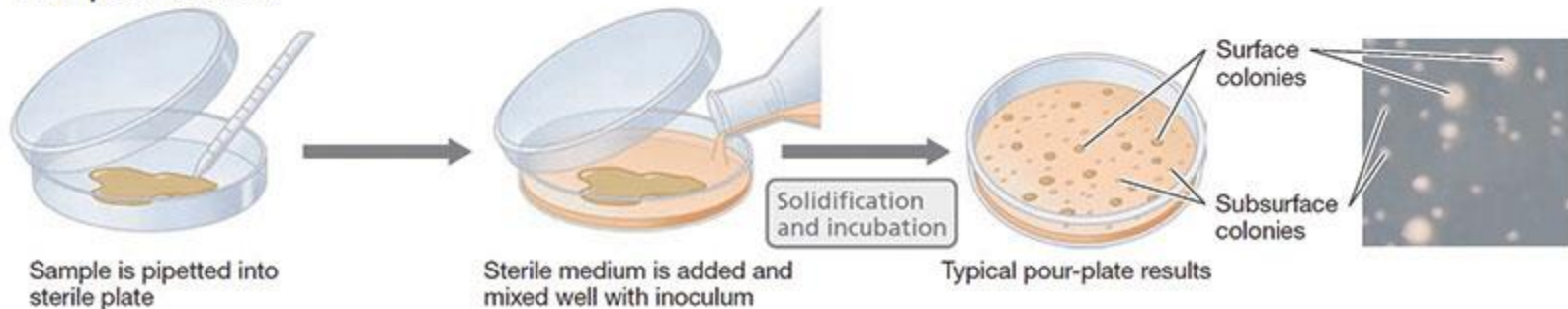
- APC is one of most widely used indicator tests.
- APC cannot be used as a safety indicator, as there is no correlation between APCs and presence of pathogens or their toxins.
- APC may be a quality indicator.
- No indicator value for some products, eg., vegetable sprouts, which naturally have high APCs in range of 10^8 – 10^9 CFU/g, or for yoghurt, which yield high APCs due to starter cultures incorporated.
- APC of product may reflect microbial load of raw materials and ingredients, or its age and storage history.

Detection and enumeration:

- Method recommended by ISO 4833 has aerobic incubation on plate count agar at 30°C for 72 h.
- SPC which is used for estimating bacterial populations in dairy products, strictly specifies 32°C for 48 h.
- ❖ Pour plate method for APC is officially recognized (AOAC 966.23C; ISO 4833).
- ❖ Spread plate technique have advantages: different colony morphologies recognized, translucent media are not required, and microorganisms are not exposed to heat of molten agar.
- Use of hydrophobic grid membrane filter (HGMF; AOAC 986.32), pectin gel (AOAC 988.18), and dry rehydratable film (AOAC 990.12).
- SimPlate[®] Total Plate Count, which uses colorimetric detection of growth in micro wells to determine most probable number of microorganisms, is the most recent method to receive official status (AOAC 2002.07).



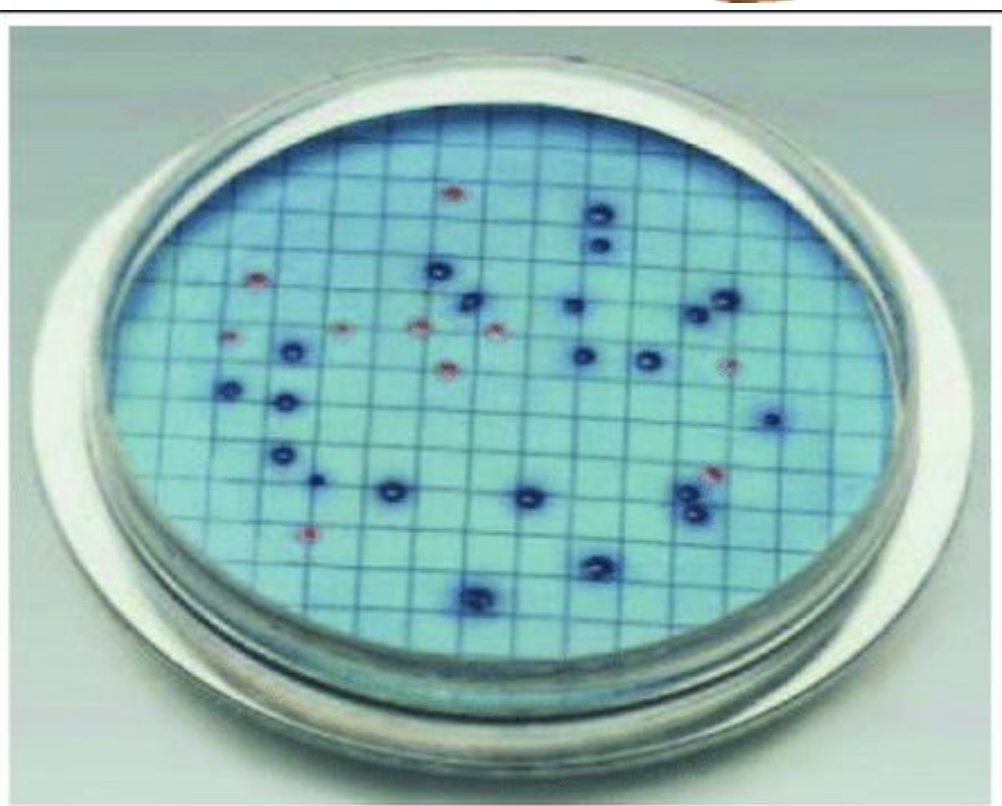
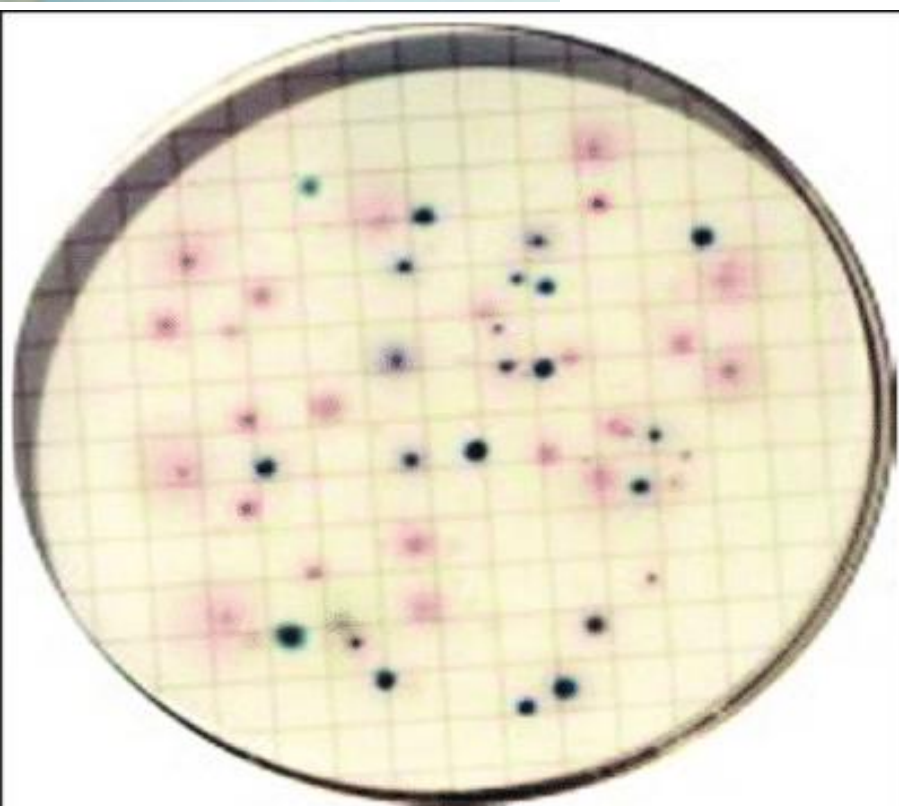
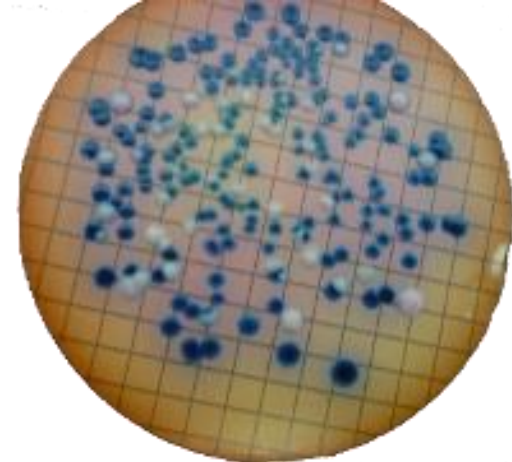
Pour-plate method



Coliforms and *E. coli*:

- Coliform group: Gram-negative, oxidase-negative, aerobic or facultative anaerobic non-spore-forming rods, able to grow in presence of bile salts, and ferment lactose to produce acid and gas within 48 h at 37°C.
- *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella*.
- As *Citrobacter*, *Enterobacter*, *Klebsiella* are normal inhabitants of environment; positive coliform test does not indicate fecal contamination.
- Faecal coliform test: Faecal coliforms (thermotrophic or thermotolerant) fermentation proceed at 44.5–45.5°C.
- *E. coli* is present in all mammalian faeces at high concentrations; it does not multiply but can survive in water for 4–12 weeks, and so it is an indicator of faecal pollution of drinking water systems.
- Coliform groups and *E. coli* are applied in food industry as sanitation and process integrity indicators and for HACCP verification.

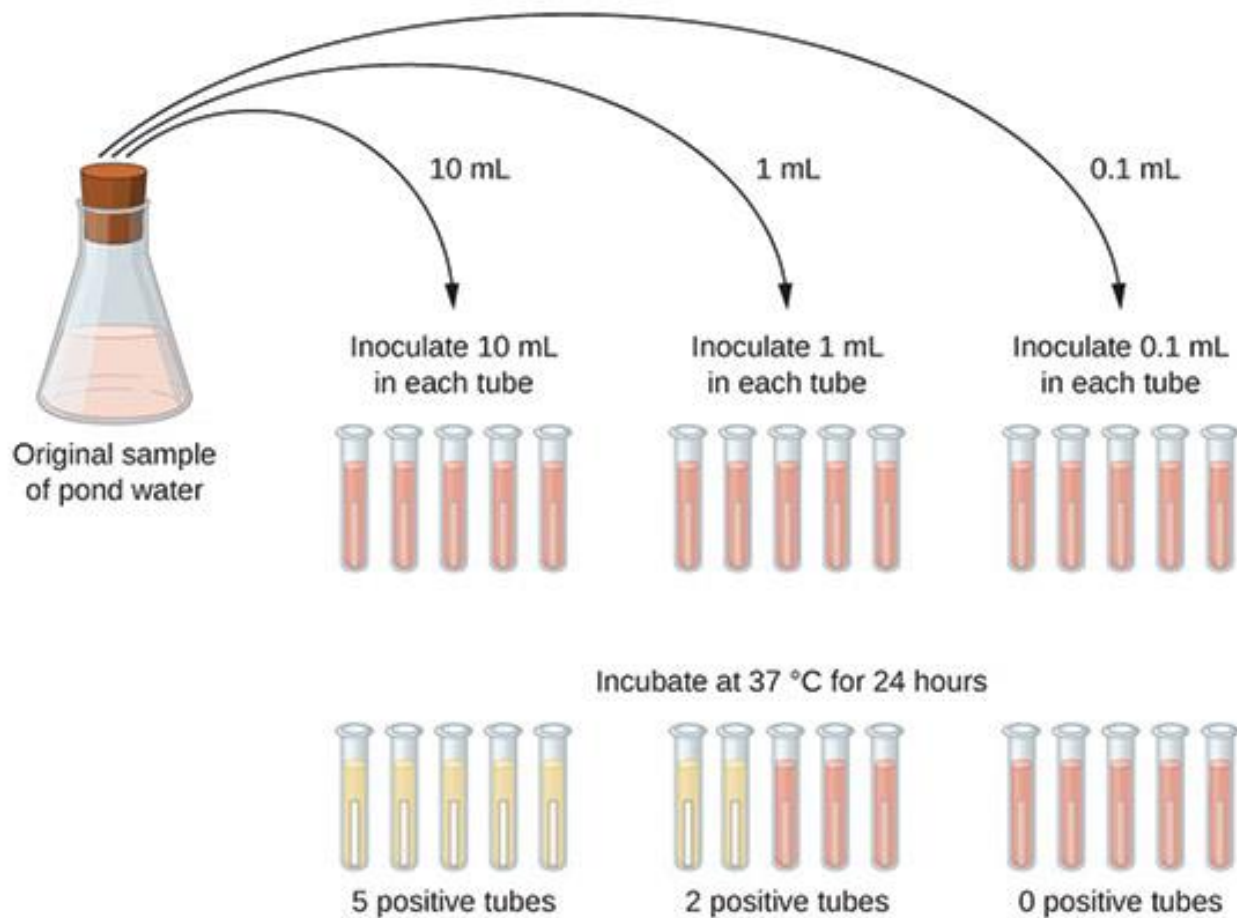




Thermo-tolerant (faecal) coliforms colonies appear as purple dots and total coliforms as pink dots using Coli scan broth.

E. coli colonies appear as blue dots using m-Coli blue broth. The red dots indicate total coliform colonies.

- **Coliform testing i.e. lactose fermentation and resistance to bile salts.**
- **Colony counts of the coliform group are obtained from violet red bile lactose (VRBL) agar (ISO 4832; ISO 5541/1).**
- **Injured coliform populations may be recovered by first inoculating the sample onto a non-selective agar medium, incubating for several hours to allow resuscitation, followed by a VRBL agar overlay for selection.**
- **MPN method for enumeration of coliforms uses Lauryl sulfate tryptose broth in first step, with confirmation of positive tubes, indicated by gas production, in brilliant green bile lactose broth.**
- **Membrane filtration, which allows analysis of larger sample volume than other methods, is recommended for coliform counts in 100 ml water (ISO 9308-1).**
- **Appropriate enzymatic treatments for foods are necessary to allow filtration of 0.5–2.0 ml sample volumes in application of HGMF for coliform determinations (AOAC 983.25).**



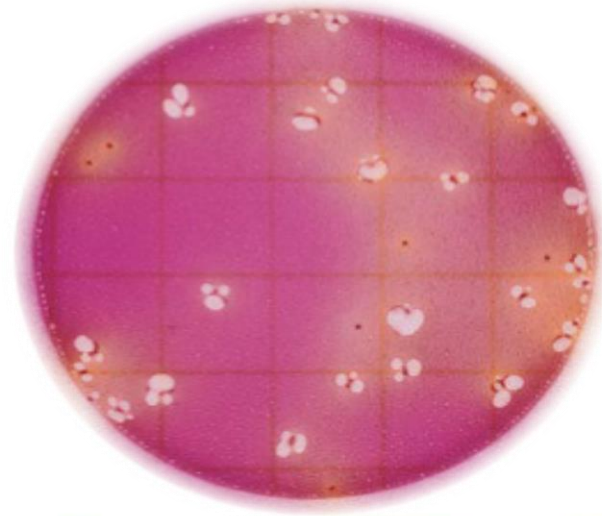


Enterobacteriaceae:

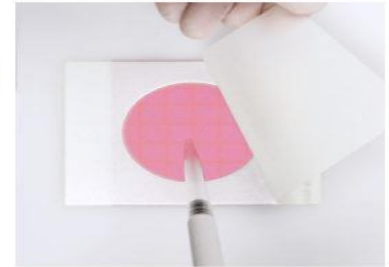
- Include *E. coli*, coliform group; food-borne pathogens *Salmonella*, *Shigella*, *Yersinia* etc.
- Lactose, specified in coliform test, is not fermented by *Salmonella*, *Shigella*, or *Yersinia*, so their presence would not be detected by test.
- But substituting glucose for lactose would allow detection of pathogens.
- Useful as process integrity indicators.
- Enterobacteriaceae may be superior to coliforms as indicators of sanitation GMPs because they have greater resistance to environment than coliforms, can colonize where sanitation has been inadequate, and are sensitive to sanitizers.
- Useful for monitoring sanitation in food manufacturing plants.

Detection and enumeration:

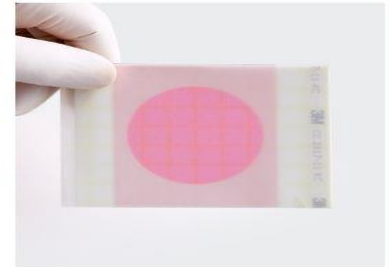
- Ferment glucose.
- Enumerated on **violet red bile glucose (VRBG) agar** or by **MPN determination using brilliant green bile glucose (BGBG) broth (ISO 7402)**.
- **Petrifilm method** for determining **Enterobacteriaceae** counts has recently received **AOAC Official Method SM status (AOAC 2003.01)**.
- Commonly used as sanitation indicators.
- As subjected to various environmental stresses, a method for their recovery by pre-enrichment in non-selective buffered peptone water, followed by selective growth in BGBG broth and isolation on VRBG agar (ISO 8523).



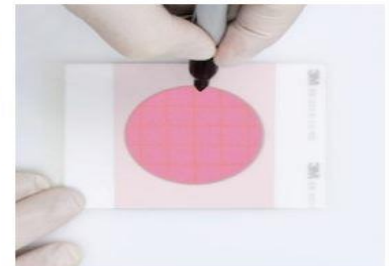
1. Inoculate
Eliminate media prep — plates are ready to inoculate.



2. Incubate
A compact incubator is all you need.



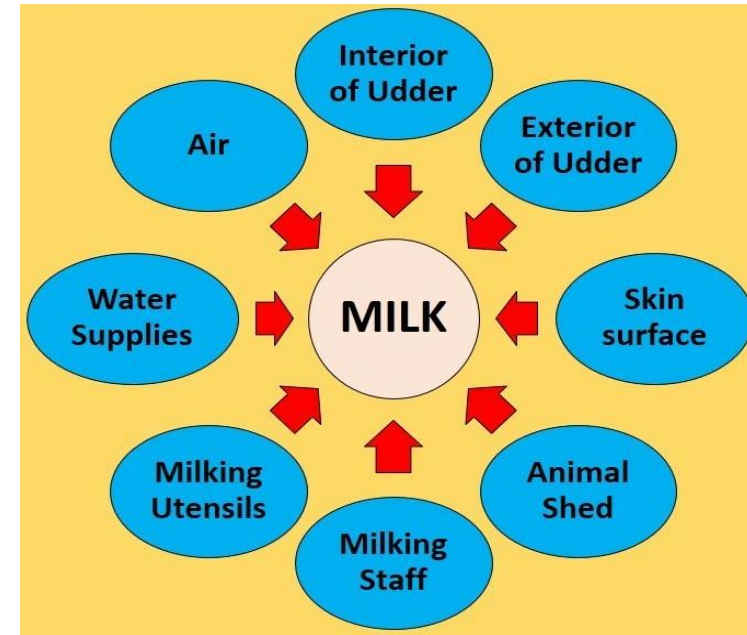
3. Interpret
Indicator dyes facilitate colony counting.



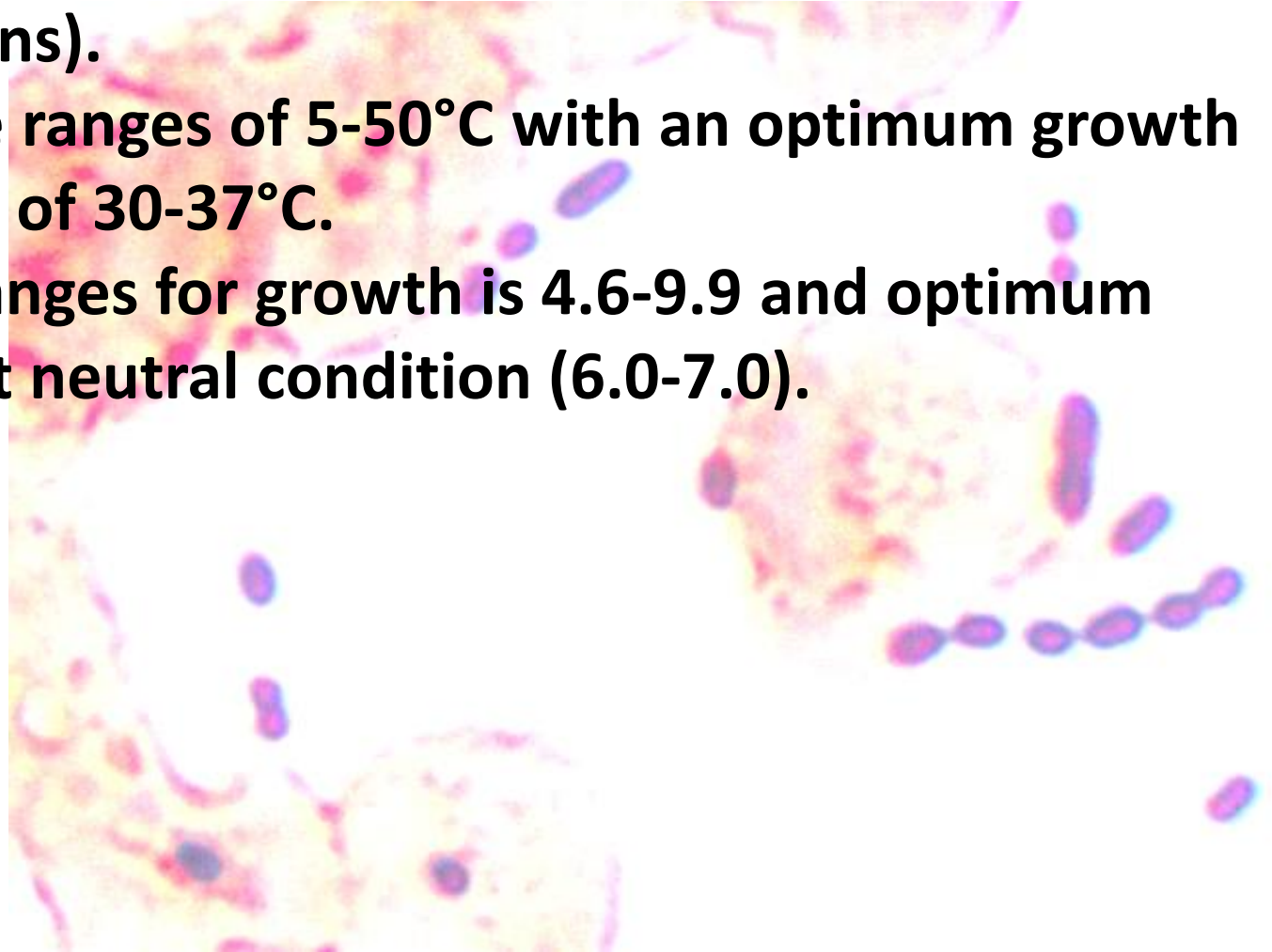
How to use 3M™ Petrifilm™ Plates.

Enterococci:

- Serves as valuable bacterial indicator for determining faecal contamination.
- Spoilage micro-organisms and cross-contaminants during food processing, when their initial numbers in raw milk are high, pasteurization is poor, pasteurized milk is not stored properly indicates poor hygiene during milk handling and processing.
- Problem in dried milk products and infant feed (faecal streptococci should be absent in 0.1 g).
- HTST pasteurization (72°C/15 secs) followed by proper storing conditions ensures no enterococcal re-growth.



- **Gram-positive, non spore forming, non-motile, facultative anaerobic, gamma-haemolytic on blood agar, catalase negative, homofermentative ovoid cocci (pairs to short chains).**
- **Temperature ranges of 5-50°C with an optimum growth temperature of 30-37°C.**
- **Typical pH ranges for growth is 4.6-9.9 and optimum growth pH at neutral condition (6.0-7.0).**



Enumeration of enterococci from dairy products:

Bile tolerance up to
40%

Sodium chloride
tolerance up to 6.5%

Survive heating at
60°C for 30 mins

Hydrolyse esculin in
presence of bile salts
by enzymatic action of
 β -glucosidase

High level of endemic
antibiotic resistance

Reduces 2,3,5-
triphenyltetrazolium
chloride (TTC)

Tolerance to sodium
azide

Enzymatic action of β -
glucosidase on
chromogenic and
fluorogenic substrates

Conventional methods:

- Culture media employed for estimation of enterococci in water, foods, feeds and clinical specimens such as **Slanetz Bartley (Membrane *Enterococcus* agar)**, **kanamycin esculin azide (KAA) medium**, **citrate azide agar (CAA)** and **bile esculin azide agar (BEA)** are applied in selective enumeration of enterococci if enterococci are only microbial component in product.

List of various selective agents commonly used for enterococci and their mode of action

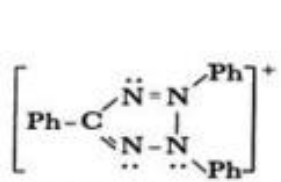
Selective Agents	Mode of action
Sodium azide	Inhibition of enzyme systems (catalase, cytochrome c oxidase) in electron transport
2,3,5-triphenyltetrazolium chloride (TTC)	Reduction of TTC leads to the insoluble red formazan compound and it depends on pH of the medium
Antibiotics (gentamicin, kanamycin)	Less selective than sodium azide, act on different sites (ribosomes, proteins, etc.)
Esculin	β -D-glucosidase enzyme hydrolyzes esculin to esculetin and dextrose; esculetin combines with ferric ammonium citrate to form black color complex
Chromogenic/ Fluorogenic substrates	β -D-glucosidase enzyme acts upon these substrates to form different colored/ fluorescent compounds

Citrate azide agar:

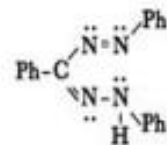
- Used for identification of enterococci in meat, meat products, dairy products etc.
- High concentrations of citrate and azide almost completely inhibit growth of accompanying microbial flora.
- Enterococci reduce colorless 2,3,5-triphenyltetrazolium chloride dye present in medium to red formazan, their colonies thus become red in color.

Bile esculin azide agar:

- Bile esculin agar modified by adding sodium azide and reducing concentration of bile.
- Organisms positive for esculin hydrolysis, hydrolyze esculin to esculetin and dextrose (glucose).
- Esculetin reacts with ferric citrate to form dark brown or black complex.
- Oxbile is used to inhibit Gram-positive bacteria and streptococci, while sodium azide inhibits Gram-negative bacteria.
- Enzymatic digest of casein and yeast enriched meat peptone used for general growth requirements in bile esculin agar.
- Sodium chloride maintains osmotic balance of medium.

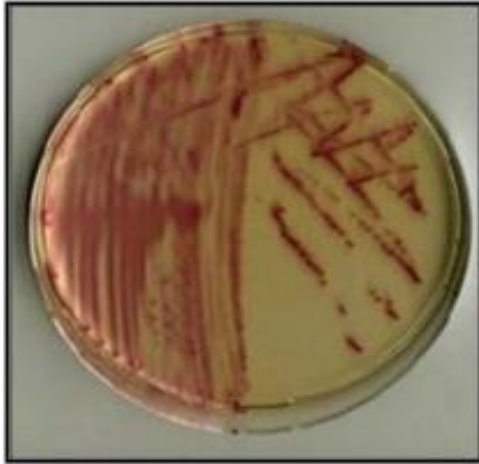


2,3,5-triphenyltetrazolium



1,3,5-triphenylformazan (RH)

Red Coloured Compound

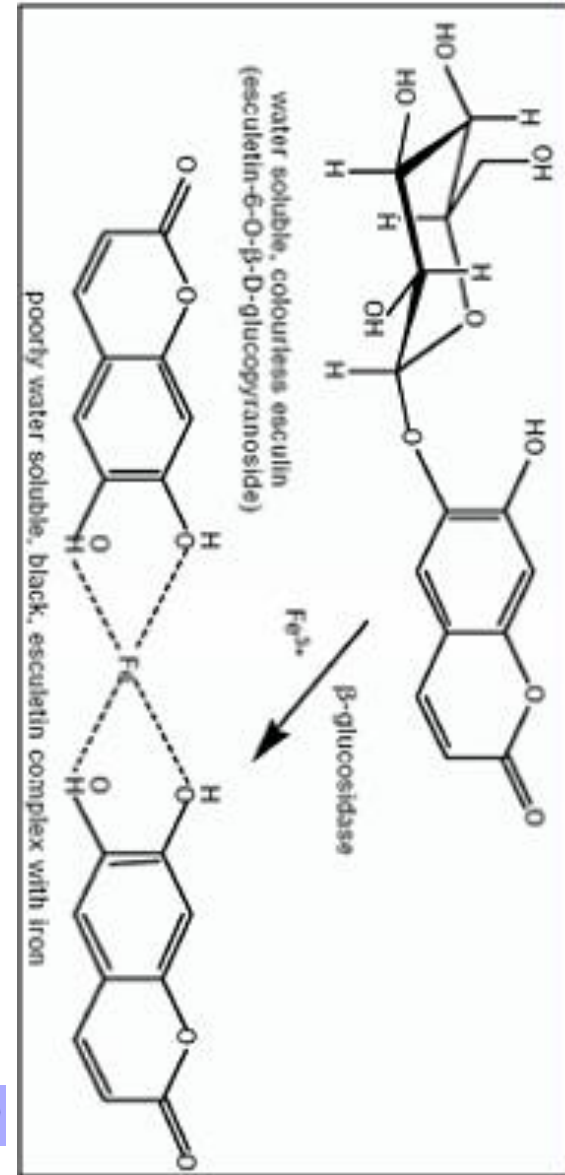


Red coloured colonies on citrate azide agar



Black color colonies on bile esculin azide agar

Reaction catalysed



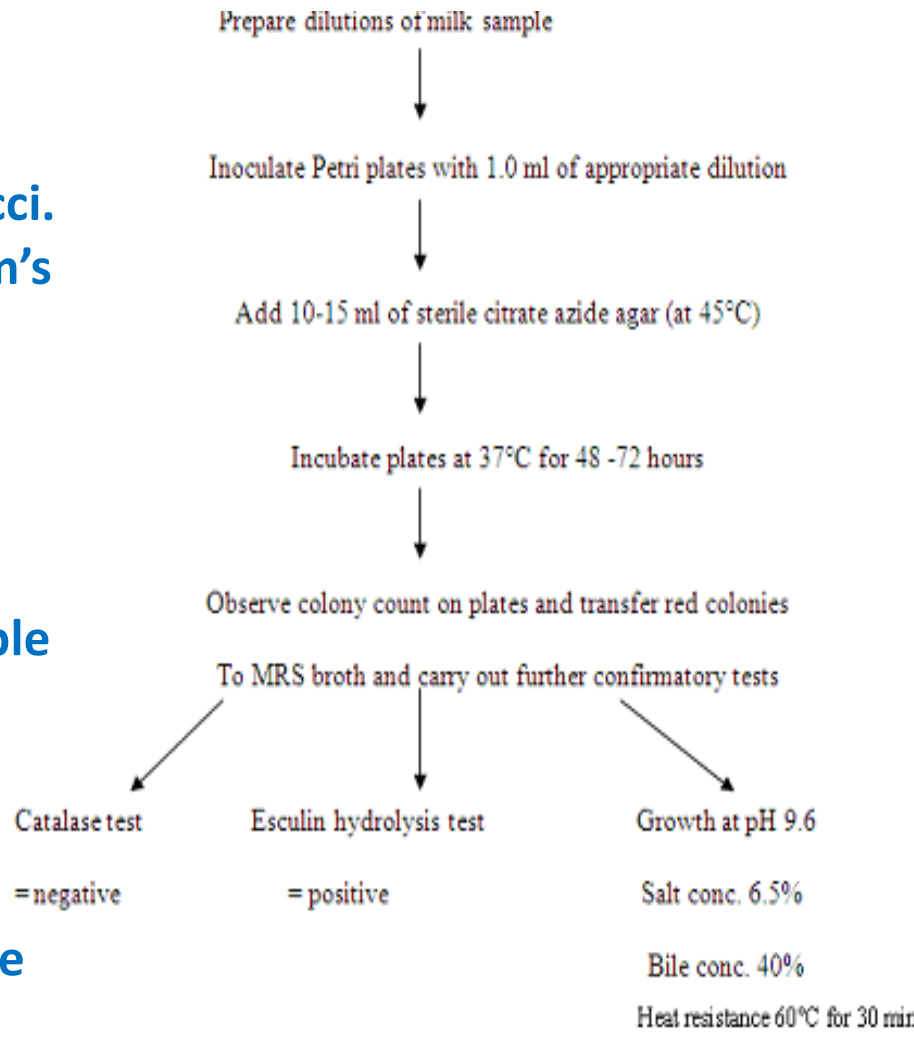
Kanamycin-esculin-azide (KAA)

medium:

- Sodium azide and kanamycin provide selective inhibition whilst esculin and iron salts form an indicator system for presumptive identification of enterococci.
- Incubation at 42°C will increase medium's selectivity.

Slanetz-bartley (Membrane enterococcus agar)

- Enterococci reduce 2, 3, 5-triphenyltetrazolium chloride to insoluble red dye formazan, producing dark red colonies on surface of agar membrane.
- Colonies may be confirmed as enterococci by demonstrating esculin hydrolysis using kanamycin esculin azide agar.

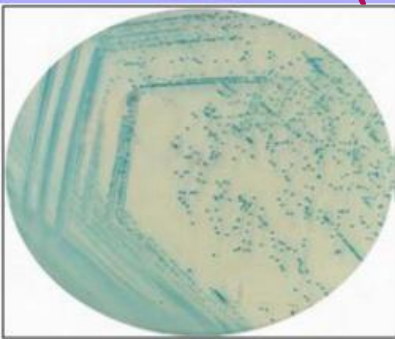


Enzyme/substrate based detection method:

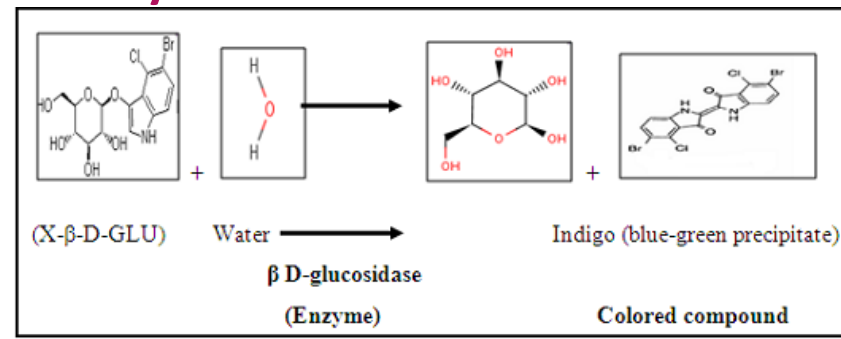
- Unique biochemical pathways that characterize each genus, species or strain and are based on unique key enzymes that participate in such metabolisms.
- Enzymes are referred as **'marker enzymes'**.
- Chromogenic/fluorogenic substrate complex is hydrolyzed by marker enzyme and free chromogen or fluorogen is released which is detected colorimetrically.

Hi chrome rapid enterococcus agar (Chromogenic based):

- Rapid detection of enterococci from water samples.
- β -glucosidase, marker enzyme for enterococci cleaves chromogenic substrate X-GLU (5-bromo-4-chloro-3-indolyl- β -D-glucopyranoside), resulting in intensive bluish green color colonies due to formation of indigo.
- Special peptone provides nitrogenous compounds and other essential nutrients.
- Sodium chloride maintains osmotic balance of medium.
- Sodium azide inhibits Gram negative organisms.
- Tween 80 (Polysorbate 80) acts as a source of fatty acids.



Bluish green color colonies on Hi chrome rapid enterococcus agar





Yeasts and molds:

- Enumerated in foods as quality indicators.
- Survives in pH 2–9; temperatures of 5-35 C; and water activity (a_w) of 0.85 or less.
- As quality indicators, can be used to assess ingredient acceptability, organoleptic characteristics, stability, and shelf life of product.
- Osmophilic yeasts can grow down to a_w of 0.65 and are used as indicators in low a_w foods, e.g., jams, syrups, juice concentrates.



Detection and enumeration:

- **Enumerated by a plate count procedure that uses agar supplemented with agents (Chloramphenicol, rose bengal, and dichloran) inhibitory to bacteria.**
- **Spread/pour plates, incubated at 25 C for 3–7 days, are recommended (ISO 7954).**
- **Rapid official methods using HGMF (AOAC 995.21) and dry rehydratable film (AOAC 997.02) recommend 50 h or 5-day incubation, respectively.**
- **A method using SimPlate colorimetric format determines yeast and mold counts in 56–72 h (AOAC 2002.11).**