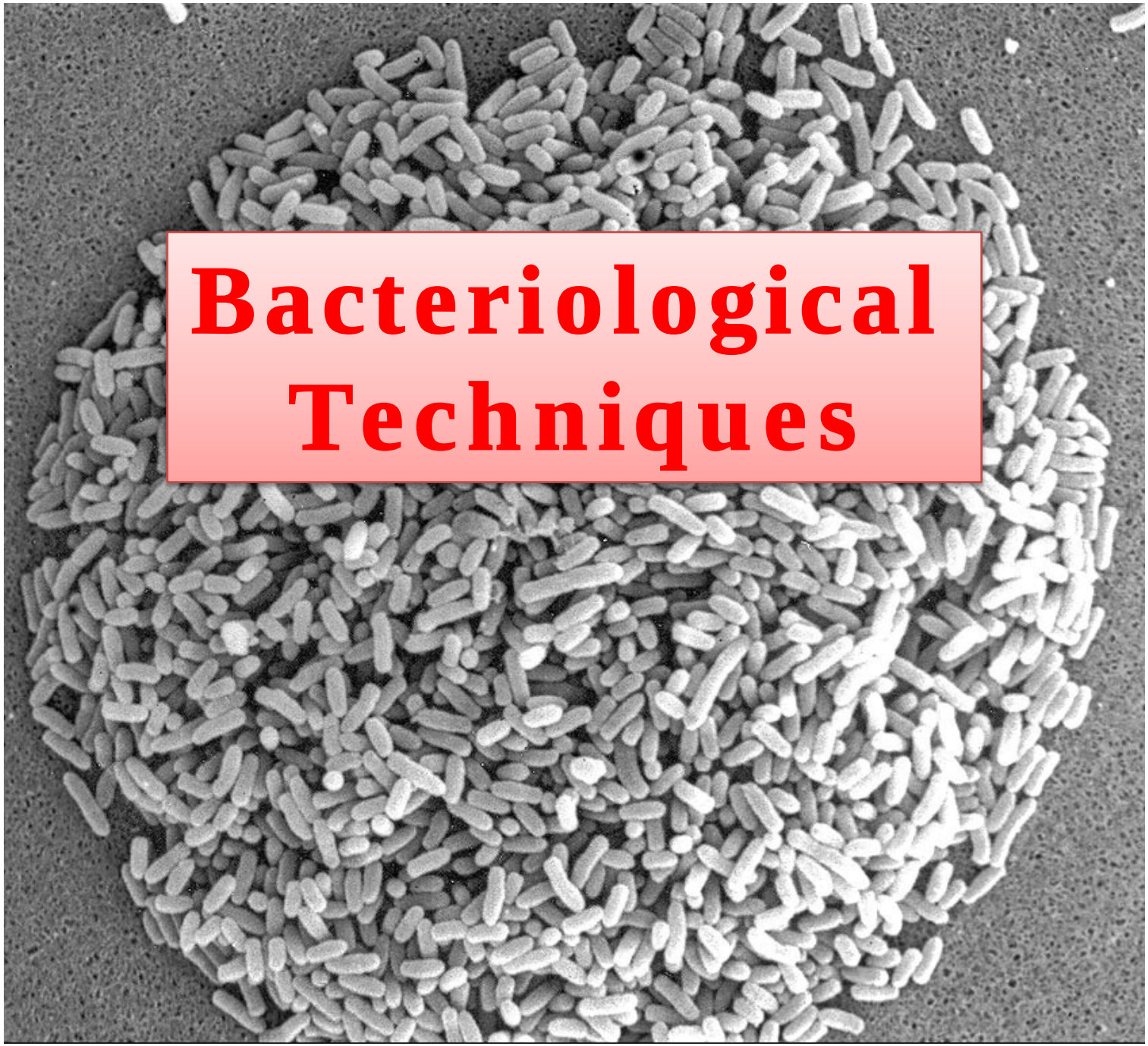
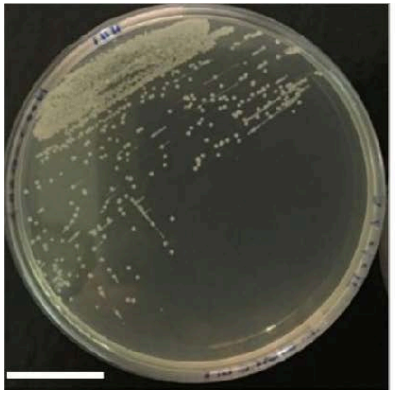
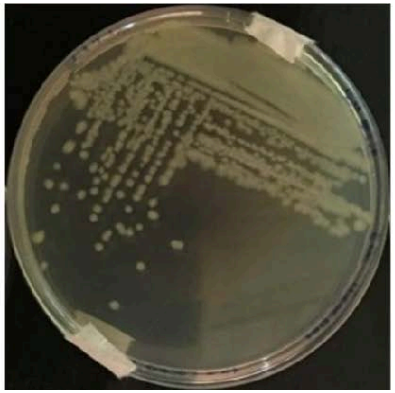
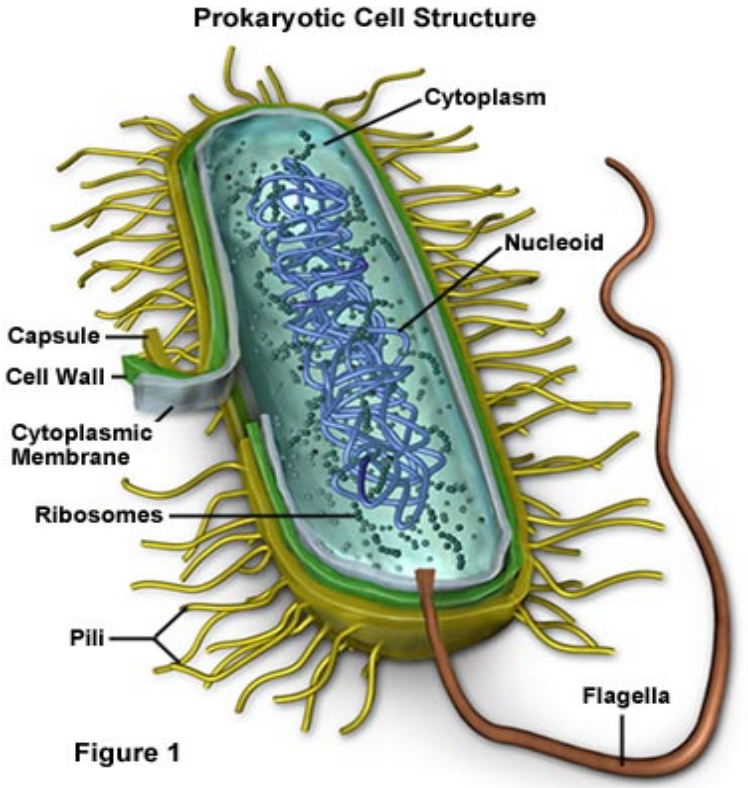


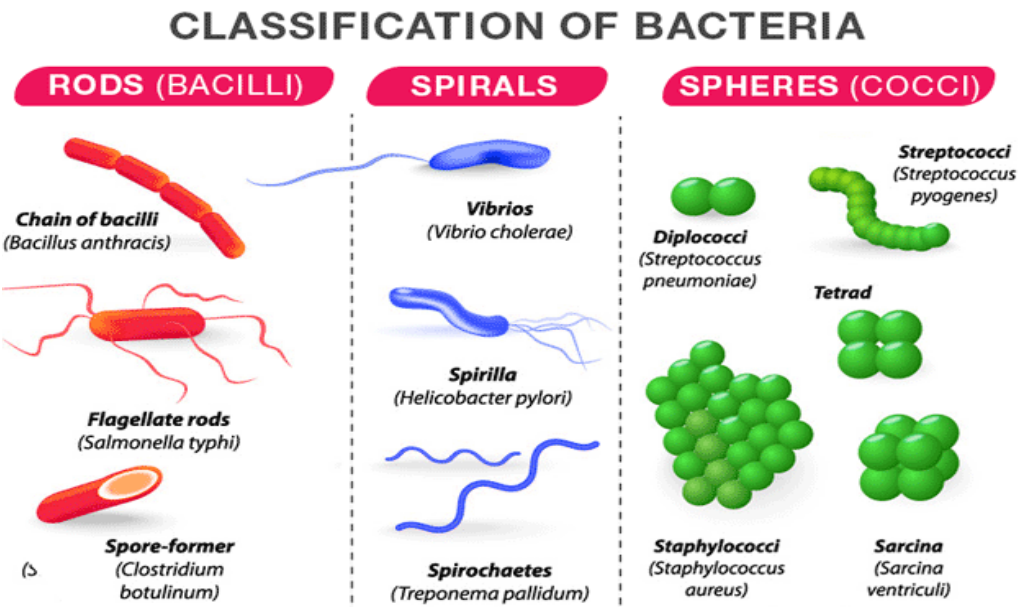
Bacteriological Techniques

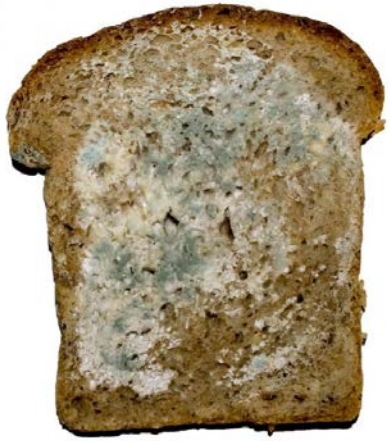




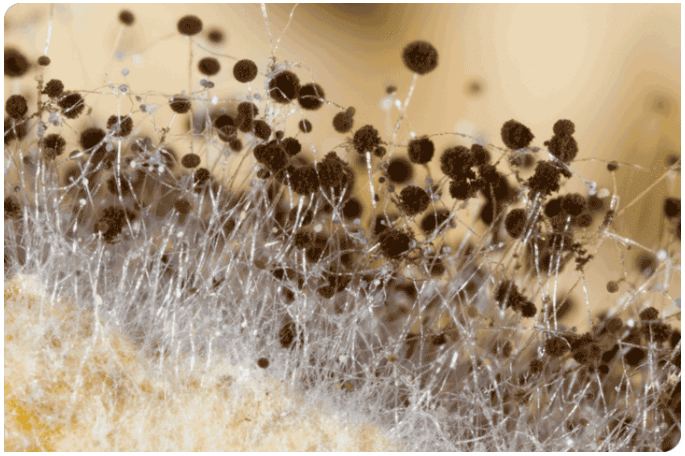
Soil Microflora



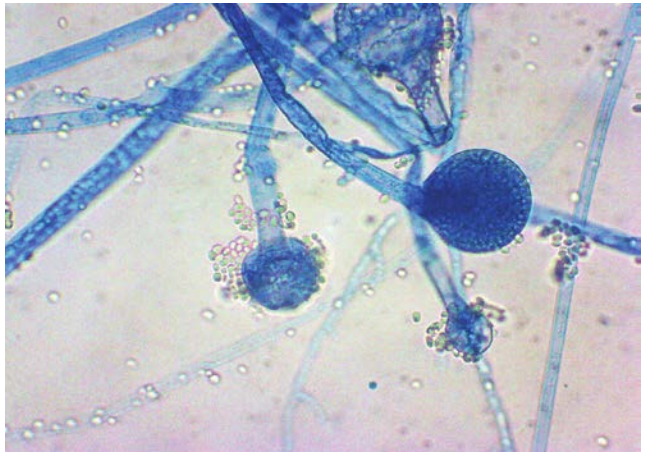




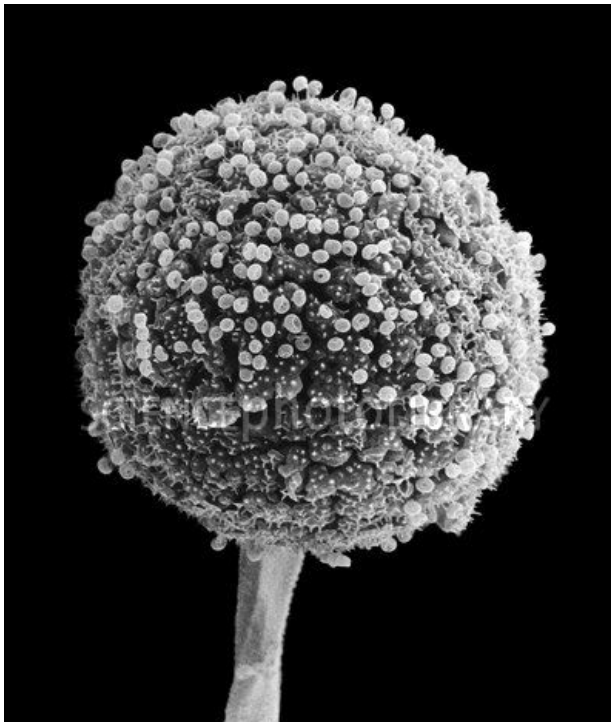
Bread Mould



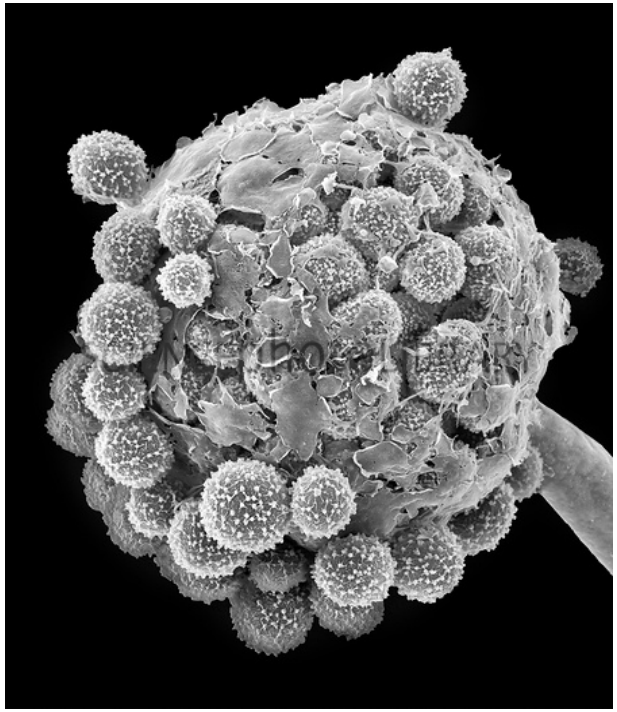
Mucor sp.



Mucor under light microscope



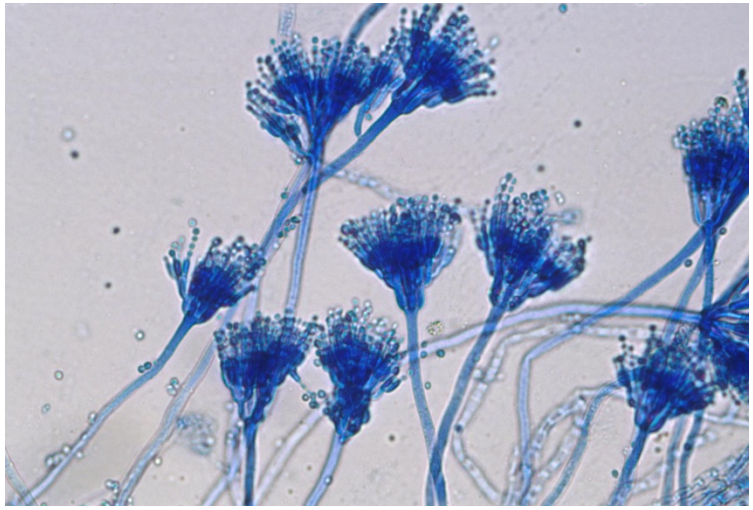
Mucor under SEM



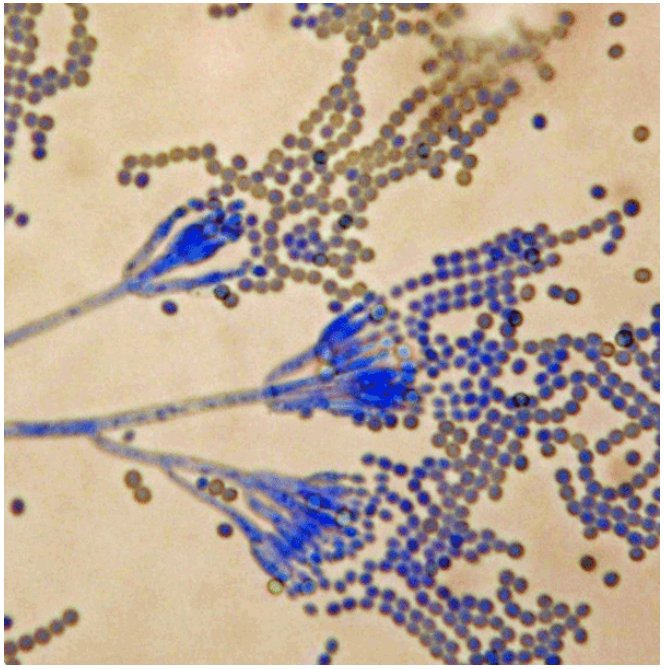
Mucor spores under SEM

Green Mould

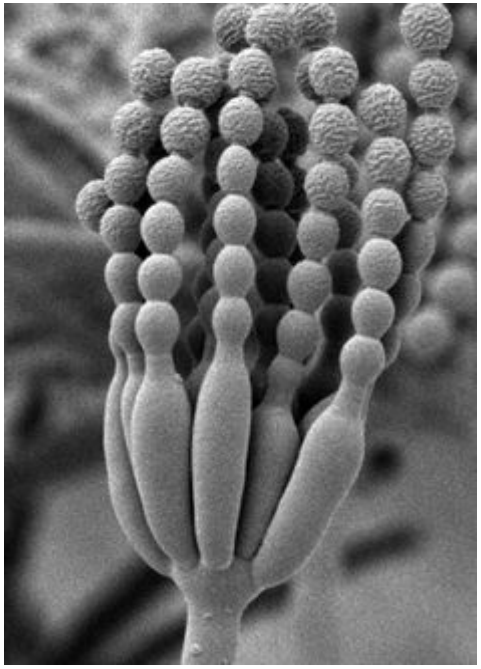
Green Mould



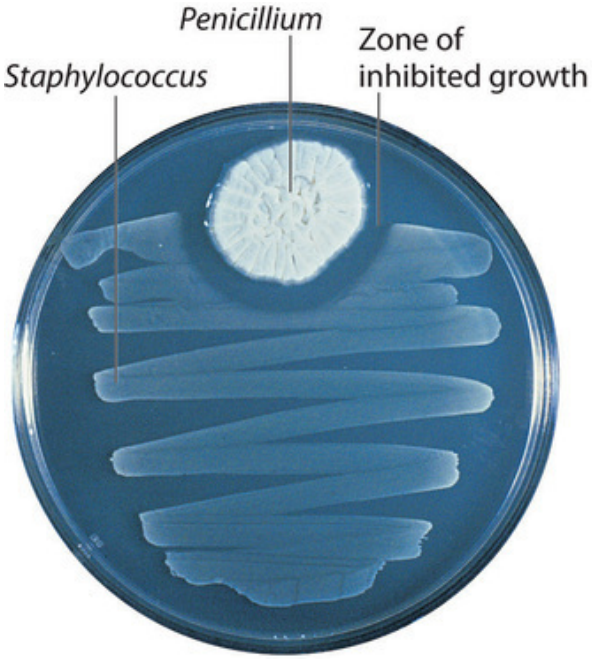
Penicillium chrysogenum

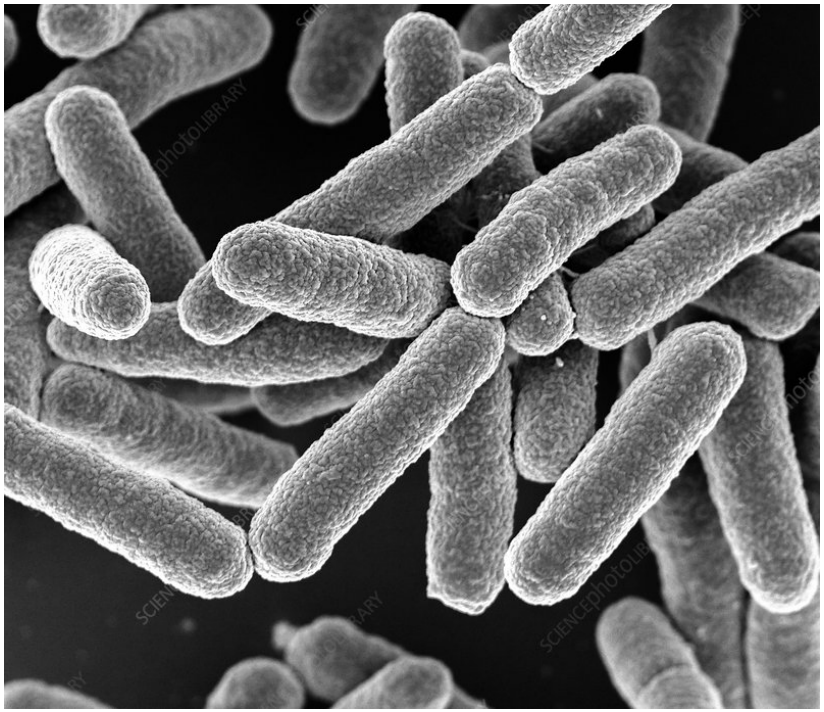


Light Micrograph

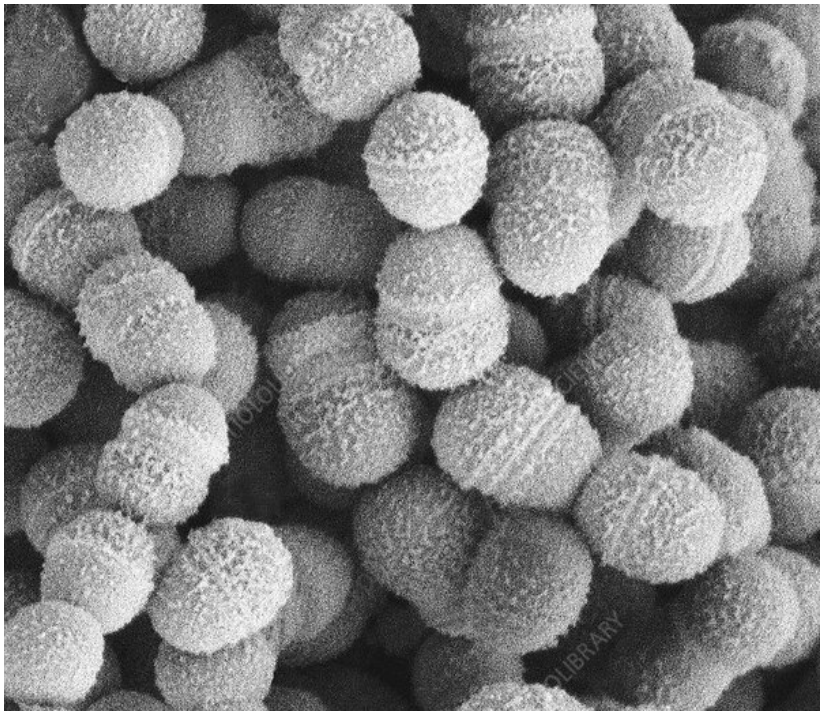


Scanning Electron Micrograph

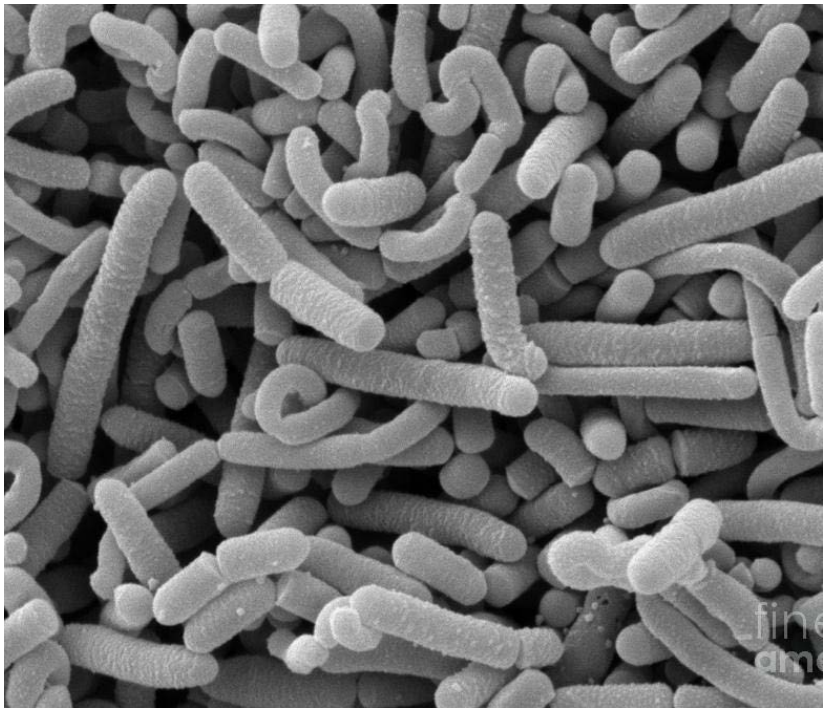




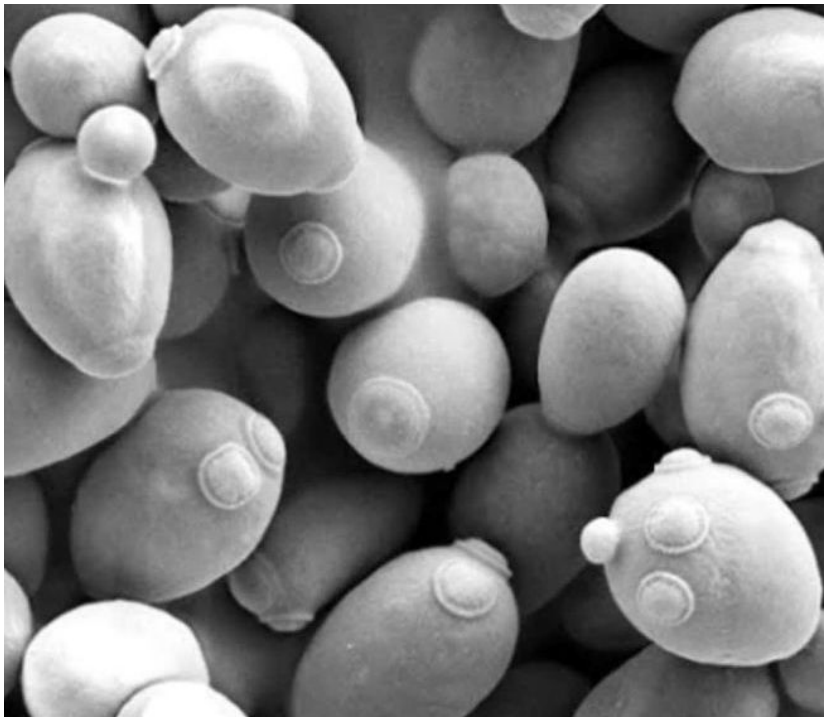
Escherichia coli



Streptococcus



Lactobacillus



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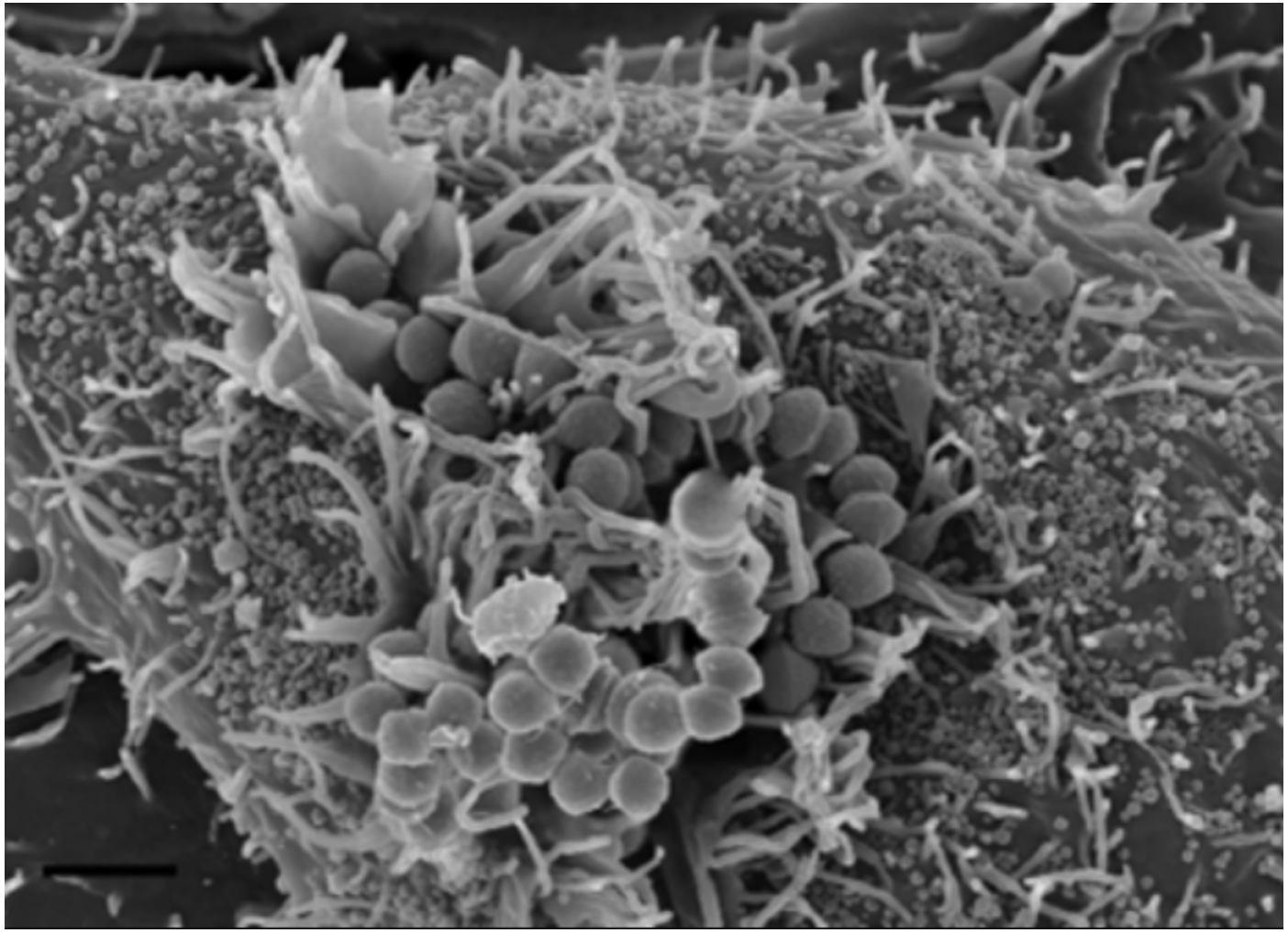

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What is Pure Culture ?

A pure (or axenic) culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another.

Methods of Pure Culture Isolation :

Solid Culture - 1) Streaking. 2) Pour Plating. 3) Spread Plating.

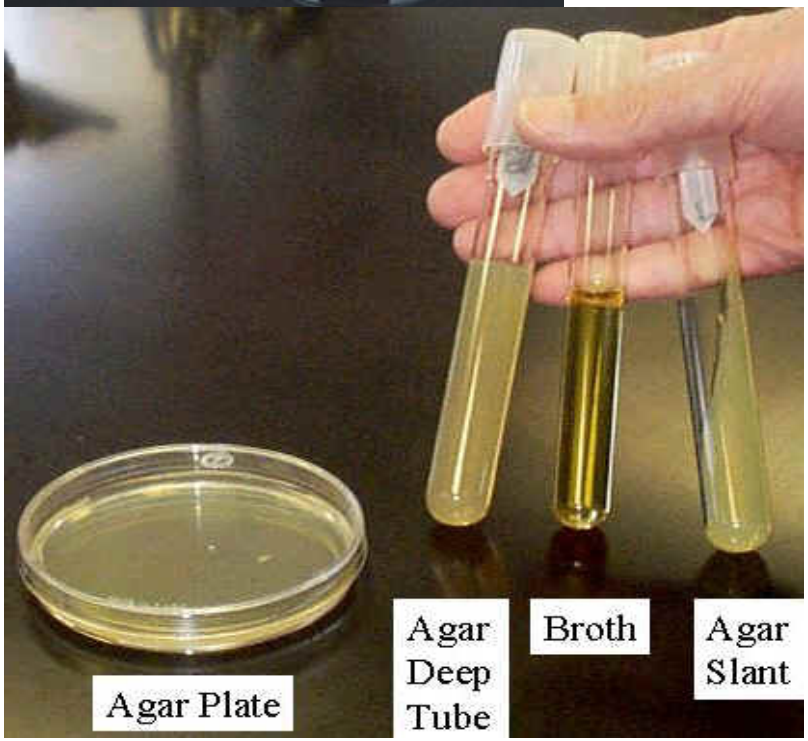
Liquid Culture - 1) Serial Dilution. 2) Enrichment Culture.

Methods of Streaking :

Streaking is a technique used to isolate a pure strain from a single species of microorganism, often bacteria. Samples can then be taken from the resulting colonies and a microbiological culture can be grown on a new plate so that the organism can be identified, studied, or tested. The modern streak plate method, first developed in **Robert Koch's laboratory**, involves the dilution of bacteria by systematically streaking them over the exterior of the agar in a petridish to obtain isolated colonies which will then grow into quantity of cells, or isolated colonies.

If the agar surface grows microorganisms which are all genetically same, the culture is then considered as a pure culture. The technique is to dilute a comparatively large concentration of bacteria to a smaller concentration. When the bacteria are sufficiently spread apart they grow into isolated colonies. Streaking is done using a sterile tool, such as a cotton swab or commonly an inoculation loop. Aseptic techniques are used to maintain pure cultures and to prevent contamination of the growth medium.

Petri dish

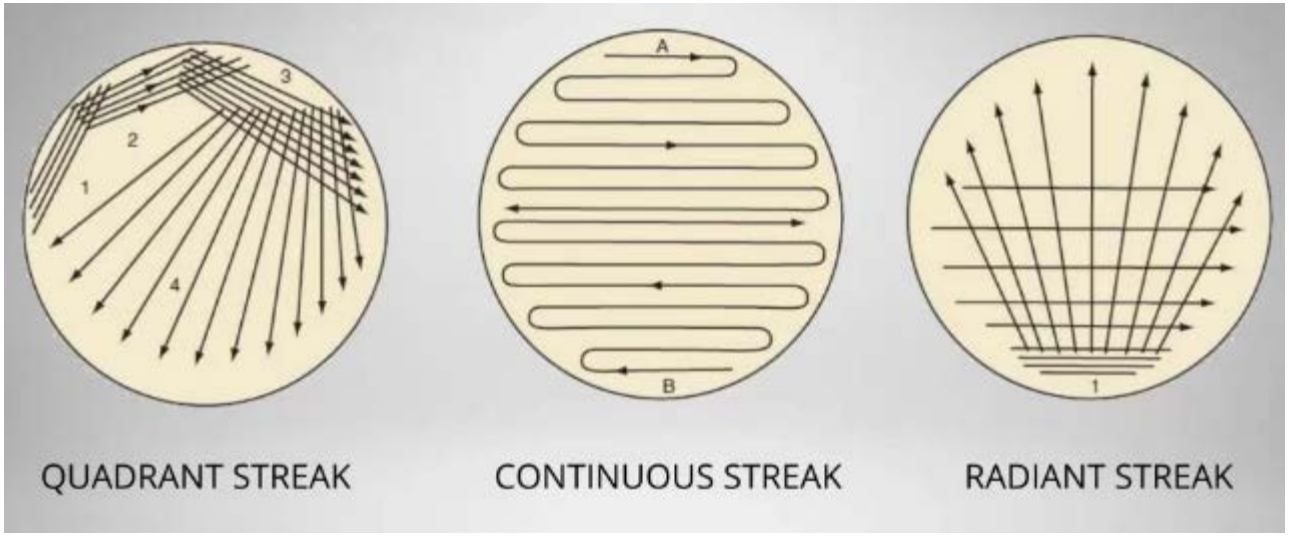


Inoculation Loop

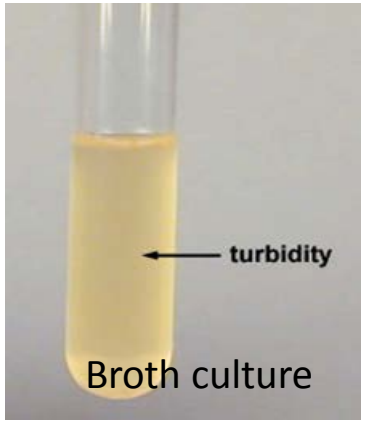
Liquid Culture Medium [Broth]

Types of Streaking :

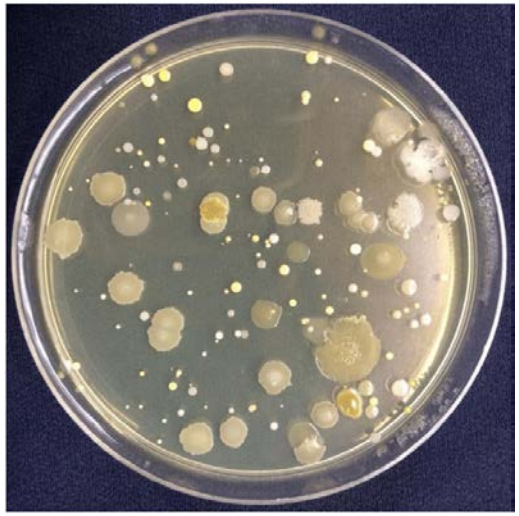
First a sterile inoculating loop is dipped into a diluted bacterial culture; then the culture-containing loop is streaked on the surface of a solidified agar plate to make a series of parallel, non-overlapping streaks. As the culture is diluted before streaking on solid agar, the organism number will decrease by the third or fourth quadrant. Therefore only a few bacterial cells are transferred on the solidified agar medium as a result it will give discrete **colony forming units (CFUs).**

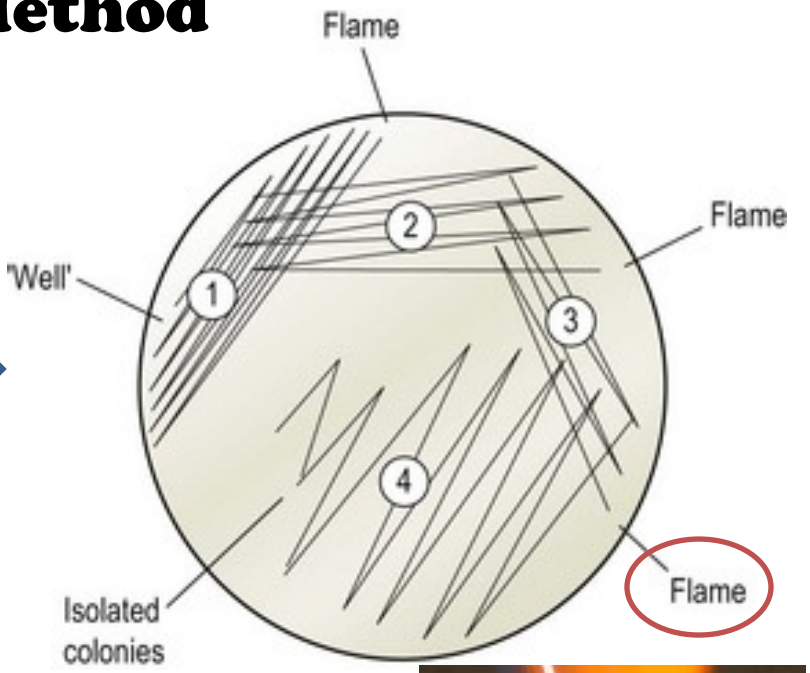


Streaking Method



Mixed Colonies





Pour-plate Method :

Pour plate method is usually the method of choice **for counting the number of colony-forming bacteria** present in a liquid specimen. In this method, fixed amount of inoculum (**aliquot volume** - generally 1 ml) from a broth/sample is placed in the centre of sterile Petri dish using a sterile pipette. Molten cooled agar (approx. 15mL) is then poured into the Petri dish containing the inoculum and mixed well. After the solidification of the agar, **the plate is inverted** and **incubated at 37°C** for generally O.N. or 24-48 hours. Microorganisms will grow both **on the surface and within the medium**. Colonies that grow within the medium generally are small in size and may be **confluent** ; the few that grow on the agar surface are of the same size and appearance as those on a streak plate. Each (both large and small) colony is carefully counted (using magnifying colony counter if needed). Each colony represents a **"colony forming unit" (CFU).**

The number of microorganisms present in the particular test sample is determined using the formula:

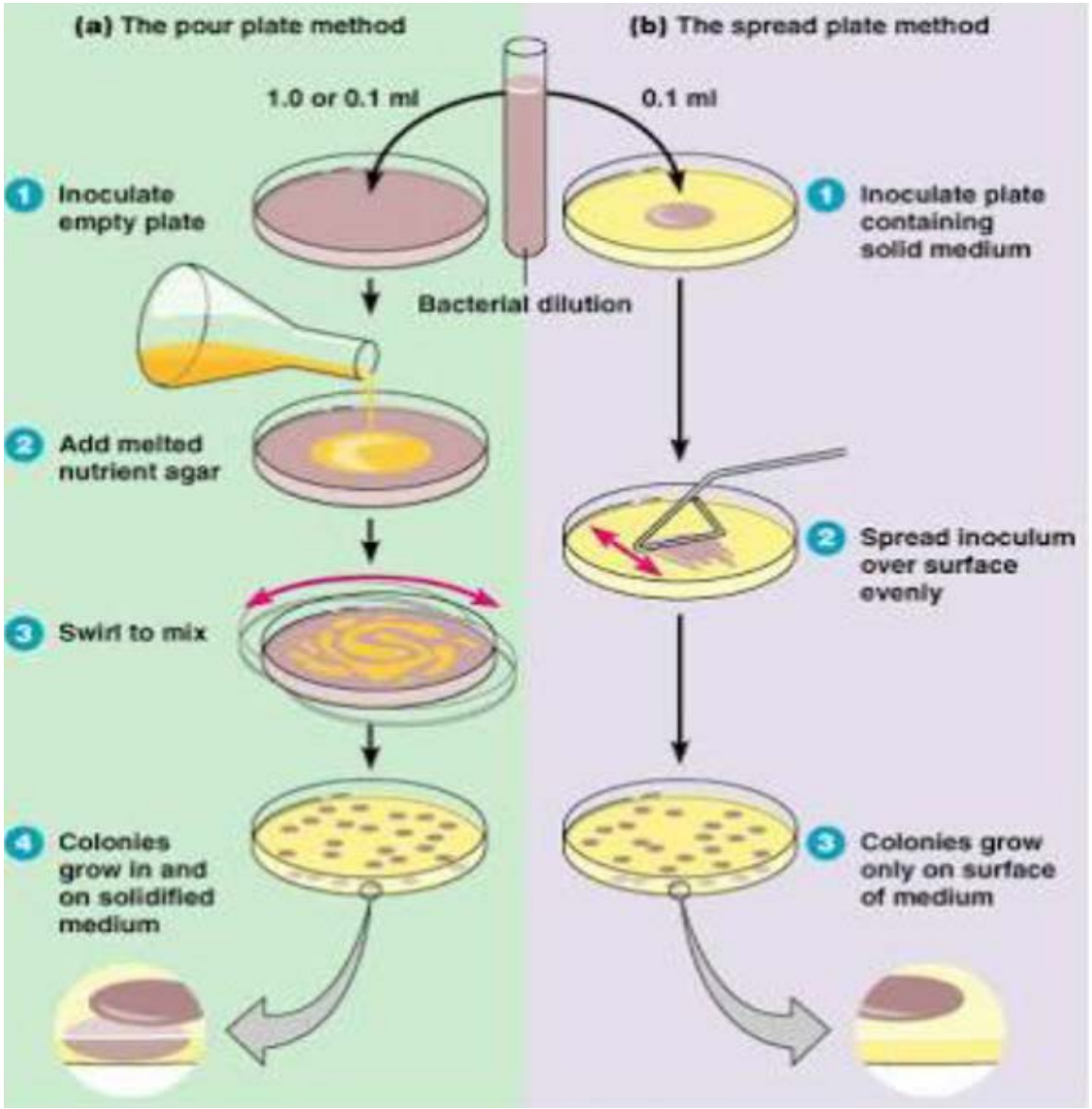
$$\text{CFU/mL} = (\text{CFU} \times \text{dilution factor}) / \text{aliquot vol. in ml}$$

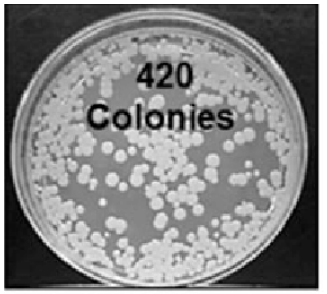
For accurate counts, the optimum count should be within the range of 30-300 colonies/plate. To insure a countable plate a series of dilutions should be plated.

Disadvantages of Pour plate method

a. Preparation for pour plate method is time consuming compared with streak plate/and or spread plate technique.
b. Loss of viability of heat-sensitive organisms coming into contact with hot agar.
c. Embedded colonies are much smaller than those which happen to be on the surface. Thus, one must be careful to score these so that none are overlooked.
d. Reduced growth rate of obligate aerobes in the depth of the agar.

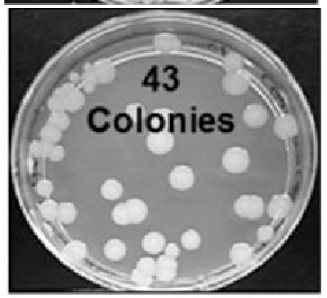
Pour-plate and Spread-plate Method :



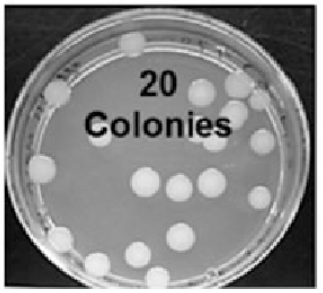


No dilution

1:4 [5x]



1:9 [10x]



1:19 [20x]

Spread-plate Method :

Spread plate technique is a method employed to plate a liquid sample for the purpose of isolating or counting the bacteria present in that sample. **A perfect spread plate technique will results visible and isolated colonies of bacteria that are evenly distributed in the plate and are countable.** The technique is most commonly applied for microbial testing of foods or any other samples or to isolate and identify variety of microbial flora present in the environmental samples e.g. soil.

To get optimum result from spread plate technique:

1. Make accurate dilutions using pipettes.
2. Apply a balanced spread technique using a glass spreader to spread the inoculum evenly on the agar surface.
3. Give a short time interval between agar inoculation and spreading.

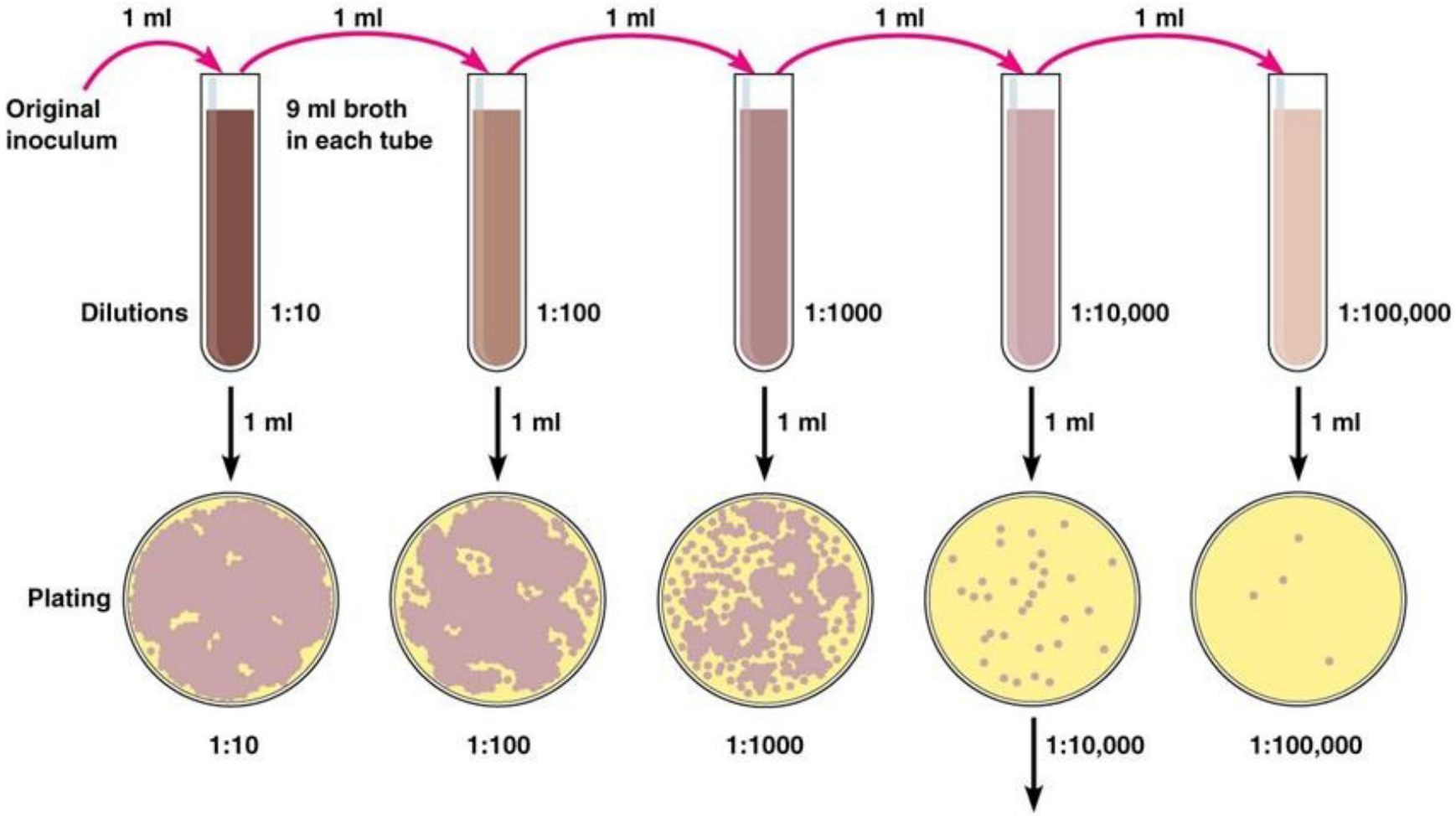
Following incubation the number of colonies present in the plate are counted. Assuming that each organism gives a single colony the number of total bacteria present in a sample are calculated.

Plating

1. Pipette out 0.1 ml from the appropriate desired dilution series onto the center of the surface of an agar plate
2. Dip the L-shaped glass spreader *(hockey stick)* into alcohol. Flame the glass spreader over a bunsen burner, let it cool before using.
3. Spread the sample evenly over the surface of agar using the sterile glass spreader, carefully rotating the Petri dish underneath at an angle of 45^{o} at the same time.
4. Incubate the plate at 37°C for 24 hours.
5. Calculate the colony forming units (CFU) value of the sample. Once you count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/mL in the original sample.

250µl of a culture when taken as aliquot and plated onto a LB-agar plate, it resulted in a confluent lawn. So the same volume of sample was diluted 4x, 10x and 50x. When 1ml of these dilutions were plated it gave 310, 120 and 27 colonies.
Find the concentration of the original culture in cells/ml.

Serial Dilution Method :



Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000 bacteria/ml in sample.)

Serial Dilution Method :

A **serial dilution** is a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration. Each dilution will reduce the concentration of bacteria by a specific amount. So, by calculating the total dilution over the entire series, it is possible to know the starting number of bacteria.

Usually the **dilution factor at each step is constant**, resulting in a **geometric progression** of the concentration in a logarithmic fashion. A ten-fold serial dilution could be 1 M, 0.1 M, 0.01 M, 0.001 M ... **Serial dilutions are used to accurately create highly diluted solutions as well as solutions for experiments resulting in concentration curves with a logarithmic scale.**

The first step in making a serial dilution is to take a known volume of STOCK (usually 1ml) and mix it into a known volume of DILUENT (media/distilled water, usually 9ml). This produces 10ml of ten-fold (10x) dilute solution. This process can be repeated to make successive dilutions.

Dilution Factor (DF) is got by dividing the final volume by the initial volume. A DF of 100 means a 1:100 dilution.

Dilution Factor = final volume/aliquot volume

Serial dilutions are multiplicative. If a 1/8 dilution of the stock solution is made followed by a 1/6 dilution what is the final dilution.

The final dilution is: 1/8 x 1/6 = 1/48

The number of microorganisms present in the particular dilution plate is determined using the formula:

CFU/mL= (CFU x DF) / aliquot volume

Types of Media

On basis of components

1. Simple
2. Complex / Undefined
3. Synthetic / Defined
4. Minimal

On basis of Microbial Growth

1. Selective
2. Differential

Simple media

The simple or basal media include nutrient broth and peptone water, which form the basis of other media. **Nutrient broth** is an example of a simple liquid medium that consists of **peptone, meat extract, sodium chloride, and water.** Addition of 0.5% glucose to nutrient broth makes it glucose broth.

Complex media

Complex media have some complex ingredients, which consist of a **mixture of many chemicals in unknown proportions**. This is an undefined medium, because the amino acid source contains a variety of compounds with the exact composition unknown. The complex media contain ***Water, a carbon source*** such as **glucose** for bacterial growth, **Various salts** needed for bacterial growth and **a source of amino acids and nitrogen** (e.g., beef and yeast extract).

e.g., chocolate agar, MacConkey agar,

TABLE 4-2 Composition and uses of some common solid media

Medium	Composition	Uses
Nutrient agar	Nutrient broth, agar 2%	Routine culture
MacConkey medium	Peptone, lactose, sodium taurocholate, agar, neutral red	Culture of Gram-negative bacteria, such as *Escherichia coli*
Blood agar	Nutrient agar, 5% sheep or human blood	Routine culture, culture of fastidious organisms, such as *Streptococcus* spp.
Chocolate agar	Heated blood agar	Culture of *Haemophilus influenzae* and *Neisseria*
Deoxycholate citrate agar	Nutrient agar, sodium deoxycholate, sodium citrate, lactose, neutral red, etc.	Culture of *Shigella* spp. and *Salmonella* spp.
Thiosulfate citrate bile salt sucrose agar	Thiosulfate, citrate, bile salt, sucrose, bromothymol blue, thymol blue	Culture of *Vibrio cholerae*
Loeffler's serum slope	Nutrient broth, glucose, horse serum	Culture of *Corynebacterium diphtheriae*
Lowenstein–Jensen medium	Coagulated hen's egg, mineral salt solution, asparagine, malachite green	Culture of *Mycobacterium tuberculosis*

Selective – Antibiotic or Nutrient Specific

Selective media contain one or more components that suppress the growth of some microorganisms without seriously affecting the ability of others to grow. Such media may also contain ingredients for differentiating among the species that do survive.

a. **Mannitol Salt Agar (MSA)** – selective for **Gram +ve**, Mannitol - differential
b. **MacConkey's Agar** - selective for **Gram –ve**, differential for Lactose Fermenters (LF)
c. **Eosin Methylene Blue (EMB Agar)** – Selects **Gram –ve** as dyes used are toxic for **Gram +ve**
d. **Hektoen Enteric Agar** - selective and differential, primarily used to recover *Salmonella* and *Shigella* from patient specimens, contains indicators of lactose fermentation and hydrogen sulfide production as well as inhibitors to prevent the growth of **Gram +ve.**
e. **Sabouraud's Agar** - selective to certain **fungi** due to its low pH(5.6) and high glucose concentration(3-4%)

Differential Media

In addition to basic nutrients, *differential media* contain one or more components, such as a **particular carbohydrate**, that can be used by some microorganisms but not by others. If the microorganism uses the component during the incubation period, a change occurs in an indicator (typically a pH indicator) that is also included in the medium.

a. Distinguish one microorganism type from another growing on the same medium by their growth characteristics.
b. Uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as **neutral red, phenol red, eosin y, or methylene blue**) added to the medium to visibly indicate the defining characteristics of a microorganism

Example :

1. **Blood Agar** - contains bovine heart blood that becomes transparent in the presence of β-hemolytic organisms such as *Streptococcus pyogenes* and *Staphylococcus aureus*.
2. Eosin methylene blue (EMB), differential for lactose and sucrose fermentation
3. EMB , MacConkey's, MSA etc

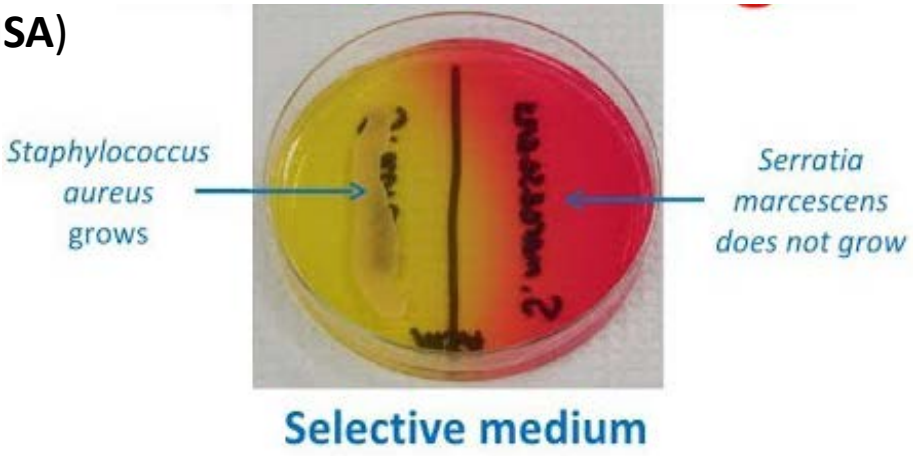
SELECTIVE MEDIA
VERSUS
DIFFERENTIAL MEDIA

SELECTIVE MEDIA	DIFFERENTIAL MEDIA
Selective media refer to a type of growth media that allows the growth of selected microorganisms in the medium	Differential media refer to a type of growth media that allows the differentiation of closely-related microorganisms
Used to isolate a particular strain of microorganisms	Used to identify and differentiate closely-related microorganisms
Use specific growth characteristics of a particular microorganism to select it from the others	Use unique growth patterns of microorganisms to differentiate them from others
Only allow the growth of a single microorganism in the medium	Allow several closely-related microorganisms to grow in the medium
Do not use indicators	Use indicators

Enrichment Culture Method :

Mannitol Salt Agar Medium (MSA)

For **Gram +ve** Bacteria

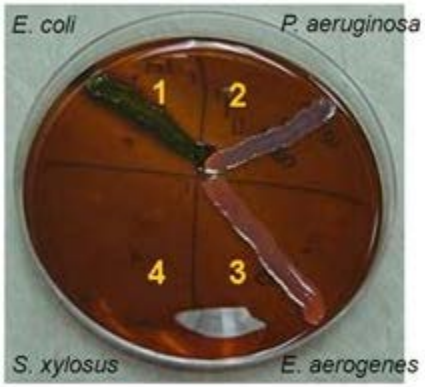


Selective medium

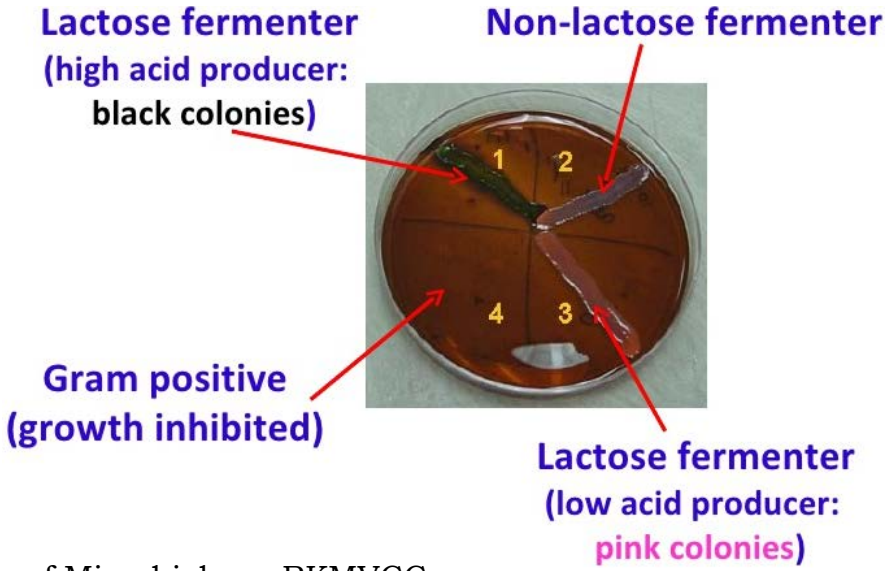
High salt (NaCl) concentration in medium favors organisms that tolerate high salt concentration, e.g. *Staphylococcus*.

Eosin Methylene Blue Agar (EMB)

For **Gram -ve** Bacteria

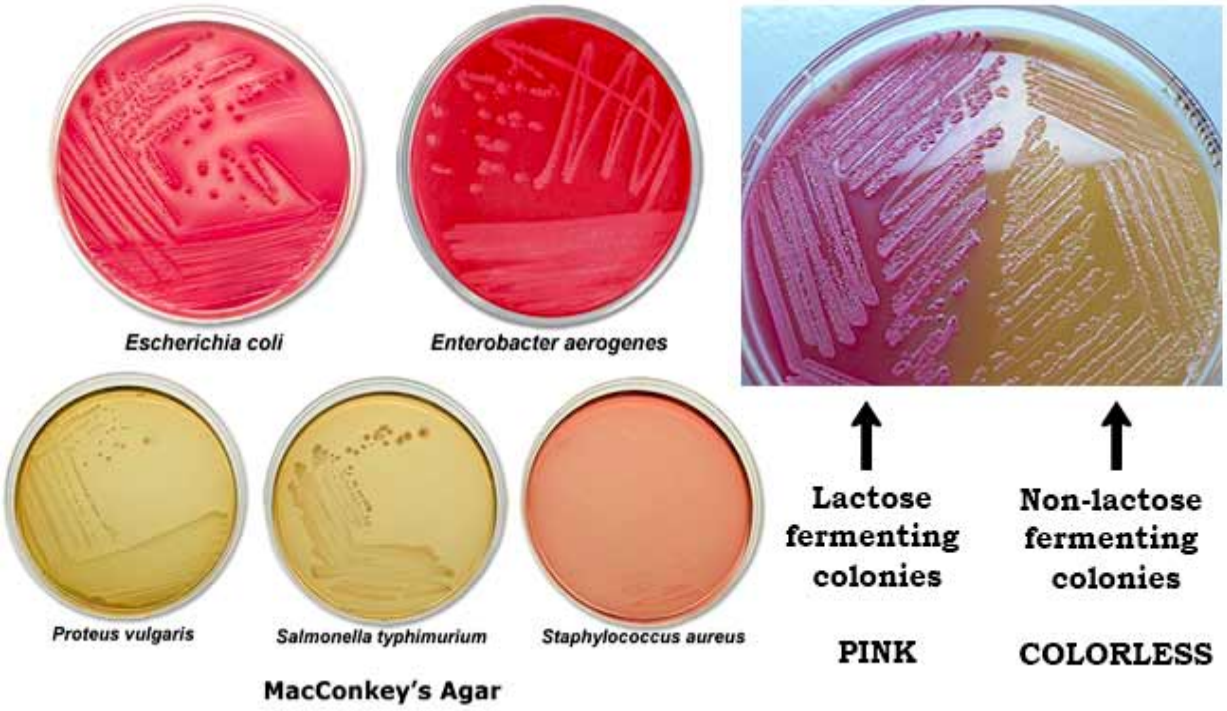


- Selective
- Differential
- Special features:
  - Eosin (dye)
  - Methylene blue (dye)
  - Lactose (fermentation substrate)
- Used for:
  - Selective culture of Gram-negative species
  - Distinguishing between Gram-negatives based on lactose fermentation



MacConkey Agar

selective for **Gram –ve**, differential for **Lactose Fermenters (LF)**



Used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae and the genus *Pseudomonas*.

Lactose monohydrate is the fermentable source of carbohydrate. The selective action of this medium is attributed to **crystal violet** and **bile salts,** which are inhibitory to most species of gram-positive bacteria. **Neutral red** is a pH indicator that turns red at a pH below 6.8 and is colorless at any pH greater than 6.8.

Composition of MacConkey Agar

Ingredients	Amount
Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 g
Agar	13.5 gm
Distilled Water	Add to make 1 Liter

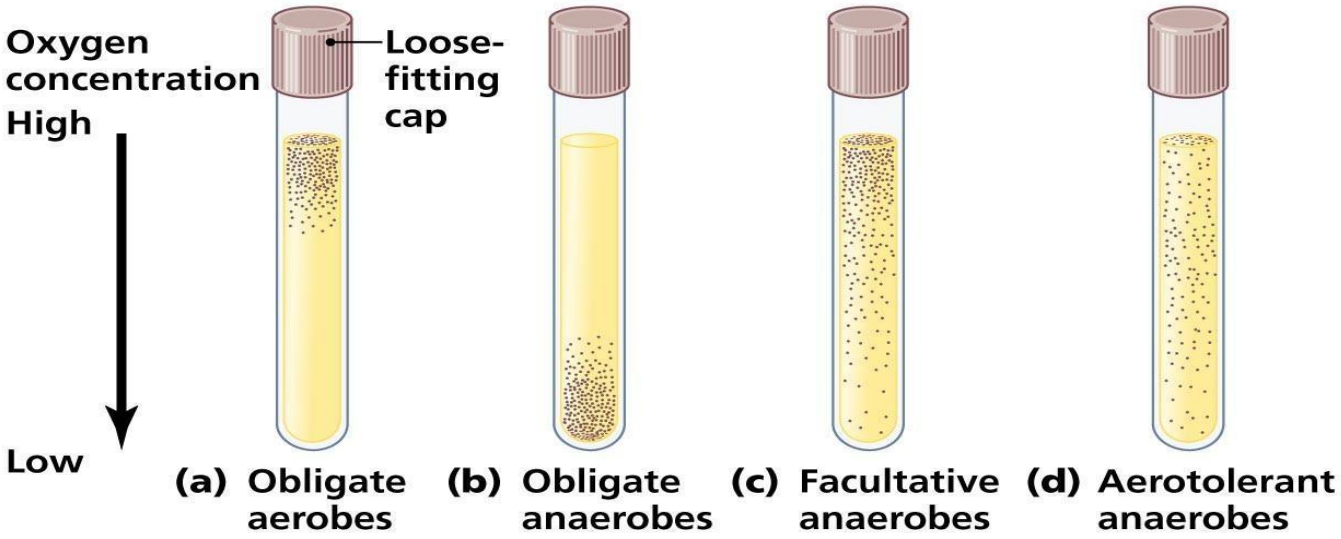
Cultivation of Anaerobic Bacteria :

Reduce the O_2 content of culture medium and remove any oxygen already present inside the system or in the medium.

Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stopper. Suitable for organisms not too sensitive to small amounts of oxygen.

Addition of a reducing agent that reacts with oxygen and reduces it to water e.g., **Thioglycolate** in **Thioglycolate broth**. After thioglycolate reacts with oxygen throughout the tube, oxygen can penetrate only near the top of the tube where the medium contacts air.

A redox indicator dye called **Resazurin** is added to the medium because the dye changes colour in the presence of oxygen and thereby indicates the degree of penetration of oxygen into the medium.



TGA Broth + Resazurin

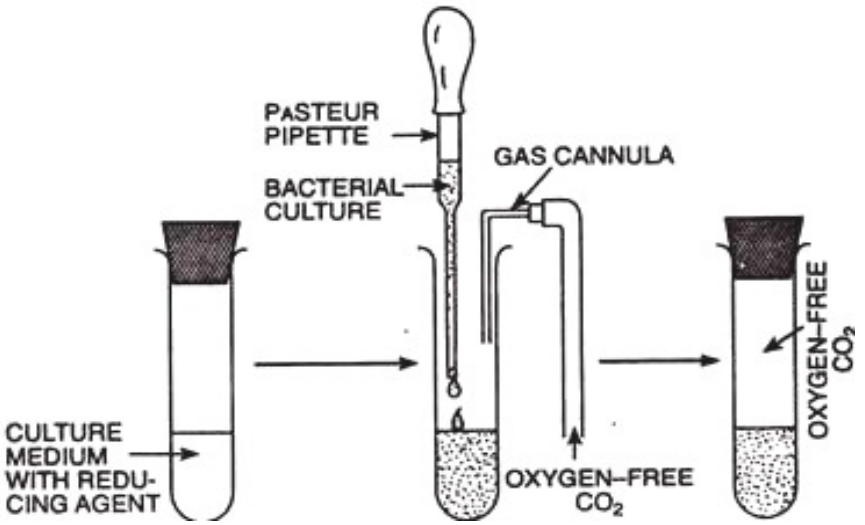
Strict anaerobes, such as methanogenic bacteria can be killed by even a brief exposure to O_2. In these cases, a culture medium is first boiled to render it oxygen free, and then a reducing agent such as **H_2S** is added and the mixture is sealed under an oxygen- free gas. All manipulations are carried out under a tiny jet of oxygen free **hydrogen** or **nitrogen** gas. For **extensive research** on anaerobes, special boxes fitted with gloves, called **anaerobic glove boxes**, permit work with open cultures in completely anoxic atmospheres.

Stringent anaerobes can be grown only in special environments, established by using one of the following methods:

Pre-reduced media : During preparation, the culture medium is boiled for several minutes to drive off most of the dissolved oxygen. A reducing agent e.g., cysteine, is added to further lower the oxygen content. Oxygen free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are being flushed with oxygen-free nitrogen, stoppered tightly, and sterilized by autoclaving. Such tubes are continuously flushed with oxygen free CO_2 by means of a cannula, restoppered, and incubated.

Cooked meat broth (CMB; original medium known as 'Robertson's bullock-heart medium'). CMB is suitable for growing anaerobic bacteria in air and also for the preservation of their stock cultures. Other media which can be used :

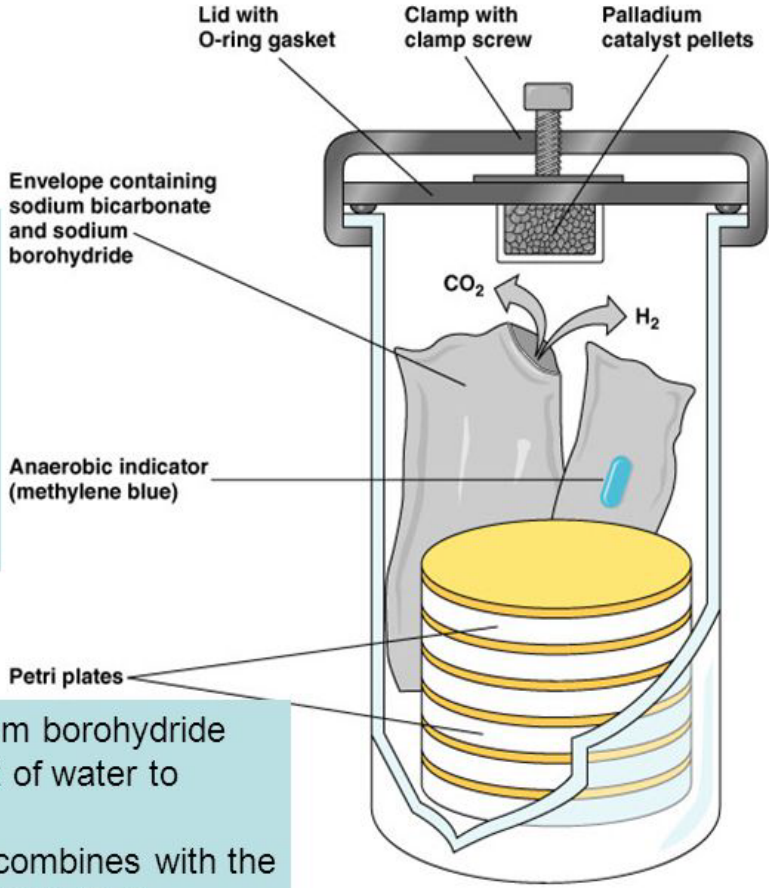
Brucella blood agar, Bacteroides **bile aesculin agar**, Phenylethyl alcohol agar, Kanamycin blood agar, etc. Anaerobic bacteria have special nutritional requirements for **vitamin K**, **hemin** and **yeast extract**.



Anaerobic Chambers : Anaerobic Chamber-This refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Culture media are placed within the chamber by means of an air lock which can be evacuated and refilled with N_2. Any oxygen in the media is slowly removed by reaction with hydrogen, forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

Anaerobic Jar : Anaerobic jar is a heavy- walled jar with a gas tight seal within which tubes, plates, or other containers to be incubated are placed along with H_2 and CO_2 generating system (**GasPak system**). After the jar is sealed, **oxygen** present in the atmosphere inside jar and dissolved in the culture medium, **is gradually used up** through reaction with the hydrogen in the presence of catalyst. The air in the jar is replaced with a mixture of H_2 and CO_2, thus leading to anoxic conditions.

anaerobic jar is an instrument used in the production of an anaerobic environment. This method of *anaerorbios is* as others is used to culture bacteria which die or fail to grow in presence of oxygen



Sodium bicarbonate and sodium borohydride are mixed with a small amount of water to produce CO 2and H .+
A palladium catalyst in the jar combines with the O 2in the jar and the H +to remove O .2

What are MICROAEROPHILEs ?

A **microaerophile** is a microorganism that requires oxygen to survive, but requires environments containing lower levels of oxygen than are present in the atmosphere (i.e. <21% O_2; typically 2–10% O_2). Many microaerophiles are also capnophiles, requiring an elevated concentration of carbon dioxide (e.g. 10% CO_2 in the case of *Campylobacter* species. These facultative anaerobes can respire **aerobically**, use **alternative terminal electron acceptors** for anaerobic respiration or grow via **fermentation**.

Many bacteria that grow optimally at saturating concentrations of O_2 also have the potential to respire under micro-oxic conditions. These bacteria can harvest O_2 present at low, even nanomolar, concentrations using high-affinity terminal oxidases.

Organisms	Aerobic growth	Low-affinity oxidase	Microaerobic growth	High-affinity oxidase	ROS defence	Anaerobic growth	Representative species
Obligate aerobes	+	+	–	–	+	–	*Mycobacterium leprae*
Microaerophiles	+	–	+	+	+	–	*Helicobacter pylori*
Facultative anaerobes*	+	+	+	+	+	+	*Escherichia coli*
Nanaerobes	+	–	+	+	+	+	*Bacteroides fragilis*
Aerotolerant anaerobes	–	–	–	–	+	+	*Streptococcus pneumoniae*
Obligate anaerobes‡	–	–	–	–	–	+	*Clostridium tetani*

ROS, reactive oxygen species.

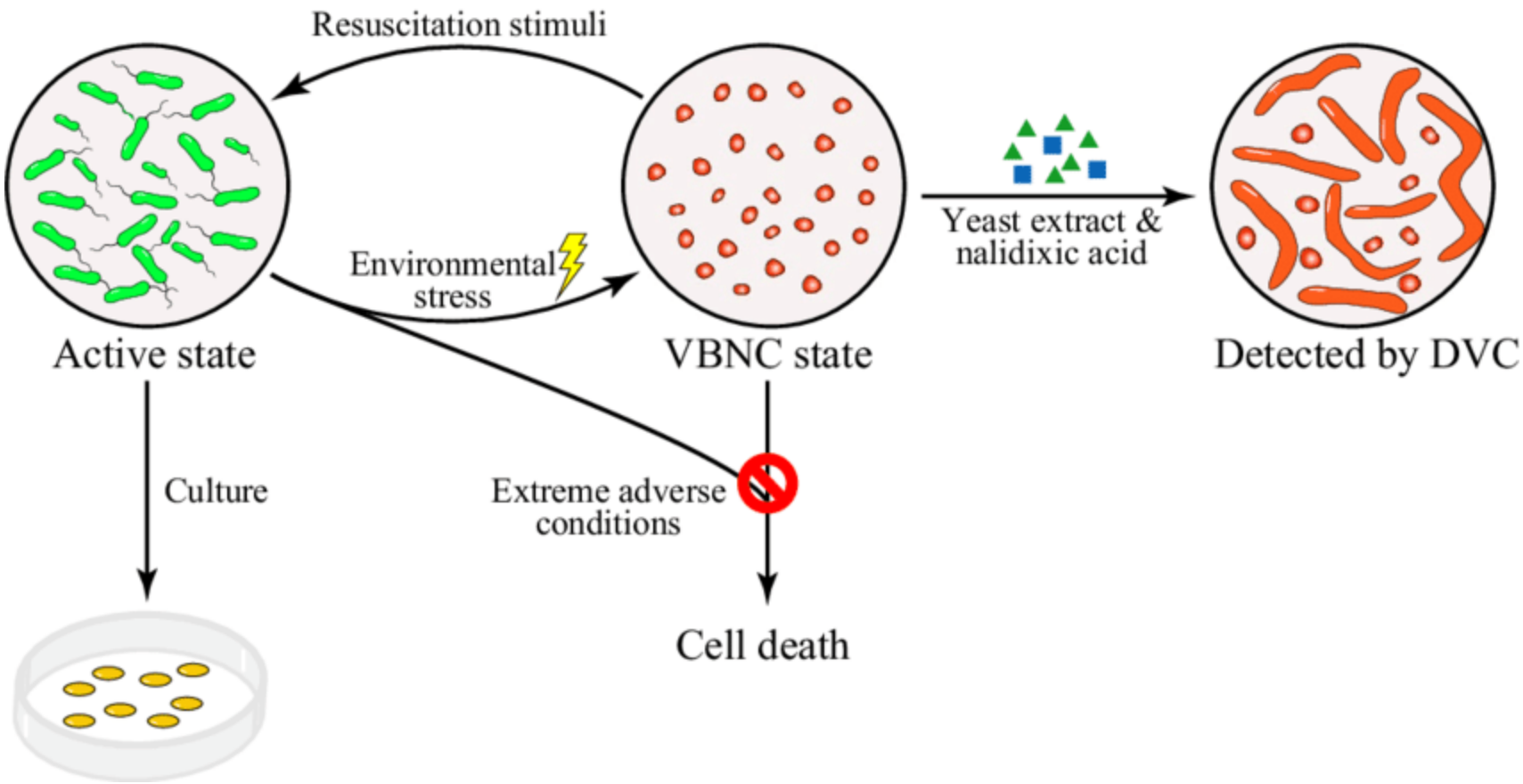
*Not all facultative anaerobes have high-affinity oxidases or grow microaerobically.

‡Many obligate anaerobes tolerate transient or low levels of O_2.

Accessing Non-Culturable Bacteria

Viable but non-culturable cells (VBNC) are defined as live bacteria that are in a state of very low metabolic activity and do not divide, but are alive and have the ability to become culturable once resuscitated. Such bacteria cannot be cultivated on conventional media (they do not form colonies on solid media, they do not change broth appearance), but their existence can be proved using other methods. The switch to the VBNC stage has been described and documented for several bacterial species: *Vibrio cholerae, Escherichia coli* (including EHEC), *Campylobacter jejuni, Helicobacter pylori* etc.

The ability to enter the VBNC state may be advantageous for bacteria, but poses a risk to human health. If VBNC cells are present, the total number of viable bacteria in a sample will be underestimated by the CFU count method due to the inherent non-culturability of VBNC cells. Even worse, if all bacteria in the sample are in VBNC state, the sample may be regarded as germ-free due to non-detection. For bacterial species causing human infections, the underestimation or non-detection of viable cells in quality control samples from the food industry and water distribution systems, or clinical samples may pose serious risks to the public. The risks emerge from the fact that pathogenic bacteria can be avirulent in the VBNC state but regain virulence after resuscitation into culturable cells under suitable conditions.



VBNC refers to a physiological state where bacteria are metabolically active, but are no longer culturable on conventional growth media. It is a survival strategy adopted by many bacteria in response to harsh environmental conditions, and the VBNC cells may return to culturable state under favorable conditions

Detection of VBNC cells - Biochemically

1. The presence and abundance of VBNC cells can be determined by comparing the number of viable cells to that of culturable cells in the sample. If the number of culturable cells drops to an undetectable level while the number of viable cells remains high, then the population in the sample has become VBNC cells. Therefore, the first major step for the detection of VBNC cells is the estimation of the remaining culturable cells in the sample by a conventional plate count technique.

2. The second major step for the detection of VBNC cells is the estimation of viable cells. Since viable cells, but not dead cells, carry out metabolic reactions and respiration, they can also be detected by the *p*-iodonitrotetrazolium violet (INT) assay based on the activity of electron transport system. INT is a soluble tetrazolium salt that can compete with oxygen as the final electron acceptor and be reduced to **iodonitrotetrazolium formazan**, a water insoluble **violet** colored dye or indicator. Hence INT can be used for the **colorimetric assay of various dehydrogenases**. in metabolically active cells. Therefore, the formation and accumulation of formazan in cells, which appear as dark red/violet precipitates under microscope, indicate the presence of an active electron transport chain, a characteristic of viable cells.

Detection of VBNC cells – Microscopy and Molecular Probes (PCR)

	Purpose of reaction	Mechanism	References
Fluorescence microscopy methods			
Acridine orange staining after the addition of nalidixic acid	Distinguishing actively growing cells from VBNC or dormant cells	Actively growing cells become elongated and fluoresce reddish orange, while VBNC or dormant cells remain normal-shaped and have greenish white fluorescence	Fakruddin et al. (2013)
Counterstaining with 5-cyano-2,3-ditolyltetrazolium chloride (CTC) and 4′,6-diamidino-2-phenylindole (DAPI)	Differential staining of VBNC and dead cells, once unculturability is confirmed	Viable cells have red fluorescence, while dead cells fluoresce blue	Ramamurthy et al. (2014)
Combination of SYTO® 9 and propidium iodide (PI) e.g. LIVE/DEAD BacLight kit	Differential staining of VBNC and dead cells, once unculturability is confirmed	Viable cells have green fluorescence, while dead cells fluoresce red	Fakruddin et al. (2013)
Molecular methods			
qPCR combined with propidium monoazide (PMA) treatment	Detection of nucleic acids exclusively from living cells, once unculturability is confirmed	PMA penetrates only damaged membranes and inhibits PCR amplification by binding to DNA of dead cells or extracellular DNA	Ramamurthy et al. (2014) and Zhao et al. (2017b)
Monitoring stress-related genes with RT-PCR	Detection of viability, once unculturability is confirmed	Bacterial mRNA has short life-time and is mainly detectable in metabolically active cells	Oliver (2010) and Ramamurthy et al. (2014)