Chapter 3

Microbial Cultivation

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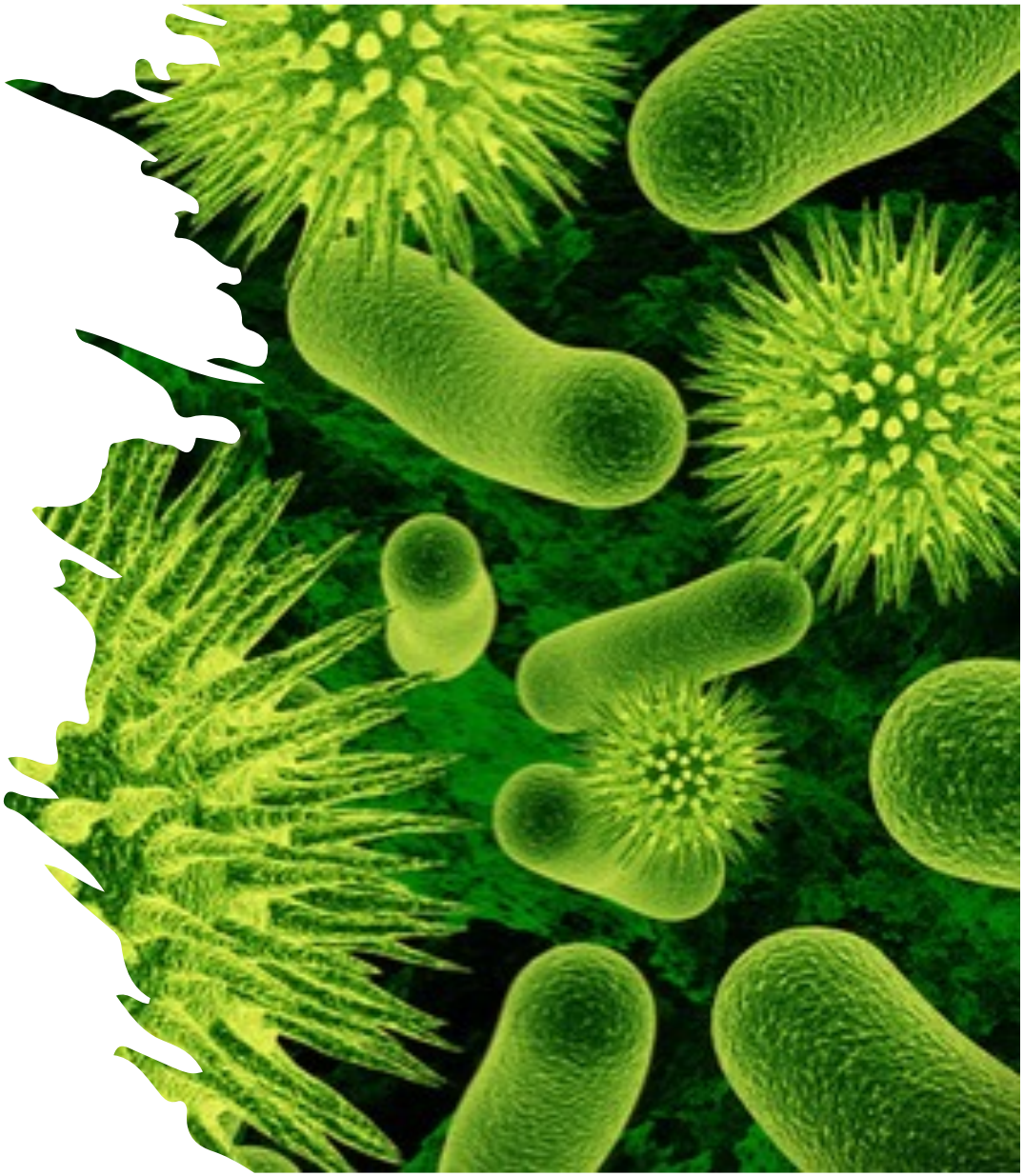


Introduction

- **Living organisms grow and reproduce.**
- When microbes are provided with nutrients & required environmental factors, they become metabolically active and grow.
- **Growth common refers to increase in a microbial size, population number, or both**
- **Growth also results when cells simply become longer and larger**
- involves an increase **in the number of cells** rather than in the size of individual cells.
- **The change in population in bacteria chiefly involves Binary fission.**
- **Note- it is not convenient to study growth and reproduction of individual mo because of their small size, therefore population no. is important**

2 levels of growth:

i. A cell synthesizes new components, increase its size

ii. Increase number of cells in the population

Increase in cellular constituents that may result in:

➢ **Increase in cell number**

- e.g., when microorganisms reproduce by budding or binary fission

➢ **increase in cell size**

- e.g., some microorganisms have nuclear divisions that are not accompanied by cell divisions

❖**Microbiologists usually study population growth rather than growth of individual cells.**

IMPORTANT POINTS

1- Bacterial Division/reproduction

2- Stages of Bacterial growth curve

3- Measurement of Microbial growth

4-Environmental factors effect microbial growth

1- Bacterial Division/reproduction

- ❑ Bacteria normally reproduce by a method called **binary fission.**
- ❑What is Binary Fission.?
- **Binary Fission is the process of bacteria reproduction where one cell become two**
- **Ex: Bacteria, paramecium, amoeba.**

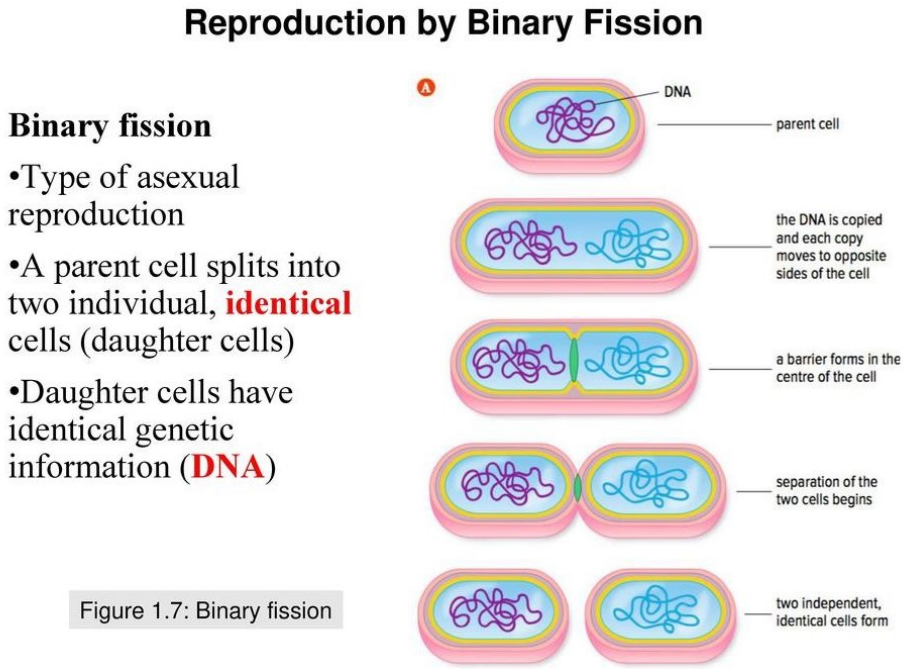


Figure 1.7: Binary fission

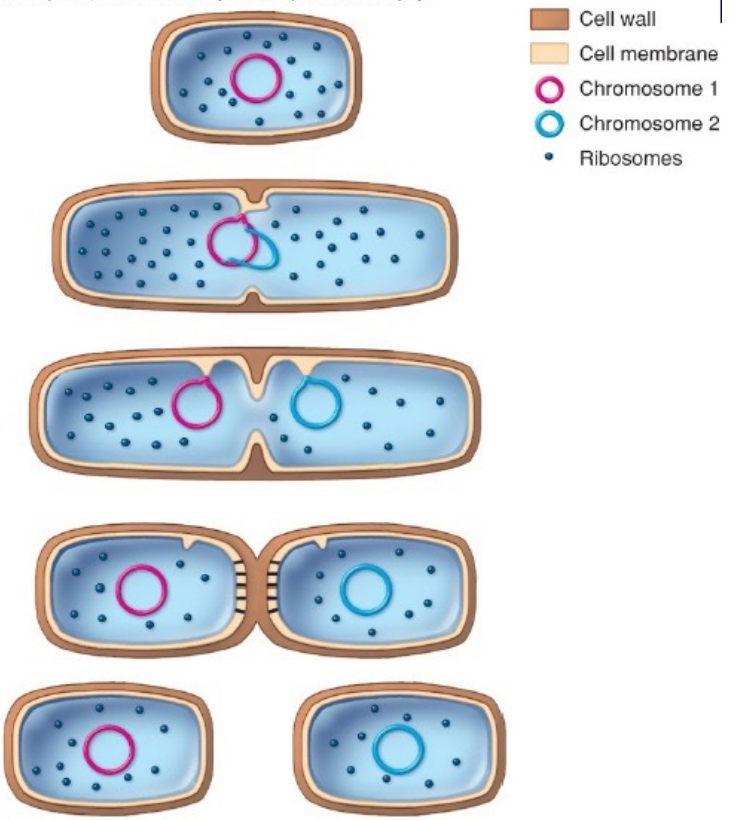
1. A young cell at early phase of cycle

2. A parent cell prepares for division by enlarging its cell wall, cell membrane, and overall volume. Midway in the cell, the wall develops notches that will eventually form the transverse septum, and the duplicated chromosome becomes affixed to a special membrane site.

3. The septum wall grows inward, and the chromosomes are pulled toward opposite cell ends as the membrane enlarges. Other cytoplasmic components are distributed (randomly) to the two developing cells.

4. The septum is synthesized completely through the cell center, and the cell membrane patches itself so that there are two separate cell chambers.

5. At this point, the daughter cells are divided. Some species will separate completely as shown here, while others will remain attached, forming chains or doublets, for example.



Stages of Binary Fission

Generation Time (Doubling time)

❑**What is Generation Time?**

➢The time required for a cell to divide (and its population to double) is called the **generation time**

❑As you seen in the picture, cell's division produces two cells, two cells' divisions produce four cells, and so on.

$$\frac{dx}{dt} = \mu x$$

$$\frac{1}{x}dx = \mu dt$$

$$\int_0^t \frac{1}{x}dx = \int_0^t \mu\, dt$$

$$lnx_t - lnx_0 = \mu(t-0)$$

$$ln\frac{x_t}{x_0} = \mu(t)$$

$$\frac{x_t}{x_0} = e^{\mu t}$$

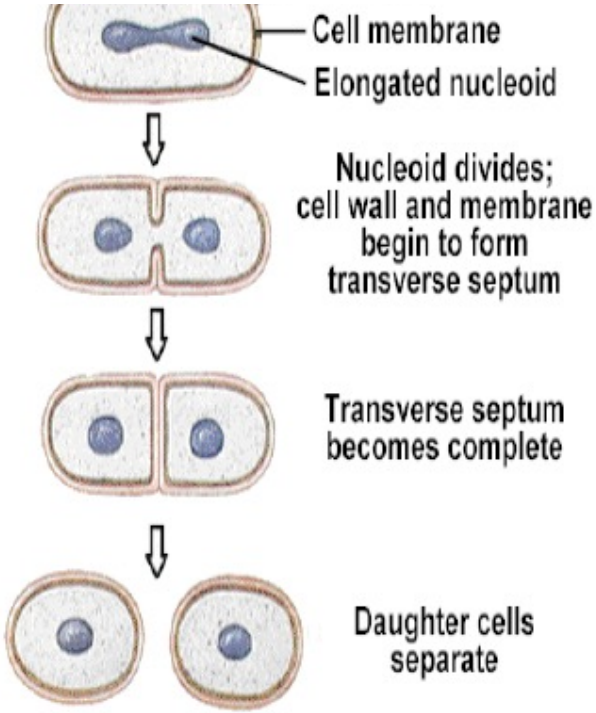
$$x_t = x_0 e^{\mu t}$$

$$t_{\frac{1}{2}};\ x_t = 2x_0$$

$$2x_0 = x_0 e^{\mu t}$$

$$\ln 2 = \ln e^{\mu t}$$

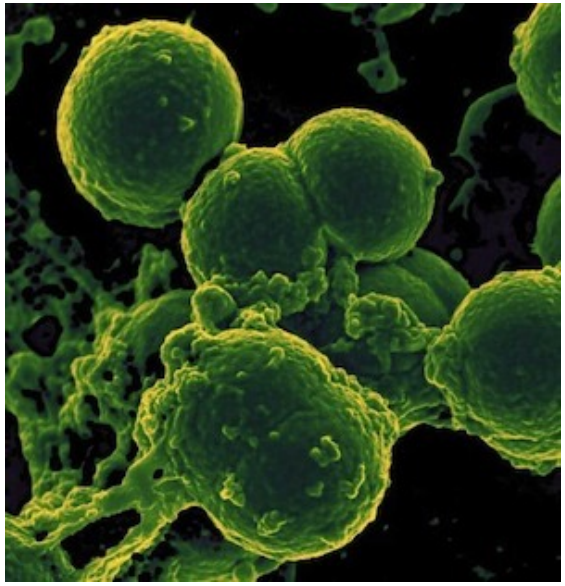
$$t_{\frac{1}{2}} = \frac{ln2}{\mu}$$



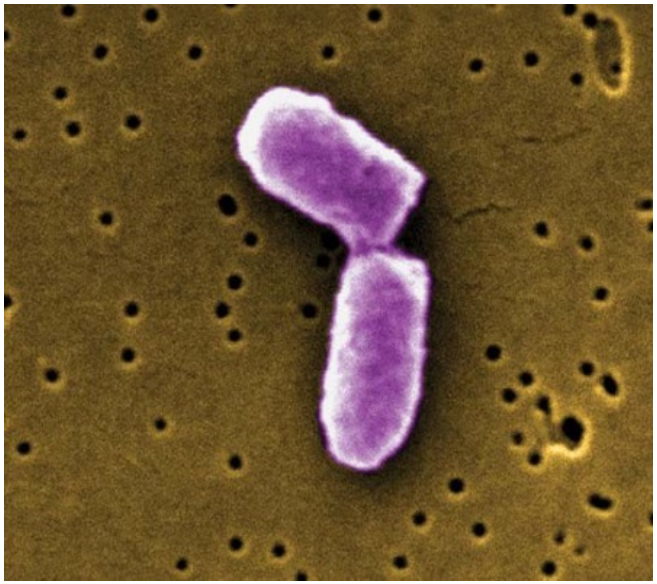
Bacterial Generation (Doubling) Time

Examples';

- *Escherichia coli and other medically important bacteria -------20 mins*
- *Staphylococcus aureus----27-30mins*
- *Bacillus subtilis------25-27mins*
- *Mycobacterium tuberculosis-----18 hours*
- *Mycobacterium leprae------14 days*
- *Treponema pallidum---1980 mins*

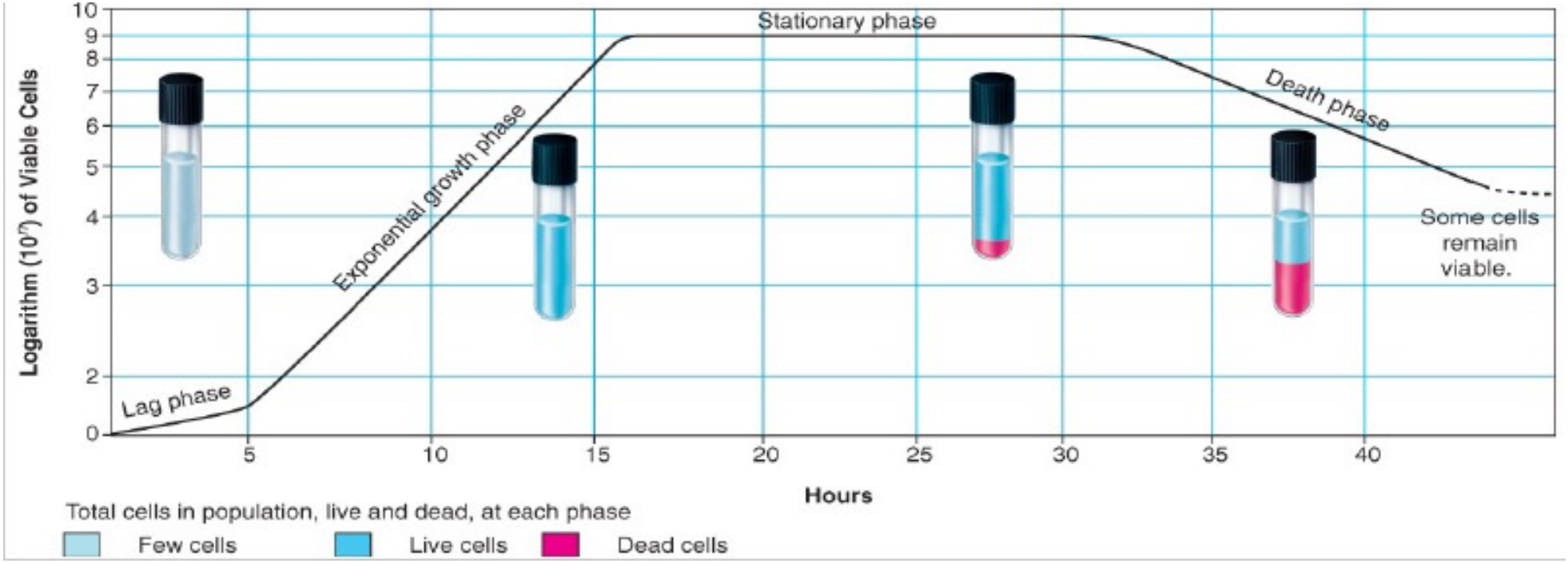


Ex) *E. coli* GT is 20 mins in optimal conditions
- After 20 generations (7 hrs) → 1 million cells
- After 30 generations (10 hrs) → 1 billion cells



Bacterial growth curve

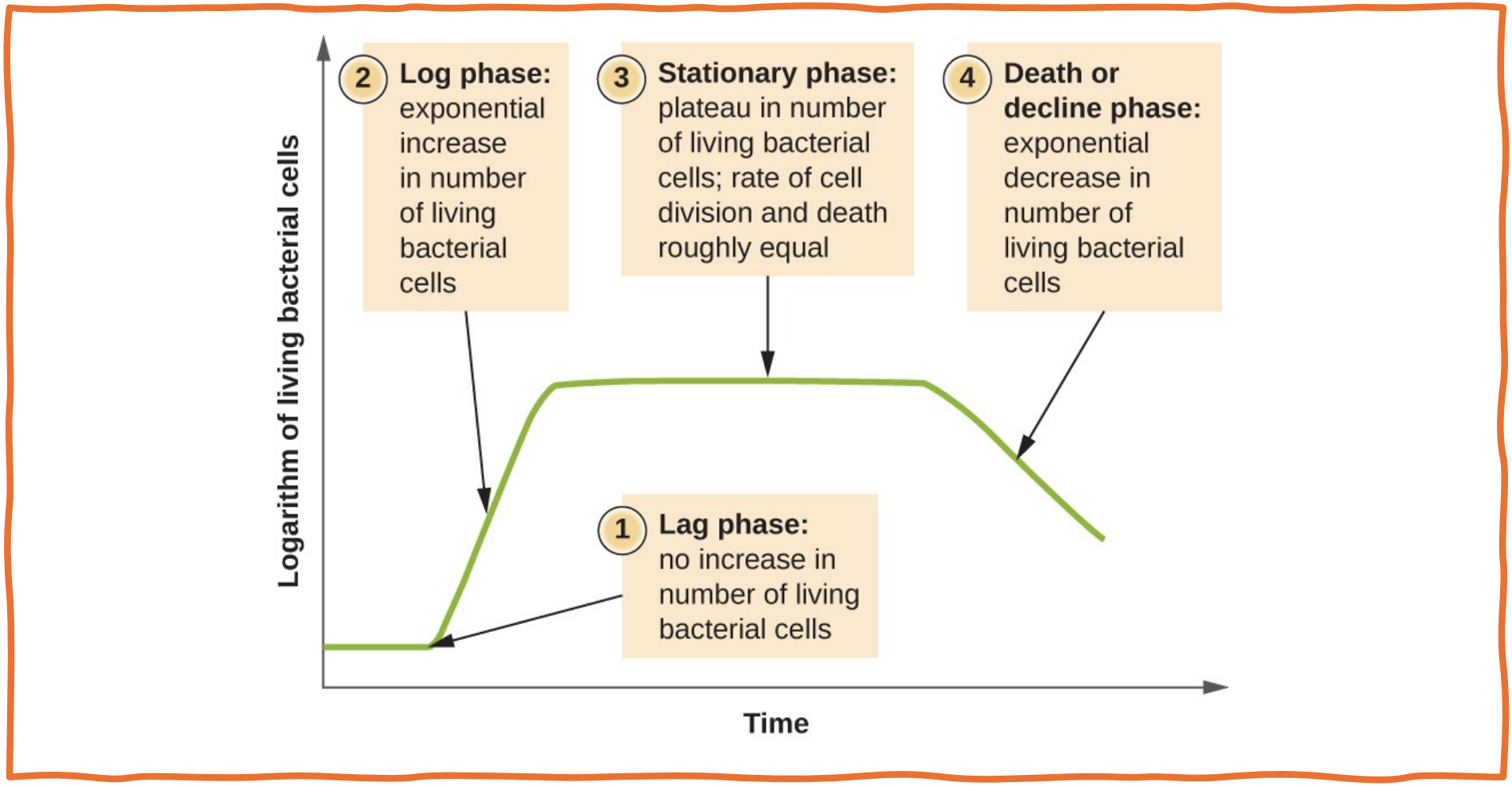
- **Batch culture (closed system)**
- **Bacteria grown in a liquid medium**
- **Single batch of medium**
- **Nutrient get exhausted and there is decline in growth**
- **When a graph is plotted as the logarithm of the no. of viable cells Vs the incubation time**



Demonstration of balanced and unbalanced growth

Shift-up experiment- bacteria when transferred from nutritionally poor medium to richer medium the lag phase is longer

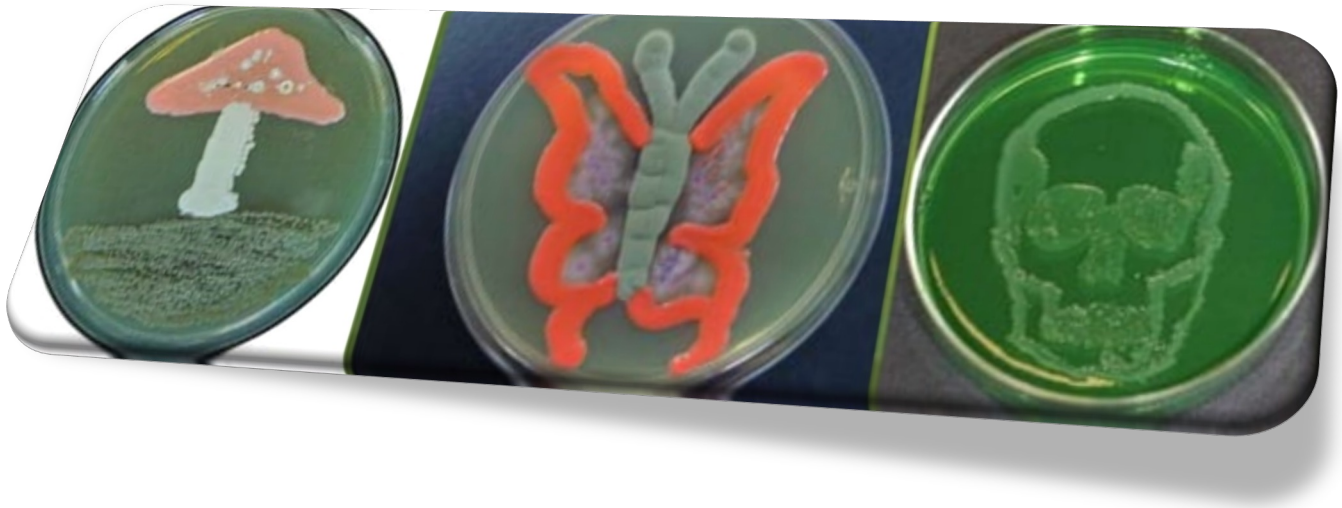
Shift-down experiment- bacteria when transferred from nutritionally rich medium to poorer medium the lag phase is shorter


2 Log phase: exponential increase in number of living bacterial cells
3 Stationary phase: plateau in number of living bacterial cells; rate of cell division and death roughly equal
4 Death or decline phase: exponential decrease in number of living bacterial cells
1 Lag phase: no increase in number of living bacterial cells
Logarithm of living bacterial cells
Time

Culture media & culture methods

Intro:

- Culture media is a liquid or solid substance required to grow the organisms from infected material to identify the causative agent.



History

The original media used by Louis pasteur (liquid med)

-Urine or Meat broth

-liquid medium = diffuse growth

-Solid medium = discrete colonies

Colony : macroscopically visible collection of millions of bacteria originatimg from single bacterial cell.

Cooked cut potato by Robert koch (solid med)

Gelatin – not satisfactory

- becomes liquid med at 24 ^{o}C

TYPES OF CULTURE MEDIA

I Based on their consistency
- a)solid medium
- b)liquid medium
- c)semi soild medium

II Based on the consistency /ingredients
- a)simple medium
- b)complex medium
- c)synthetic or defined medium
- d)Special media

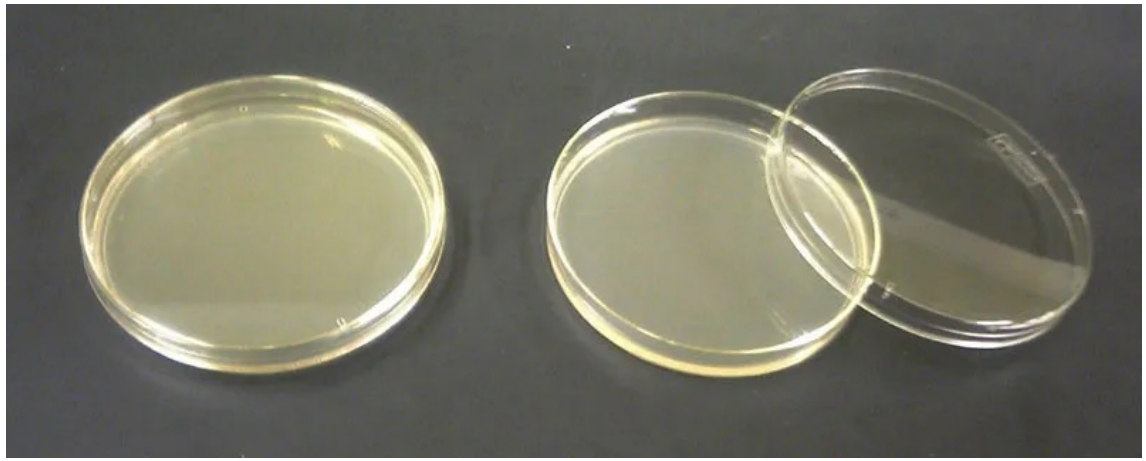
III Based on oxygen requirement
- a)aerobic media
- b)anaerobic media

I Based on their consistency

- <u>SOLID MEDIA</u>
- -CONTAINS 2% Agar
- -Colony morphology , pigmentataion,hemolysis
- can be appreciated.
- E.g; Nutrient agar, blood agar

- <u>LIQUID MEDIA</u>
- - no agar
- - For inoculum prep, blood culture, for the isolation
- of pathogens from mixture
- E.g; Nutrient broth

- <u>Semi solid medium</u>
- 0.5% agar
- E.g; Motility medium



II Based on the consistency /ingredients

SIMPLE MEDIA/BASAL MEDIA

- they contain minimum ingredient that supports growth of non- fastidious bacteria

E.g, Nutrient broth, nutrient agar

Nutrient broth consist of peptone, meat extract, NaCl and Water

Nutrient broth +0.5% Glucose =Glucose broth

Nutrient broth +2% agar. =Nutrient agar

Agar concentration reduced (0.2-0.5%)=semi solid med

Complex media

Media that contain some ingredients of unknown chemical composition are complex media such as blood agar. Complex media contain undefined components like peptones, meat extract and yeast extract.

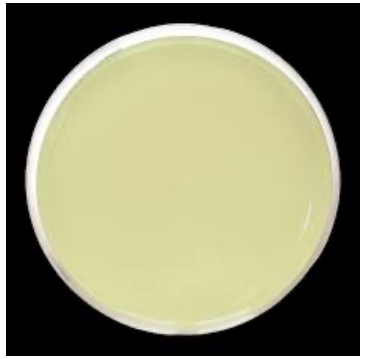
Synthetic or defined media

specially prepared media from pure chemical substances for research purpose and composition of every component is well known

- eg: peptone water -
- 1% peptone + 0.5% NaCl in water.

Nutrient agar

Potato dextrose agar



Czapek dox medium

Ingredients	Quantity /L
Sucrose ($C_{12}H_{22}O_{11}$)	30.00 g
Sodium nitrate($NaNO_3$)	2.00 g
Magnesium glycerophosphate ($C_3H_7MgO_6P$)	0.50 g
Potassium chloride (KCl)	0.50 g
Dipotassium sulfate (K_2SO_4)	0.35 g
Ferrous sulfate ($FeSO_4$)	0.01 g
(pH value regulated to 6.8 ± 0.2 at 25 ± 2°C)	

Special media

- Enriched media
- Enrichment media
- Selective media

ENRICHED MEDIA

I When basal media is added with additional nutrient such as blood, serum or egg

E.g,

blood agar: blood + nutrient agar

use for streptococus

chocolate agar: it is heated blood agar

use for Neisseria, H. inflenza

loffler's serum slope: serum + nutrient agar

use for Corneybacterium diptheria

Blood agar

Alpha Hemolysis

•**Effect on blood agar**: Green-grey or brown color around the bacterial colony caused by incomplete lysis of the blood.
•**Mechanism**: Hydrogen peroxide produced by bacteria turns hemoglobin to methemoglobin causing the green color of the medium. The cell's membrane remains generally intact.
•**Produced by**: *Staphylococcus aureus*, *Streptococcus pneumoniae*

Beta Hemolysis

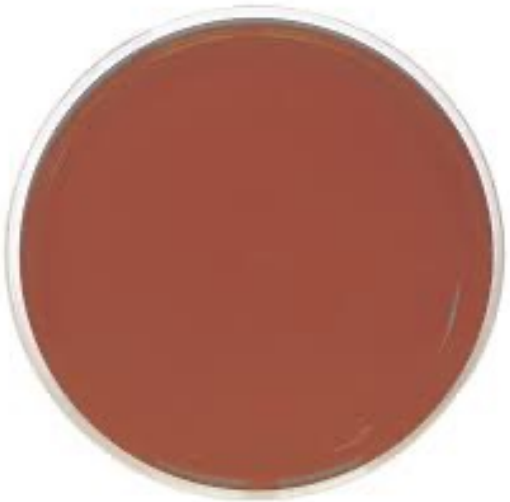
•**Effect on blood agar**: Clearing of the agar around the colony due to complete red blood cell lysis. Another type of hemolysis, alpha prime hemolysis (or wide-zone alpha hemolysis), could be confused for beta hemolysis. In alpha prime hemolysis, the agar around the bacterial colony has a small zone of unlysed red blood cells and complete lysis surrounding this.
•**Mechanism**: Beta hemolysis is caused by exotoxins called streptolysins which interact with cholesterol in cell membranes of the red blood cells to form pores.
•**Produced by**: *Streptococcus pyogenes*, *Streptococcus agalactiae*

Gamma Hemolysis

•**Effect on blood agar**: No effect.
•**Mechanism**: Gamma hemolysis is the absence of hemolysis activity.
•**Produced by**: *Neisseria meningitidis*, *Enterococcus faecalis*

chocolate agar

Aside from the “typical” blood agar plate described above, there’s another type of blood agar: chocolate agar. But unlike the name suggests, this type of agar is not made of chocolate. The agar takes on a chocolate color due to the addition of lysed blood into the medium. It’s used to grow organisms like Haemophilus influenzae, which require factor V (nicotinamide adenine dinucleotide) from the lysed red blood cells for growth. So sorry to those of you hoping for the tasty aroma of chocolate emanating from these plates!



loffler’s serum slope

Loeffler Medium is a modification of the original formula developed by Loeffler in 1887. Loeffler medium enhances primary and secondary isolation and cultivation of fastidious pathogenic microorganisms, especially from the nose and throat. It also restores virulence and other identifying properties (microscopic and colonial) after they have been lost due to prolonged incubation or repeated subculturing. The high serum content helps in determining the proteolytic activity of organisms. It is also used for the demonstration of pigmentation and ascospores.

Enrichment media

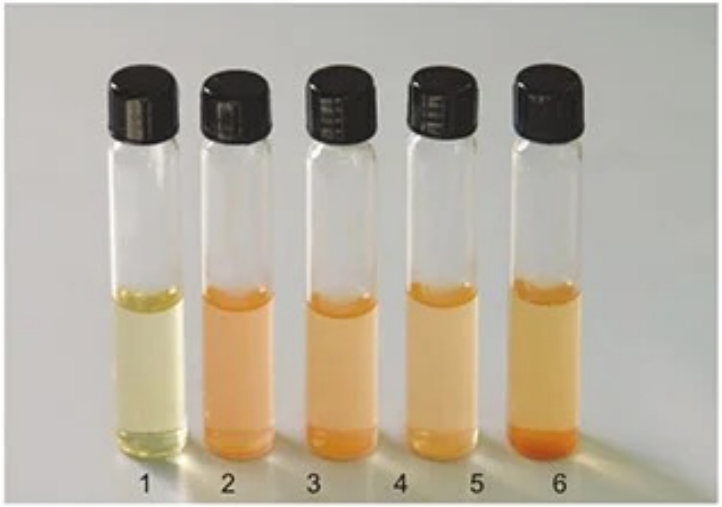
I. It is a liquid media which increase the growth of particular species or inhibits its competitors useful for isolation of pathogen

E.g,,

- Selenite F broth - Salmonella and Shigella
- Alkaline peptone water - *Vibro cholerae*
- Tetrathionate broth - Salmonella

Uninoculated

Salmonella



Fluid Selenite Cystine Medium (Selenite Cystine Medium) (Twin Pack) M025

1. Control
2. Salmonella Typhimurium ATCC 14028
3. Salmonella Choleraesuis ATCC 12011
4. Salmonella Typhi ATCC 6539
5. Escherichia coli ATCC 25922


HIMEDIA
M618-500G
Alkaline Peptone Water
Directions

SELECTIVE MEDIA

I Enriched media & selective both are same principle but they are solid media.

E.g,

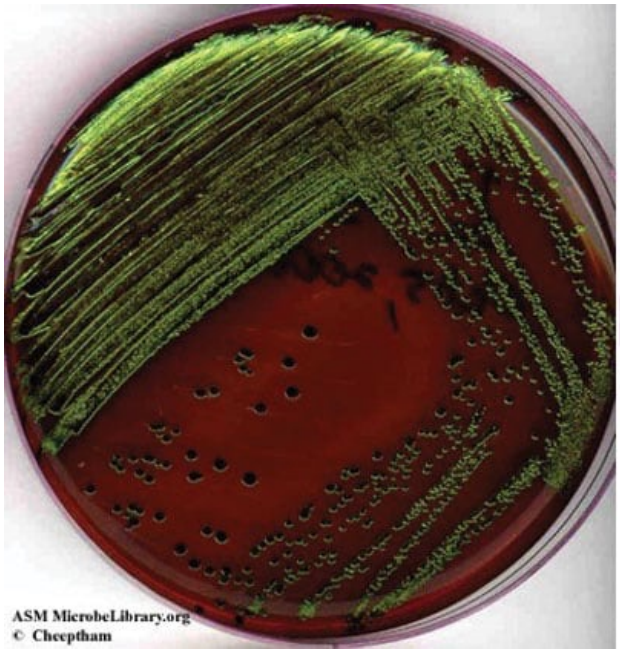
- **Eosin Methylene Blue (EMB) agar** - E. coli
- Macconkey agar – *Pseudomonnas sp.*
- TCBS – *V. cholerae*
- LJ medium – *Mycobacterium tuberculosis*
- Wlison and Blair medium – *Salmonella typhi*
- Tellurite Blood Agar– *Corynebacterium diphtheri*ae

Eosin Methylene Blue (EMB) agar

Eosin Methylene Blue (EMB) agar is a differential microbiological medium, which slightly inhibits the growth of Gram-positive bacteria and provides a color indicator distinguishing between organisms that ferment lactose (e.g., *E. coli*) and those that do not (e.g., *Salmonella, Shigella*).

EMB agar was originally devised by Holt-Harris and Teague and further modified by Levine. It is thus a combination of the Levine and Holt-Harris and Teague formulae which contains a peptic digest of animal tissue and phosphate as recommended by Levine and two carbohydrates as suggested by Holt-Harris and Teague.

The medium is important in medical laboratories to distinguish gram-negative pathogenic microbes in a short period of time.

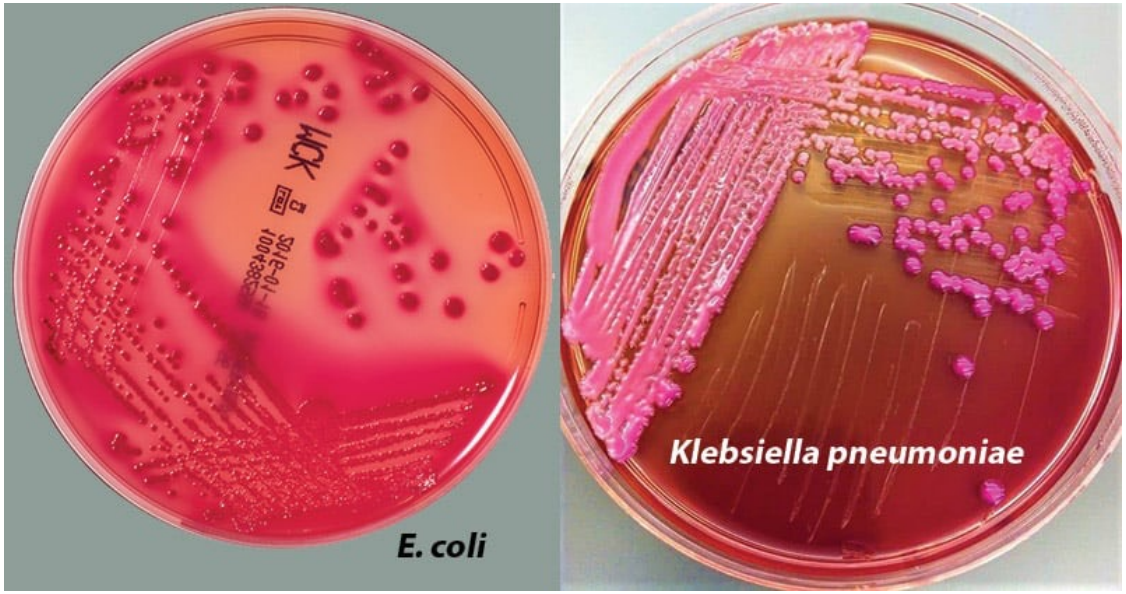


Organisms	Growth
Escherichia coli	Blue-black bull's eye; may have a green metallic sheen
Pseudomonas aeruginosa	Colorless
Enterobacter aerogenes	Good growth; pink, without sheen
Klebsiella pneumoniae	Pink, mucoid colonies
Proteus mirabilis	Luxuriant growth; colorless colonies
Salmonella Typhimurium	Luxuriant growth; colorless colonies

MacConkey agar

MacConkey agar was the first solid differential media to be formulated which was developed inthe 20th century by Alfred Theodore MacConkey. MacConkey Agar is the earliest selective and differential medium for the cultivation of coliform organisms.

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

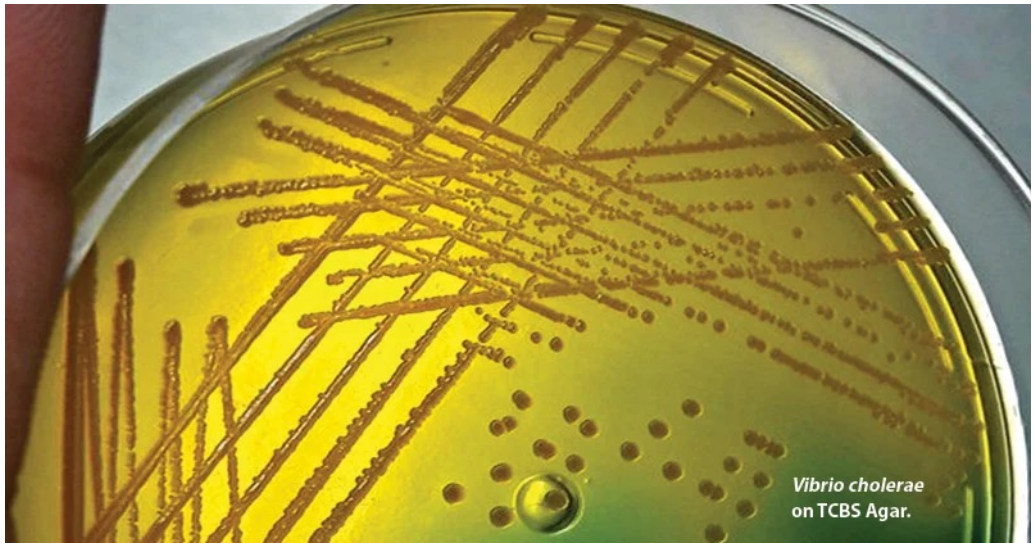


Organisms	Growth results
Escherichia coli	Pink to rose-red colonies (may be surrounded by a zone of precipitated bile)
Enterobacter, Klebsiella	Mucoid, pink colonies
Proteus	Colorless colonies, swarming growth
Salmonella, Shigella	Colorless colonies, or sometimes medium color: orange to amber
Pseudomonas	Irregular, colorless to pink colonies

Thiosulfate-citrate-bile salts-sucrose (TCBS) Agar

, is a type of selective agar that is used in microbiology laboratories to isolate *Vibrio* species. TCBS Agar is a selective differential medium for isolating and cultivating ***Vibrio cholerae*** and other *Vibrio* species from clinical specimens and other materials. TCBS Agar was developed by Kobayashi et al, who modified the selective medium of Nakanishi. Although this medium was originally designed for the isolation of *V. cholerae* and *V. parahaemolyticus*, most Vibrios grow to healthy large colonies with different colonial morphologies.

TCBS Agar is used for the isolation of *Vibrio cholerae* and other enteropathologic *Vibrio* (in particular *Vibrio parahaemolyticus*) in fish, seafood and biological samples of animal origin.



Microorganisms	Characteristics
Vibrio cholera	Flat yellow colonies, 2-3 mm in diameter
Vibrio alginolyticus	Large yellow colonies
Vibrio fluvialis, Vibrio vulnificus	Yellow or translucent colonies
Vibrio parahaemolyticus	Colorless colonies with a green center
Pseudomonas, Aeromonas	Blue colonies
Enterobacteria or others	Tiny transparent colonies

Lowenstein Jensen (LJ) Media

Lowenstein Jensen (LJ) Media is a selective medium that is commonly used for the cultivation and isolation of *Mycobacterium*, specifically *Mycobacterium tuberculosis* from clinical specimens. LJ medium was originally formulated by Lowenstein who incorporated congo red and malachite green to inhibit unwanted bacteria. The present formulation comprises a glycerated egg-based medium and is based upon Jensen's modification. Jensen modified Lowenstein's medium by altering the citrate and phosphate contents, eliminating the congo red dye, and by increasing the malachite green concentration. Gruft further modified L. J. Medium with the addition of penicillin, nalidixic acid, and ribonucleic acid (RNA) to increase selectivity. This medium supports the growth of a wide variety of *Mycobacteria* and can also be used for niacin testing.

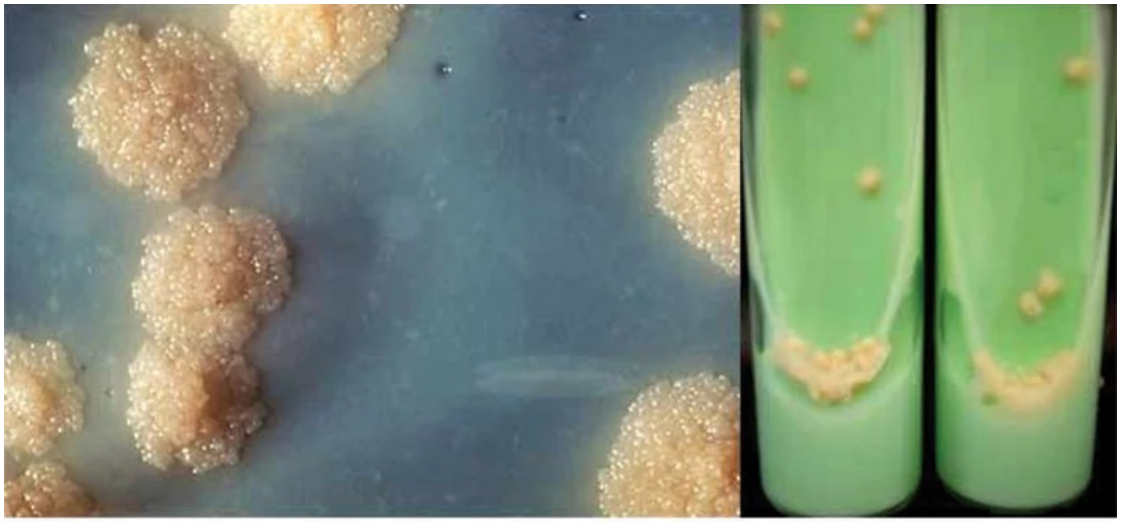


Fig: Cultural Characteristics of *Mycobacterium tuberculosis*

Tellurite Blood Agar

Tellurite Blood Agar is a selective medium used for isolation and cultivation of Corynebacterium species. Potassium tellurite acts as a selective agent and has inhibitory activity against most gram-positive and gram-negative bacteria except Corynebacterium species.

C.diphtheriae reduces potassium tellurite to tellurium and thereby produce gray-black coloured colonies.

*Corynebacterium diphtheri*ae

Special media

- Indicator media
- Differential media
- Sugar media
- Transport media
- Anaerobic medium

INDICATOR MEDIA

- These media contains of indicator which changes colour when a bacterium grows in them. S. typhi grows as black colour on wilson and blair medium.
- Eg

  blood agar

  macconkey agar

  christension urease medium

DIFFERNTIAL MEDIA

- This media differentiate between two groups of bacteria by using an indicator.
- Eg Macconkey agar
- Distinguish between lactose fermenters & non lactose fermenters

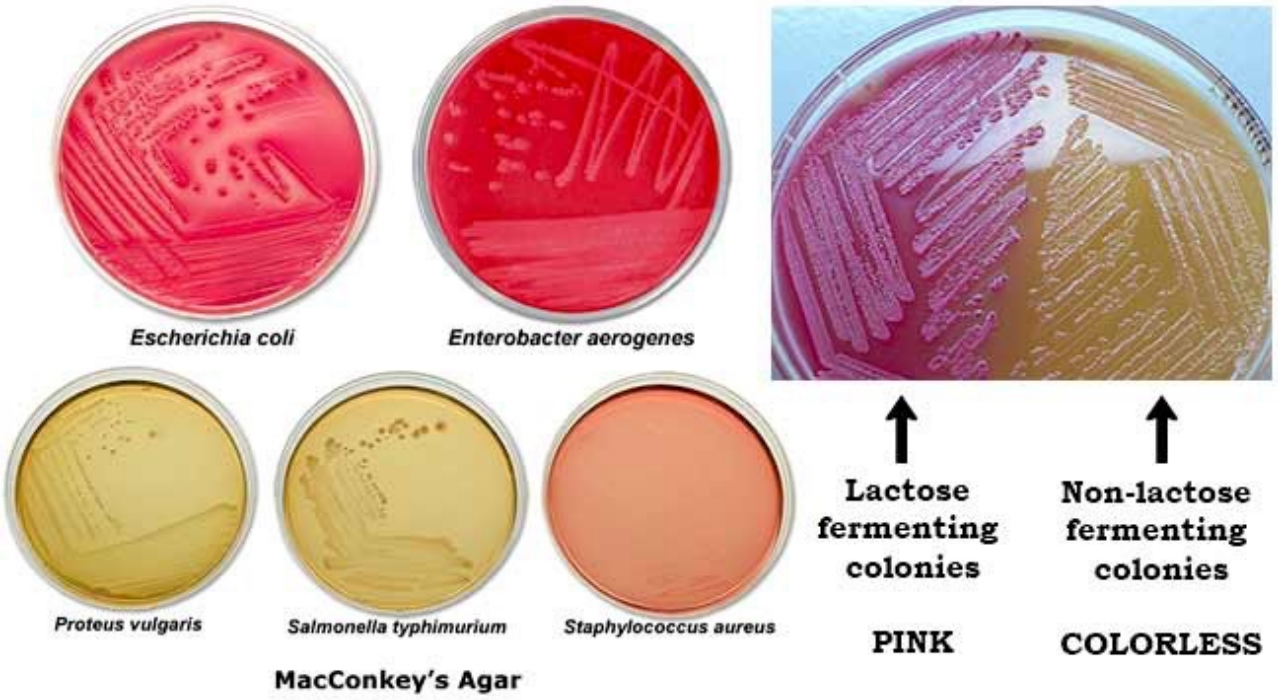
compostion: peptone

lactose

agar

Neutral red

Taurocholate



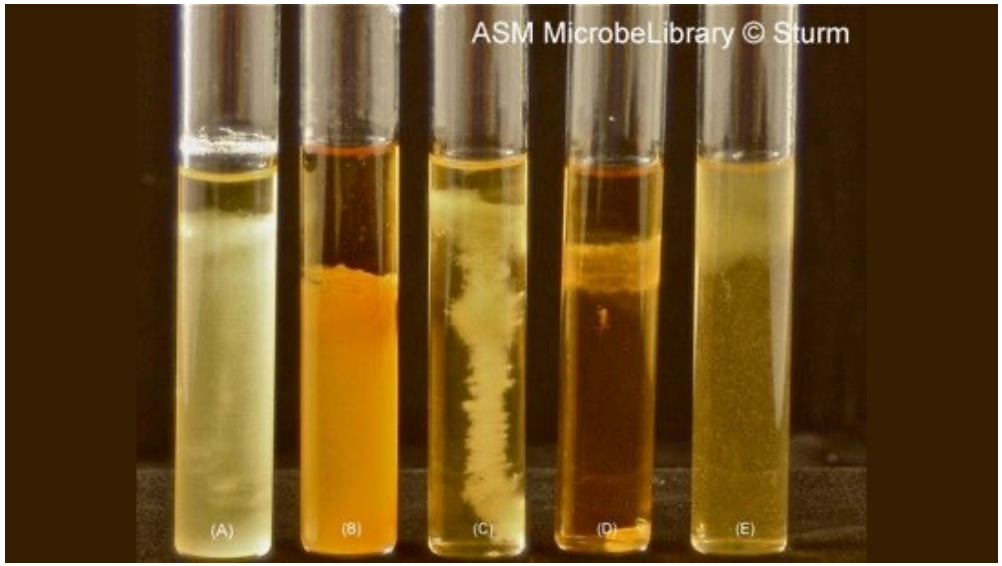
SUGAR MEDIA

- Media containing any fermentable substance.
- E.g glucose, arabionse, lactose, starch e.t.c,
- Media consist of one % of sugar

ANAEROBIC MEDIA

- these media are used to grow anaerobic organisms
- EG:,

Robertson cooked meat medium



Thioglycolate medium

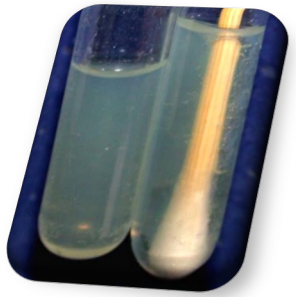
TRANSPORT MEDIA

- This is used for transport the specimen. In this media bacteria not multiply only remains viable when delay transporting the specimen from site of collection to laboratory
- Eg

  -stuart medium: non nutrient soft agar containing a reducing agent

  -bufferd glycerol saline: enteric bacilli

  -Amies media - Neisseria



Culture methods

- Culture methods employed depend on the purpose for which they are intended.
- The indications for culture are:
- To isolate bacteria in pure cultures.
- To demostrate their properties.
- For bacteriophage & bacteriocin susceptibility.
- To determine sensitivity to antibiotics.
- To estimate viable counts.
- Maintain stock cultures.

Culture methods includes:

- Solid culture
  - Streak culture
  - Lawn culture
  - Stab culture
  - Pour plate culture
  - Spread plate
- Liquid culture
- Anaerobic culture methods

STREAK CULTURE

- It is a routinely used method to isolate bacteria.
- One loopful of culture is made as a primary inoculum and is then distributed thinly over the plate by streaking it with the loop in a series of parallel lines in different segments of the plate.
- Loop flamed and cooled between the different sets of streaks.
- On incubation, growth may be confluent at the site of the original inoculation but becomes progressively thinner and well separated colonies are obtained over the final series of streaks.

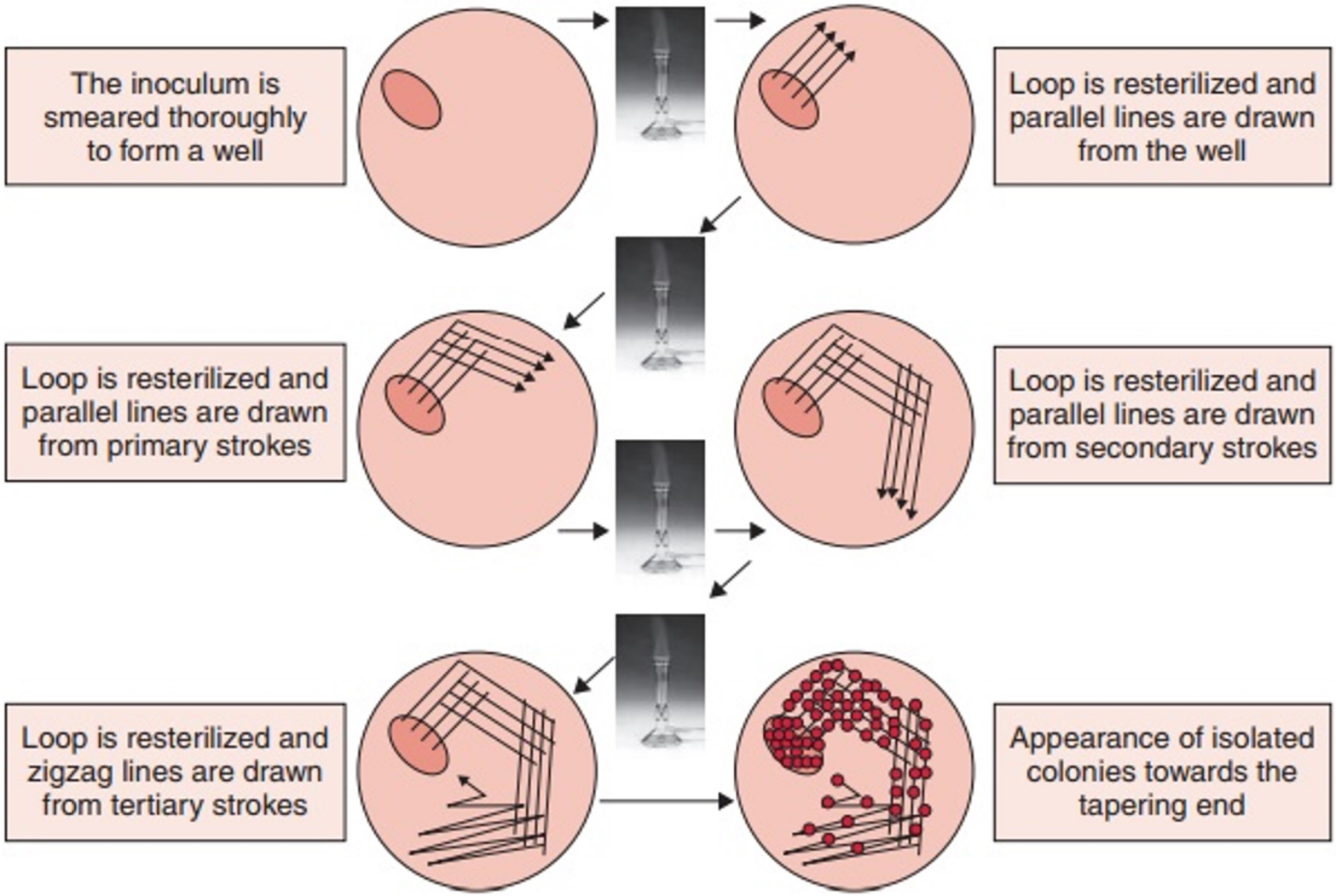
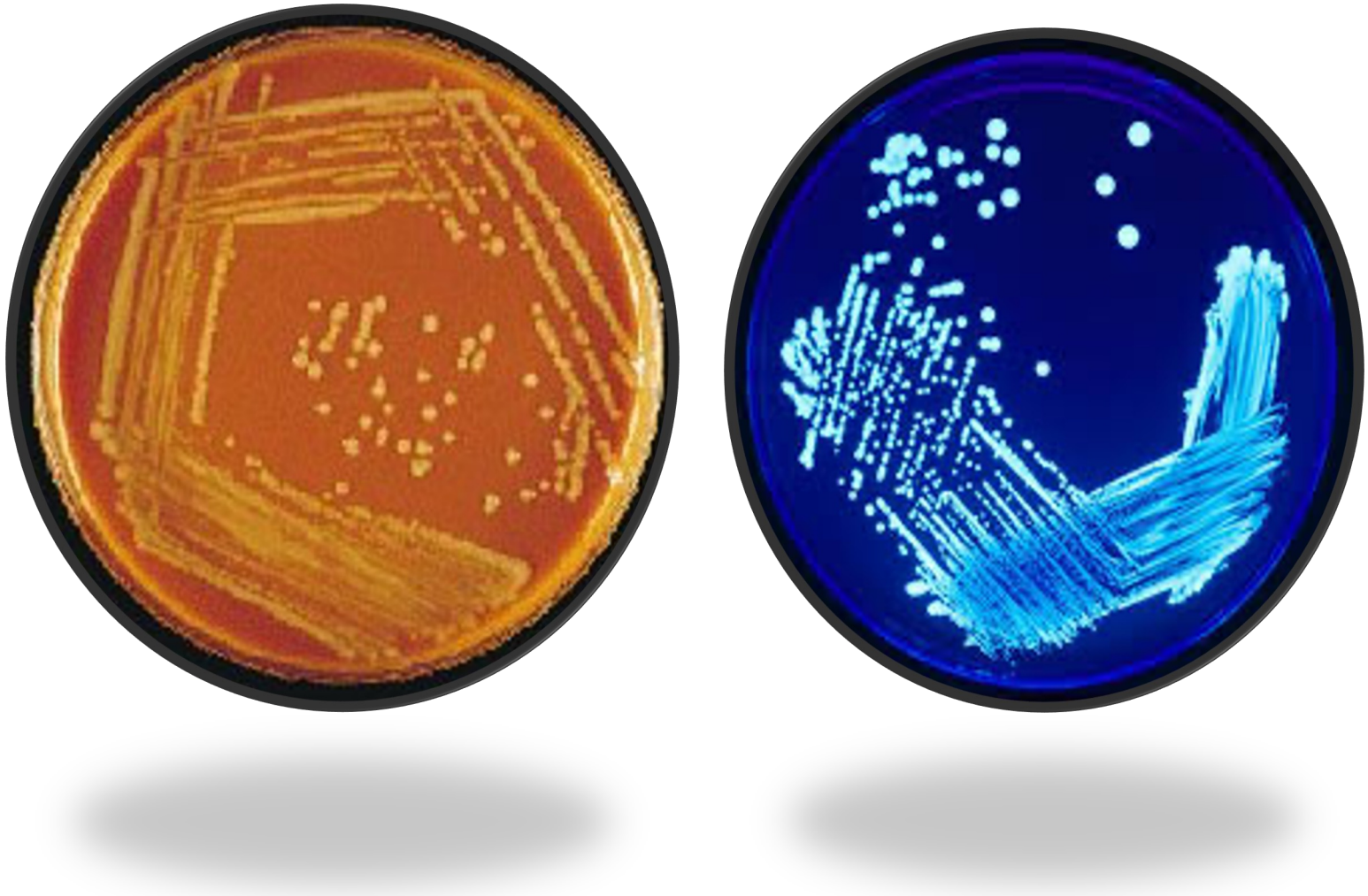
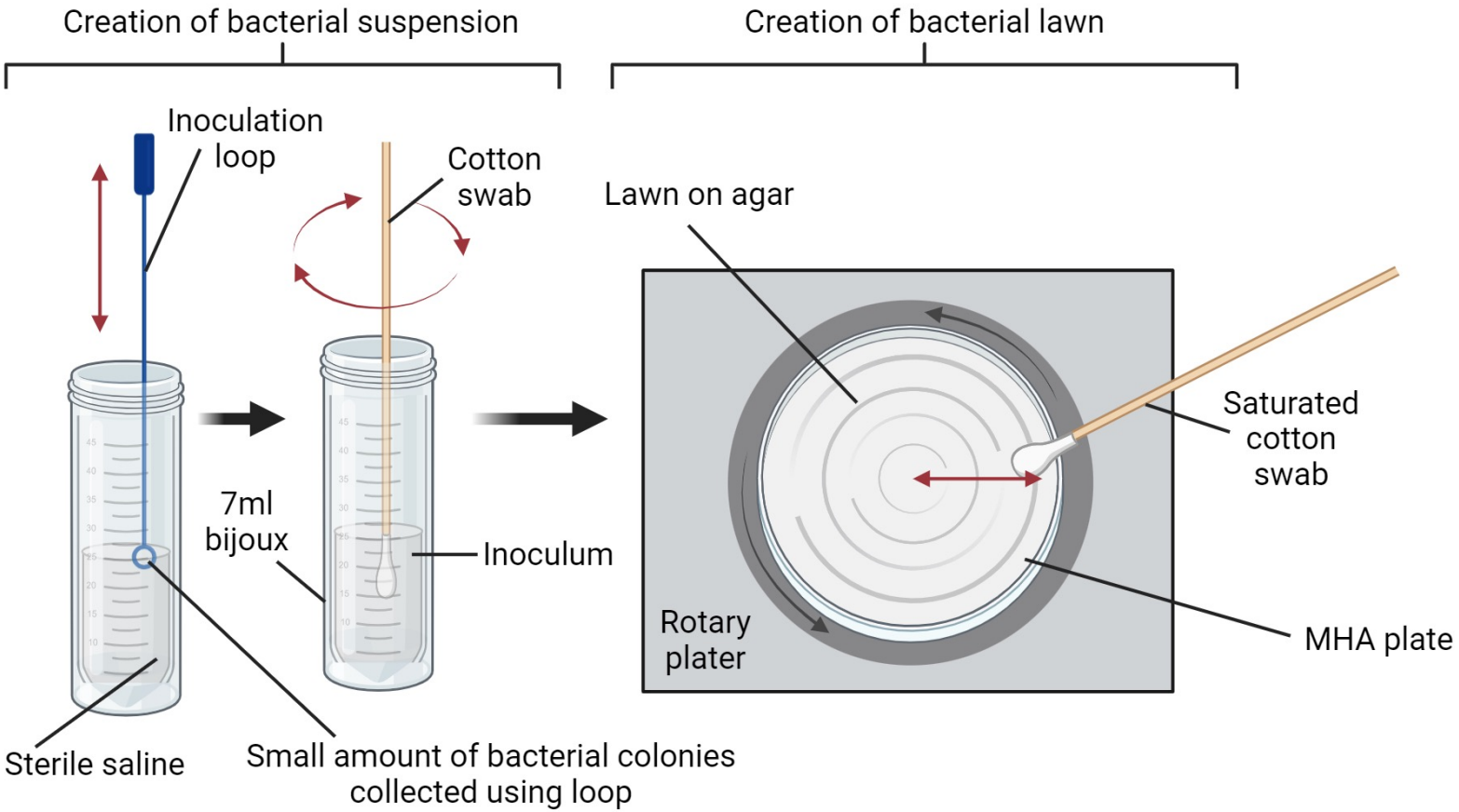


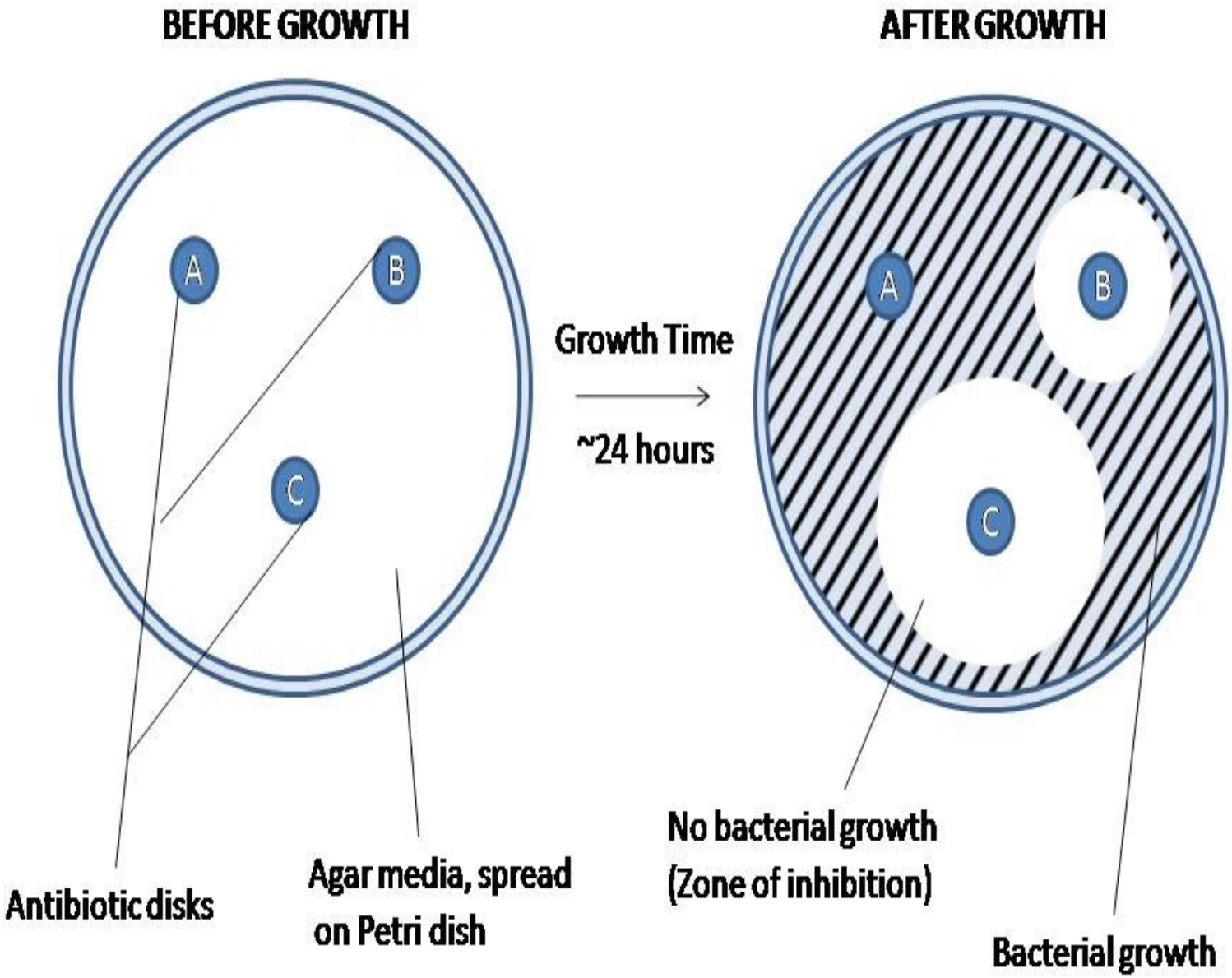
FIG. 5-1. Diagrammatic representation showing streak culture.



LAWN CULTURE

- Also called as carpet culture.
- Provides a uniform thick surface growth of the bacterium.
- Useful for bacteriophage typing and antibiotic sensitivity test.
- Also used in the preparation of bacterial antigens and vaccines.
- There are two methods of obtaining lawn culture, which are as follows:
  - (i) **SWABBING** – A sterile swab soaked in liquid bacterial culture is inoculated on to the culture plate and then incubated at 37°C overnight to obtain uniform lawn of bacterial growth on the surface of the culture plate.
  - (ii) **FLOODING**- The surface of the culture plate is flooded with a liquid culture or suspension of the bacterium and then excess material is drained out.

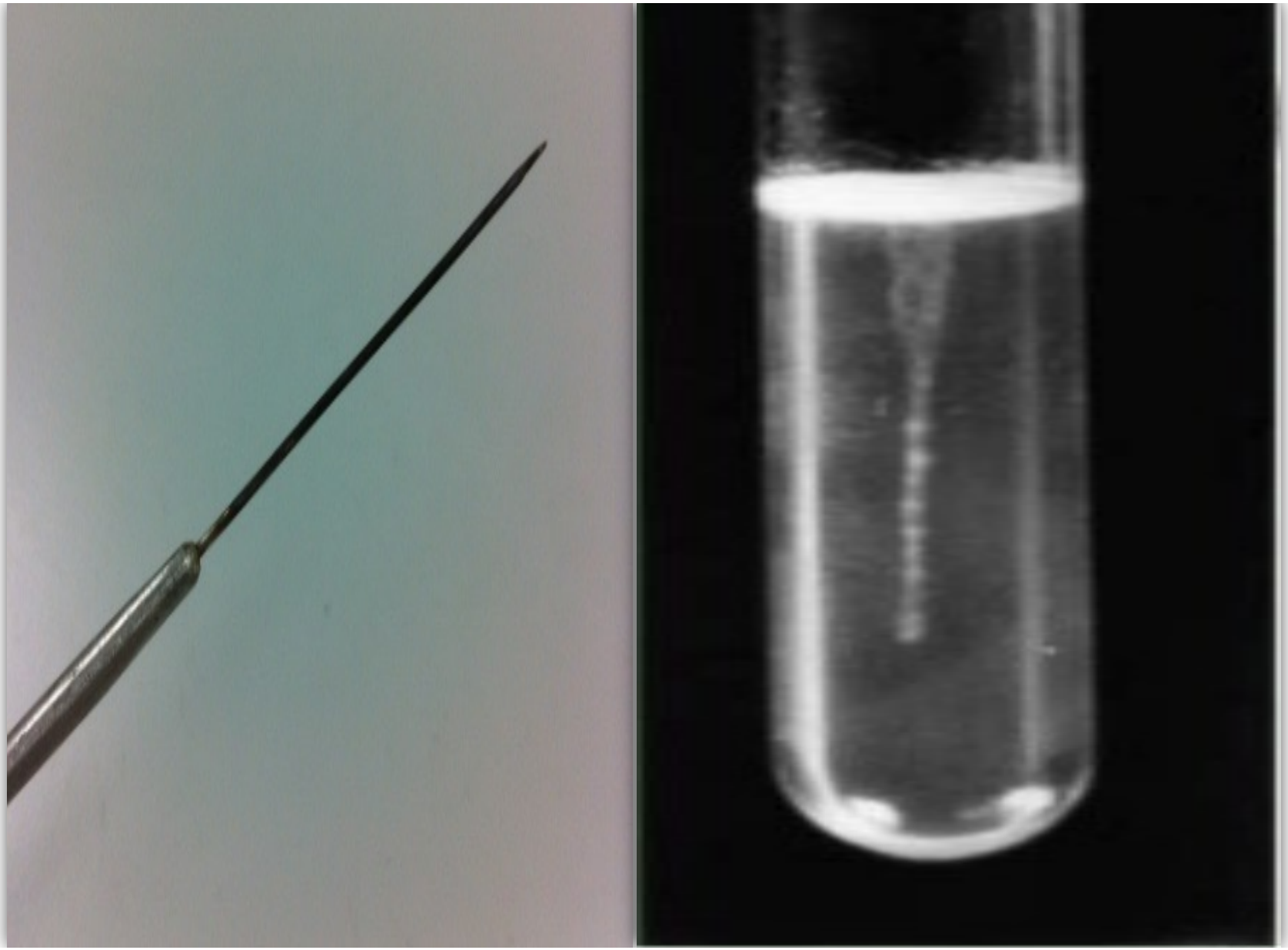

Creation of bacterial suspension
Creation of bacterial lawn
Inoculation loop
Cotton swab
Lawn on agar
Saturated cotton swab
7ml bijoux
Inoculum
Rotary plater
MHA plate
Sterile saline
Small amount of bacterial colonies collected using loop


BEFORE GROWTH
AFTER GROWTH
A
B
C
A
B
C
Growth Time
~24 hours
Antibiotic disks
Agar media, spread on Petri dish
No bacterial growth (Zone of inhibition)
Bacterial growth

STAB CULTURE

- This is performed by stabbing the semi solid agar but by a straight wire.
- stab culture is used for:
  (i) Maintaining stock culture.
  (ii) For demonstration of oxygen requirement of the bacteria by oxidative-fermentative (OF) test.
  (iii) Motility testing using semisolid agar.

- Examples where stab culture method is used are mannitol motility medium, nutrient agar semisolid butts, triple sugar iron agar test or TSI.



POSITIVE:

Organism moves away from stab

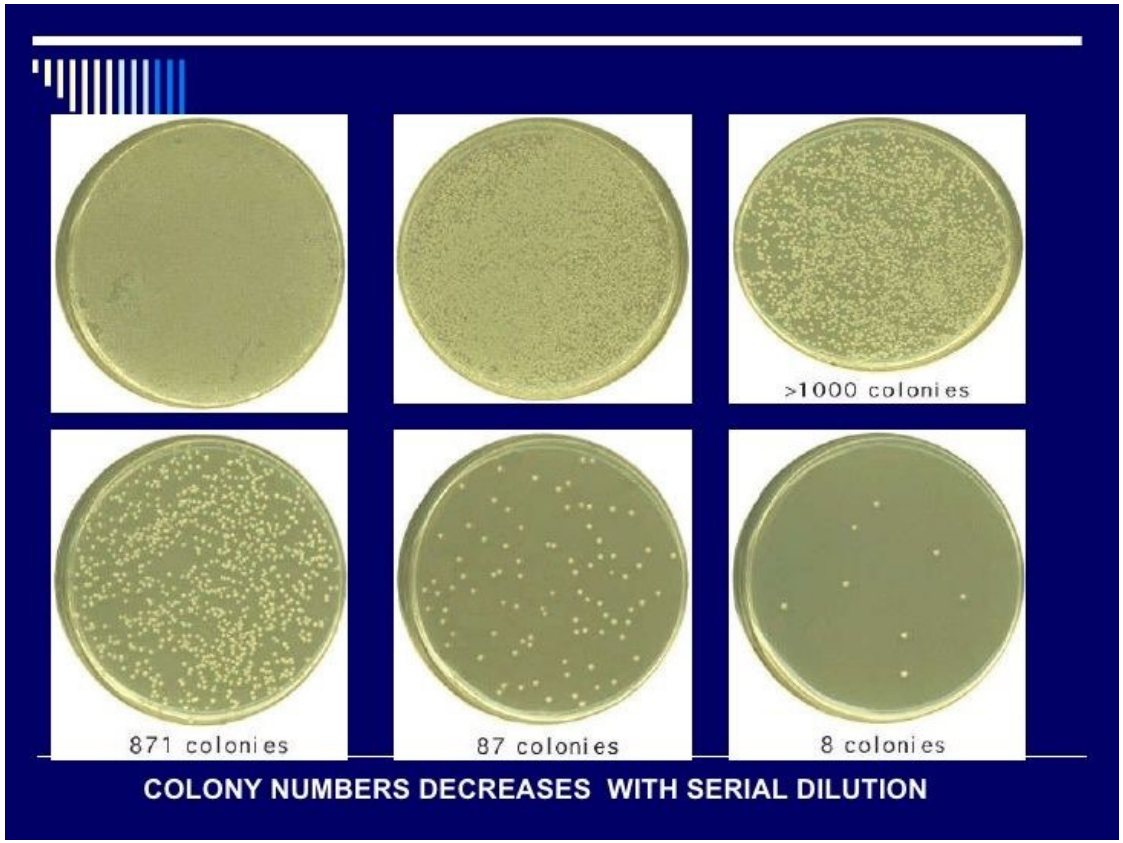
NEGATIVE:

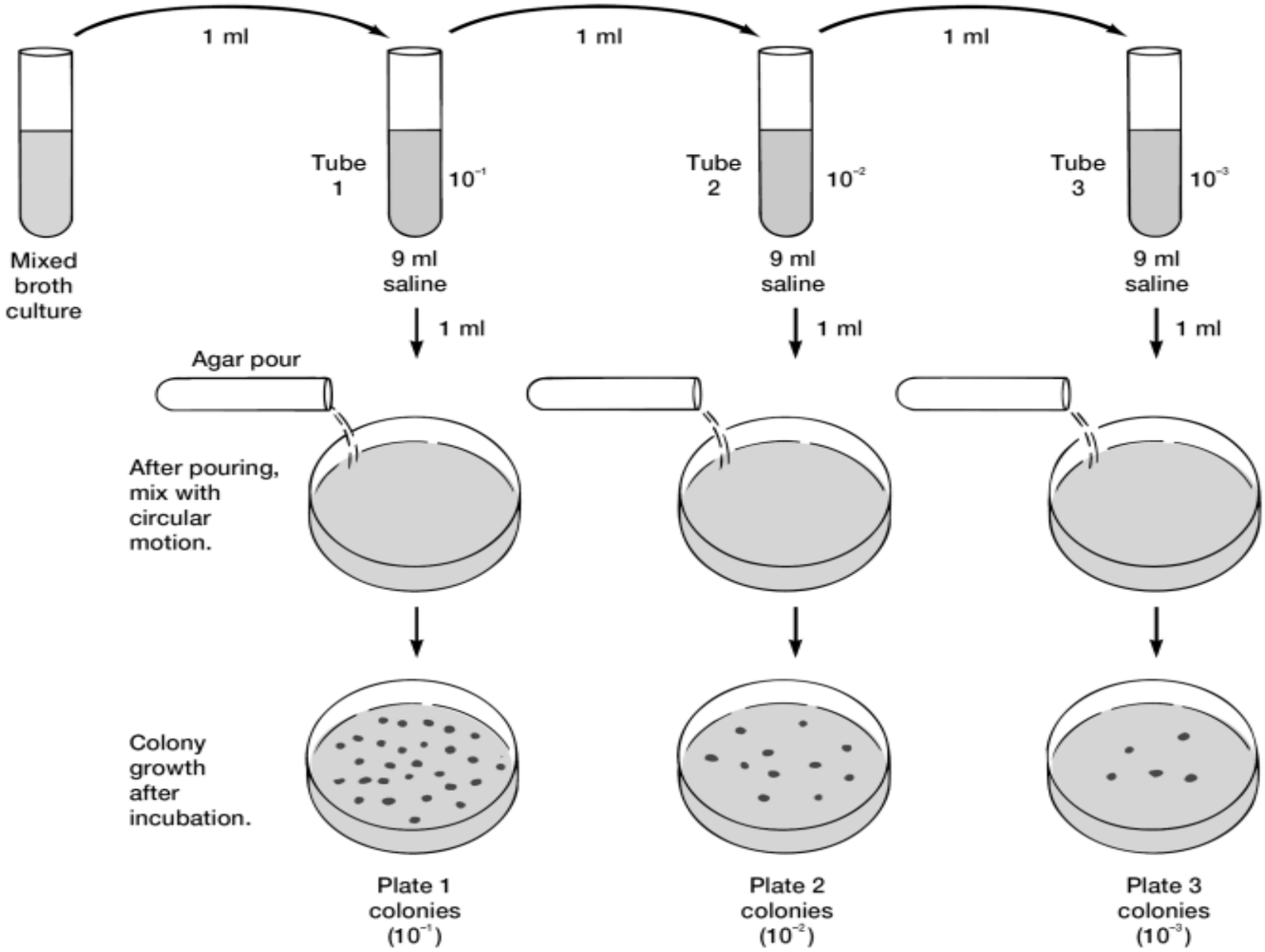
Organism does **NOT** move away from stab

MOTILITY TEST OF BACTERIA

POUR PLATE METHOD

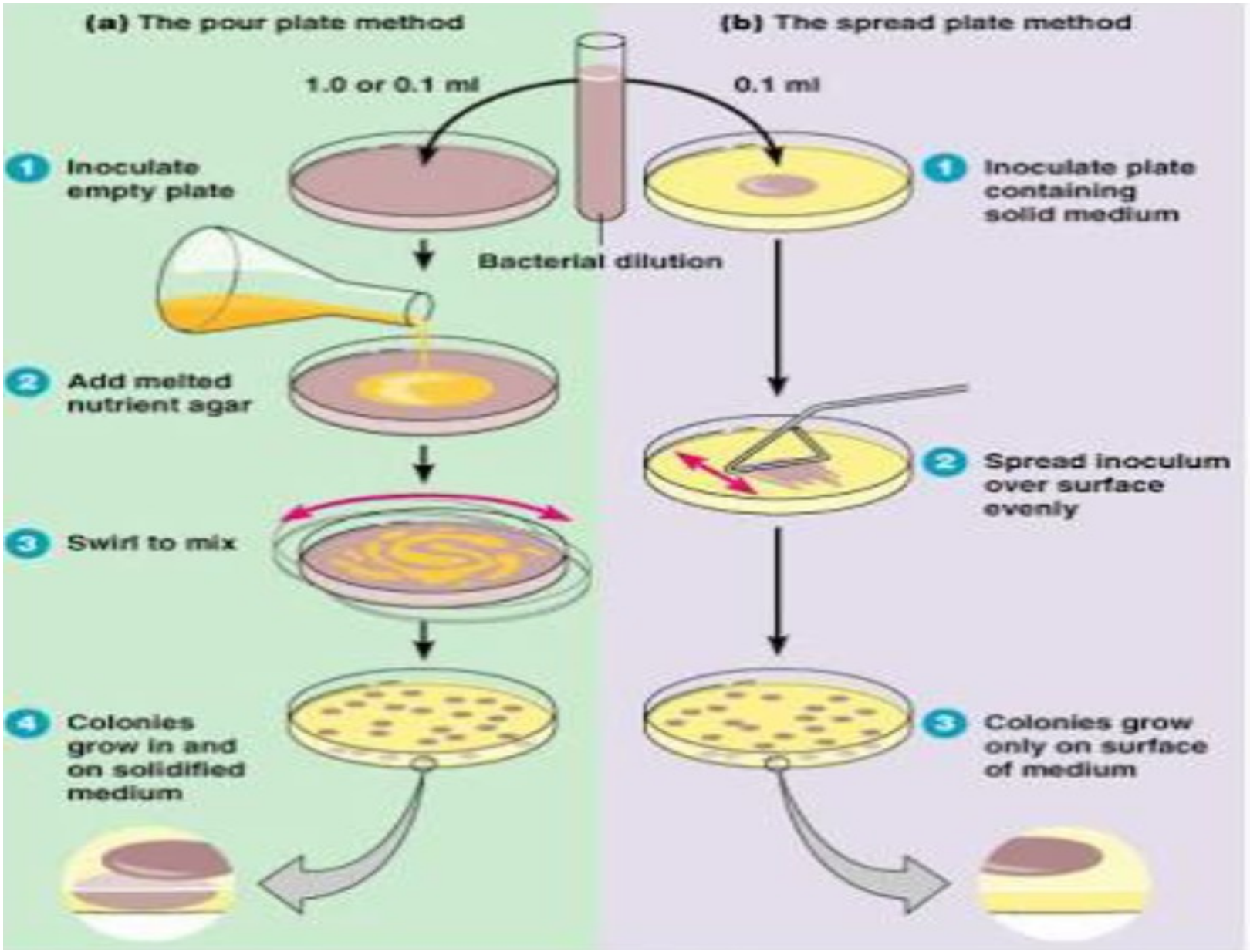
- This is a quantitative culture method, used to estimate viable bacterial count. This is one of the best method to determine the number of bacteria present per ml of liquid broth/specimen.
- **Serial 10-fold dilution** : Of the original bacterial suspensions are made. 9ml of nutrient broth is poured into a set of test tubes. 1 ml of the bacterial suspension added to the first test tube, mixed and then, 1 ml is serially transferred to the subsequent tube and so on.
- **Pour plating**: 1ml from each tube is added to a measured quantity of molten nutrient agar (which has been cooled to 45°C), mixed properly, and then is poured into a petri dish. After being cooled and solidified, the petri dishes are incubated overnight at 37°C
- **Colony counting**: Next day, the total number of colonies formed are counted from one among the plate that contains colonies between 30-300 colonies per plate. The lower dilutions will produce much crowded colonies, hence not suitable for counting. Each colony represents one bacterium in the specimen.
- **Viable count/ml**: of the specimen is calculated by multiplying the number of colonies/ plate with the dilution factor.




1 ml
1 ml
1 ml
Mixed broth culture
Tube 1
10^{-1}
Tube 2
10^{-2}
Tube 3
10^{-3}
9 ml saline
9 ml saline
9 ml saline
1 ml
1 ml
1 ml
Agar pour
After pouring, mix with circular motion.
Colony growth after incubation.
Plate 1 colonies (10^{-1})
Plate 2 colonies (10^{-2})
Plate 3 colonies (10^{-3})

SPREAD PLATE METHOD

This is another method for estimating the viable bacterial count. After serial dilution of the sample, known volume of individual dilutions are spread evenly on the surface of a suitable agar plate to obtain a lawn culture. After incubation colonies are counted and multiplied by dilution factor to estimate the colony count.


(a) The pour plate method
(b) The spread plate method
1.0 or 0.1 ml
0.1 ml
1 Inoculate empty plate
Bacterial dilution
1 Inoculate plate containing solid medium
2 Add melted nutrient agar
2 Spread inoculum over surface evenly
3 Swirl to mix
4 Colonies grow in and on solidified medium
3 Colonies grow only on surface of medium

Liquid cultures

- Liquid Culture is used for Culture of specimen Such as blood or body fluid.

Requirment:-

- Specimen
- Liquid media
- Pipette / Syringe
- Screw-Capped bottle or flask.

Procedure:-

- Inoculated by adding the Specimen directly in to the liquid medium with the help of Syringe or Pipette.
- Bacterial growth is detected by observing turbidity in the medium and some aerobic bacteria form Surface pellicles.

Uses

- blood, body fluid Culture, water analysis.

ANEROBIC CULTURE METHODS

- Anaerobic bacteria grow only in the absence of oxygen
- Anaerobiosis can be established by various methods.

Production of a Vacuum

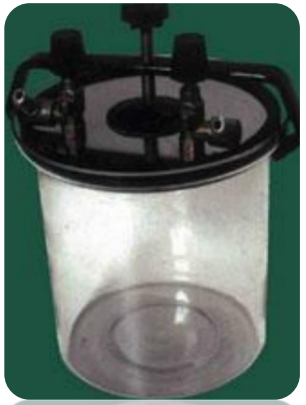
- Cultivation in vacuum was attempted by incubating cultures in a vacuum desiccator, but it proved to be unsatisfactory. This method is not in use now.

Displacement of Oxygen

- Displacement of oxygen by inert gases like hydrogen, nitrogen, carbon dioxide or helium is sometimes employed. Oxygen can never be removed completely by this method.

By Displacement and Combustion of Oxygen

- Anaerobiosis obtained by "McIntosh and Filde's" anaerobic jar is the most reliable and widely used method.



McIntosh and Filde's anaerobic jar

Gas pack

Measurement of Microbial Growth

- ❑Can measure changes in number of cells in a population
- ❑Can measure changes in mass of population
- ❑Microbial growth to determine **growth rates and generation times** can be measured by different methods.

Measurement of Cell Numbers

- **What are the lab equipment's used to measure bacteria cell numbers?**
- There are two types:-

1- Total count/Direct cell counts:-

a) Counting chambers method/ Haemocytometer method

b) Breed method/direct microscopic method

c) Electronic counters methods

d) Proportional counter method

2- Viable cell counts/Indirect method

- **Plating methods (spread, pour plate)**
- **Membrane filtration methods**

VIABLE (STANDARD) PLATE COUNT

• **Viable Plate Count** (also called a Standard Plate Count) is one of the most common methods, for enumeration of bacteria. Serial dilutions of bacteria are plated onto an agar plate. Dilution procedure influences overall counting process. The suspension is spread over the surface of growth medium. The plates are incubated so that colonies are formed. Multiplication of a bacterium on solid media results in the formation of a macroscopic colony visible to naked eye. It is assumed that each colony arises from an individual viable cell. Total number of colonies is counted and this number multiplied by the dilution factor to find out concentration of cells in the original sample.

• A major limitation in this method is selectivity. The nature of the growth medium and the incubation conditions determine which bacteria can grow and thus be counted. Viable counting measures only those cells that are capable of growth on the given medium under the set of conditions used for incubation. Sometimes cells are viable but non-culturable.

• The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted and then plated out on an agar surface in such a manner that single isolated bacteria form visible isolated colonies, the number of colonies can be used as a measure of the number of viable (living) cells in that known dilution. The viable plate count method is an indirect measurement of cell density and reveals information related only to live bacteria.

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PROCEDURE
VIABLE PLATE COUNT

- We will be testing four samples of water for the Viable Count. The samples include:
- **1) water from a drinking fountain**
2) boiled water from a drinking fountain
3) water from the local river
4) boiled water from the local river
- You will need **DATA TABLE 1** to input your data and calculate the number of CFU per ml.

Data Table 1
Viable Plate Count

Sample	Dilution Factor	# of Colonies	Dilution Factor X # of Colonies	# of CFU / ml
Faucet Water				
River Water				
Boiled Faucet Water				
Boiled River Water				

CFUs per ml of sample = The # of colonies counted X The dilution factor of the plate counted

- **1)** Take 6 dilution tubes, each containing 9 ml of sterile saline.

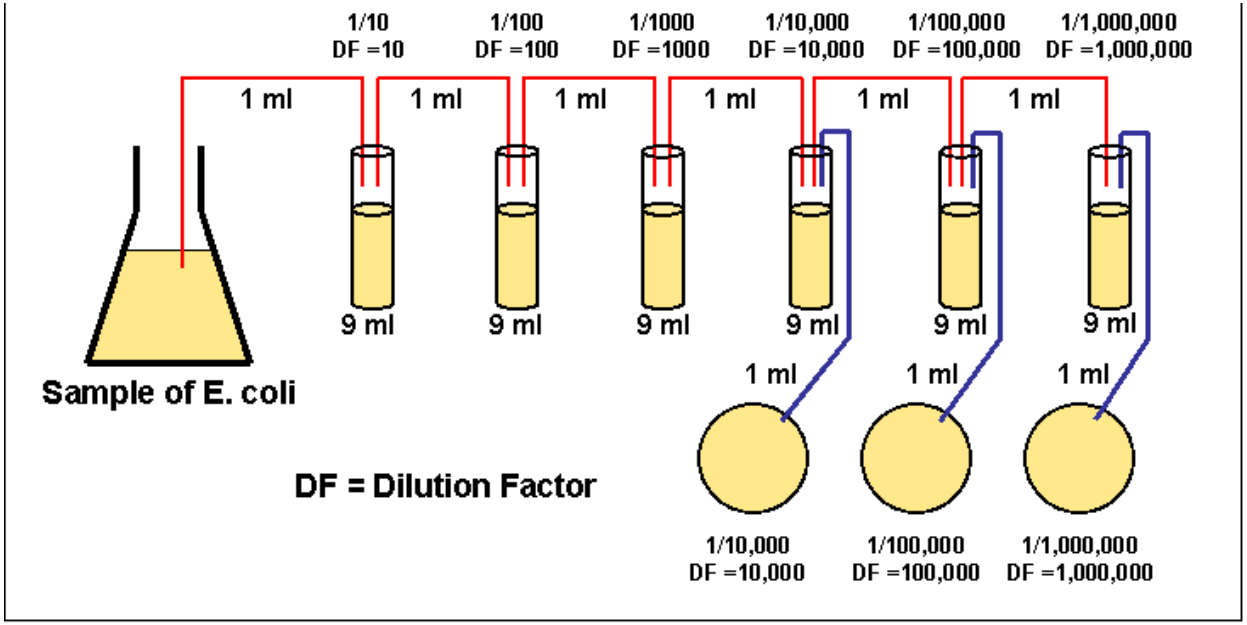
2) Dilute 1 ml of a sample by withdrawing 1 ml of the sample and dispensing this 1 ml into the first dilution tube.

3) Using the same procedure, withdraw 1 ml from the first dilution tube and dispense into the second dilution tube. Subsequently withdraw 1 ml from the second dilution tube and dispense into the third dilution tube. Continue doing this from tube to tube until the dilution is completed.

- **4)** Transfer 1 ml from each of only the last three dilution tubes onto the surface of the corresponding agar plates.

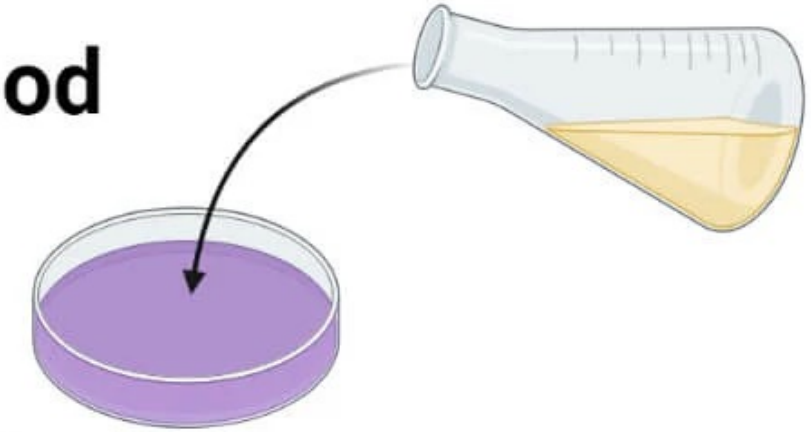
5) Incubate the agar plates at 37°C for 48 hours.

6) Choose a plate that appears to have between 30 and 300 colonies.

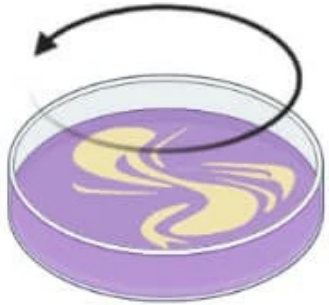


Pour Plate Method

1 Pipette bacterial sample onto petri dish

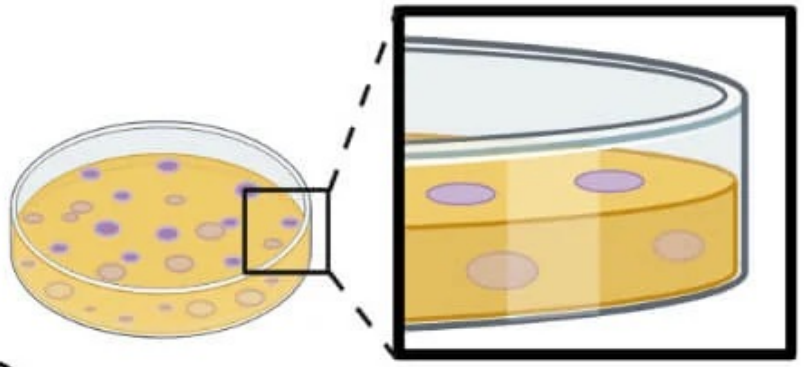


2 Pour liquid nutrient agar



3 Swirl to mix

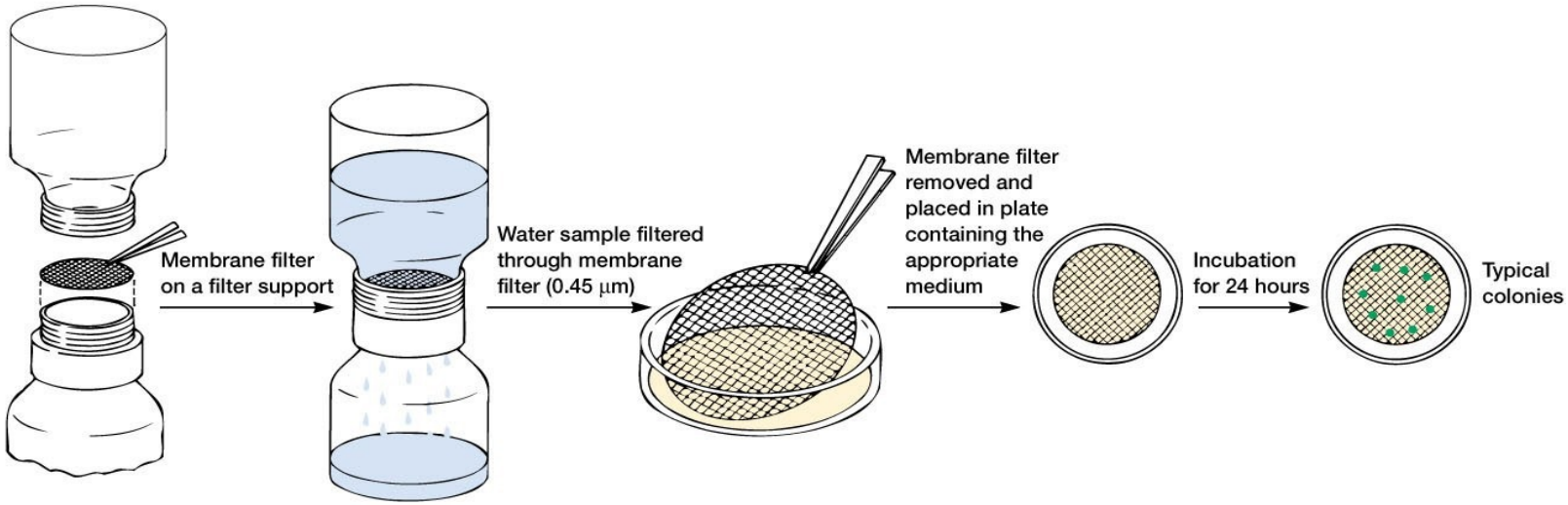
Incubate



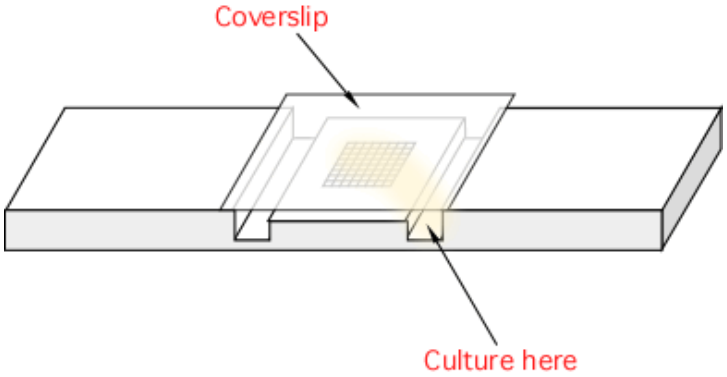
4 Colonies grow on agar surface and subsurface

D) Membrane filters

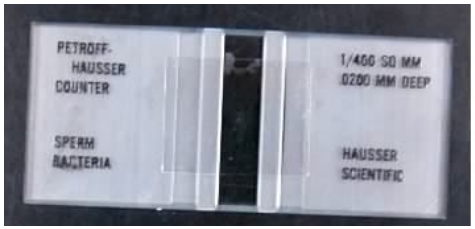
- ✓A diluted suspension of microorganism/Cells is filtered through special membrane that provides dark background for observing cells
- ✓Cells are retained on filter
- ✓The disc is placed in a culture media in a petri plate
- ✓Incubated at ideal growth factors
- ✓Useful for counting bacteria
- ✓With certain dyes, can distinguish living from dead cells

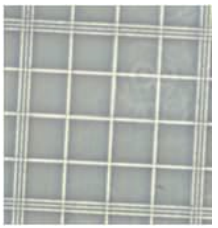


DIRECT MICROSCOPIC CELL COUNT

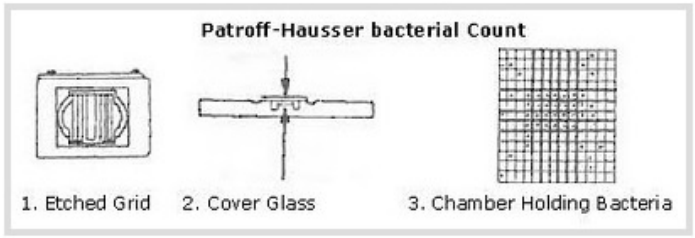


- In the direct microscopic count, a counting chamber with a ruled slide is employed. It is constructed in such a manner that the ruled lines define a known volume. The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.
- The Petroff-Hausser counting chamber for example, has small etched squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep. The volume of one small square therefore is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc). There are 16 small squares in the large double-lined squares that are actually counted, making the volume of a large double-lined square 1/1,250,000 cc. The normal procedure is to count the number of bacteria in five large double-lined squares and divide by five to get the average number of bacteria per large square. This number is then multiplied by 1,250,000 since the square holds a volume of 1/1,250,000 cc, to find the total number of organisms per ml in the original sample.

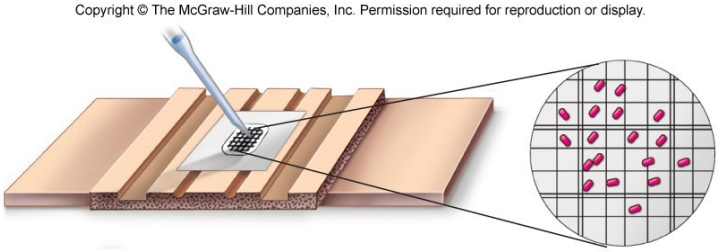




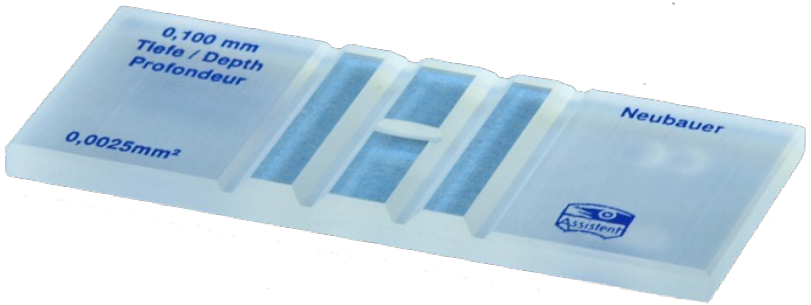
B) Counting chambers/ Petroff-Hausser counting chamber

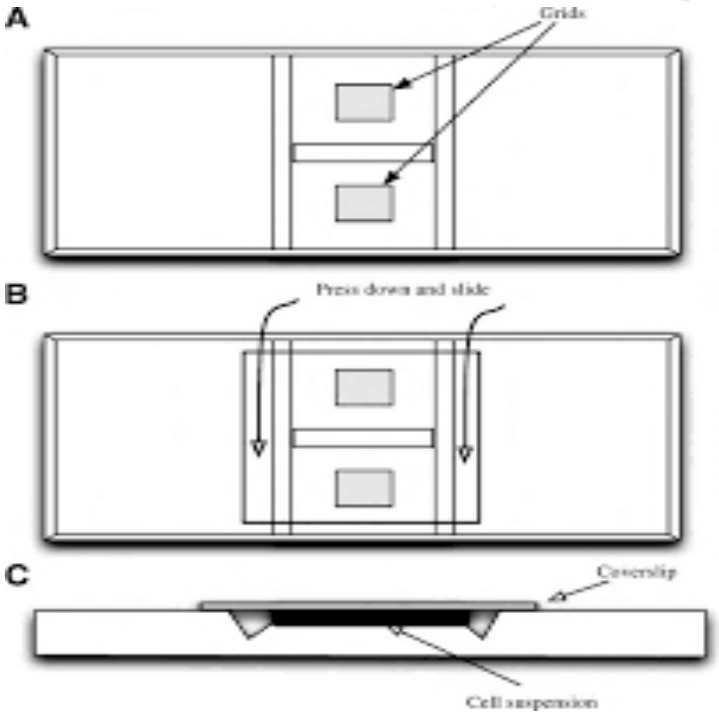


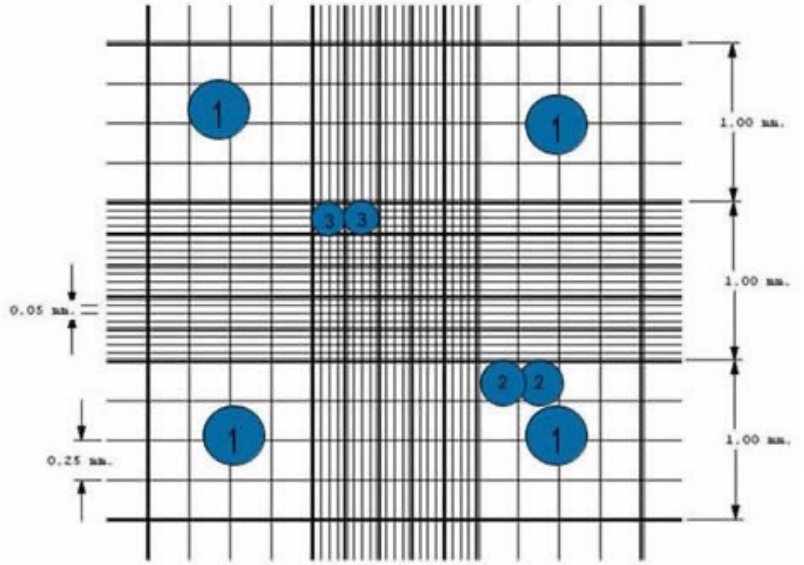
- **Easy, inexpensive, and quick**
- Useful for counting both **Eucaryotes(Haemocytometer) and Procaryotes (Petroff-Hausser counter)**
- More easily counted if they are stained or if phase I contrast of florescence microscope is employed.
- Cannot distinguish living from dead cells
- Very small cells are usually missed.
- Calculated taking the count of number of bacteria per unit area of grid and multiplying it by a conversion factor (depending on chamber volume and sample dilution used).



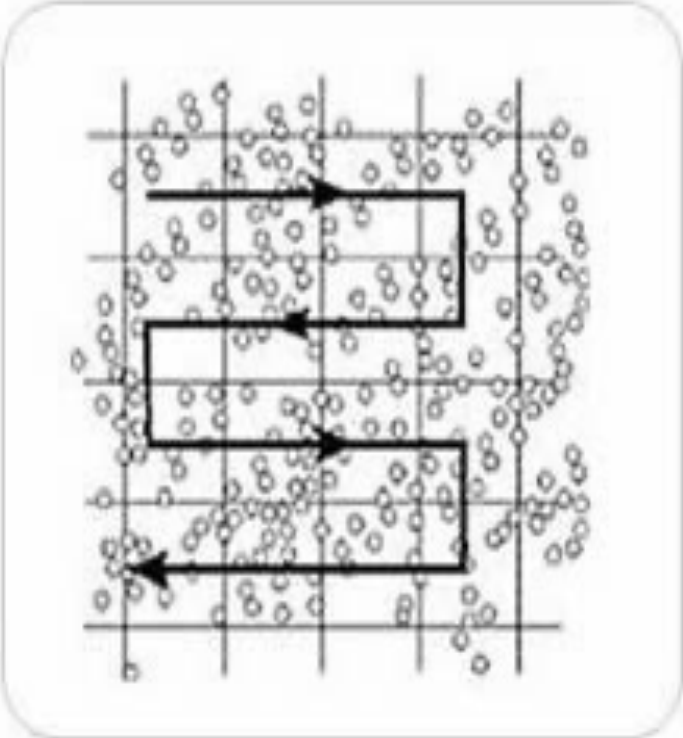
Neubauer's slide/Haemocytometer

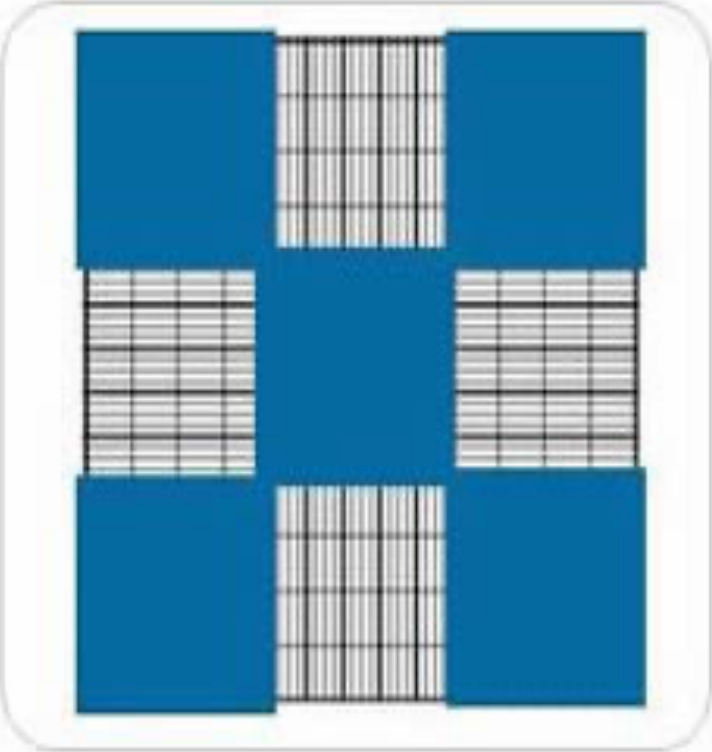




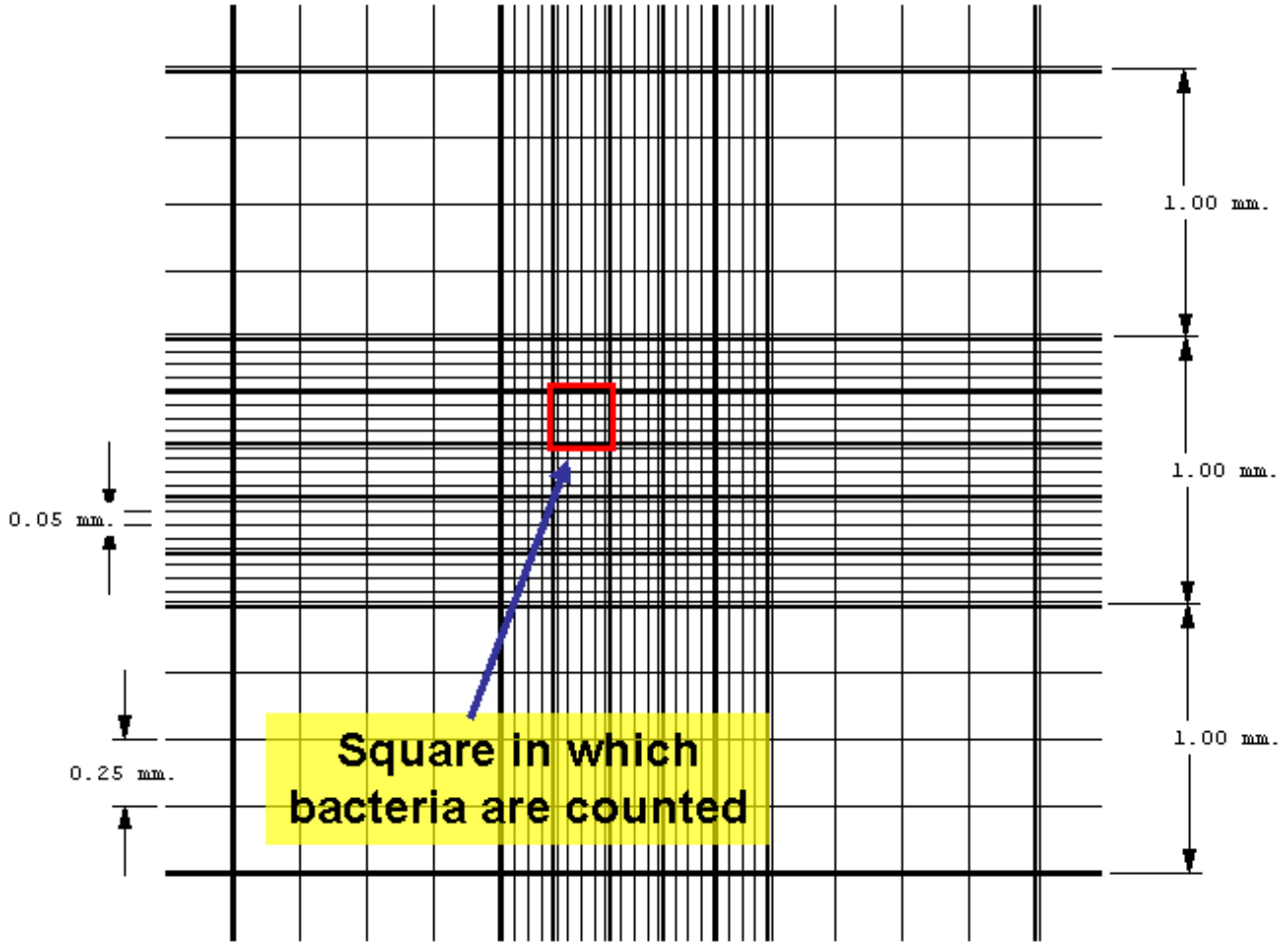


Reading style





Petroff-Hausser counting chamber



PROCEDURE
DIRECT MICROSCOPIC COUNT

- Add 1 ml of the sample into a tube containing 1 ml of the dye methylene blue. This gives a 1/2 dilution of the sample.
  2) Fill the chamber of a Petroff-Hausser counting chamber with this 1/2 dilution.
  3) Place the chamber on a microscope and focus on the squares using 400X.
  4) Count the number of bacteria in one of the large double-lined squares. Count all organisms that are on or within the lines.

- Calculate the number of bacteria per cc (ml) as follows:
- **The number of bacteria per cc (ml)**

=
The number of bacteria per large square
X
The dilution factor of the large square (1,250,000)
X
The dilution factor of any dilutions made prior to placing the sample in the counting chamber, such as mixing it with dye (2 in this case)

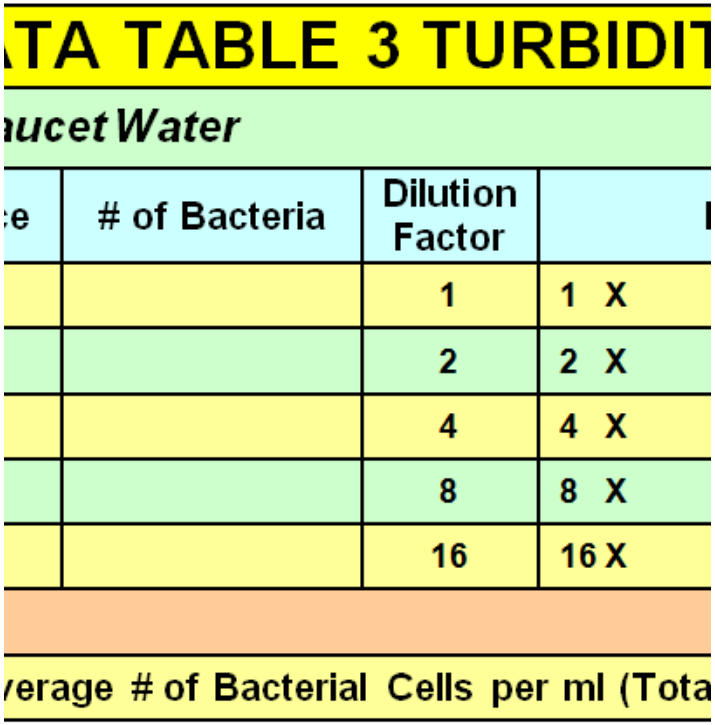
TURBIDITY COUNT

- When you mix the bacteria growing in a liquid medium, the culture appears turbid. This is because a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. By measuring the amount of light absorbed by a bacterial suspension, one can estimate and compare the number of bacteria present. Spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead and alive.

- The instrument used to measure turbidity is a spectrophotometer.

PROCEDURE
TURBIDITY COUNT

- We will be testing only <u>two</u> samples of water for the turbidity enumeration test. One of the samples has been drawn from a drinking water faucet while the other was taken from the local river. You will need **DATA TABLE 3** and a printable version of the **STANDARD CURVE CHART** to enumerate your samples bacteria.

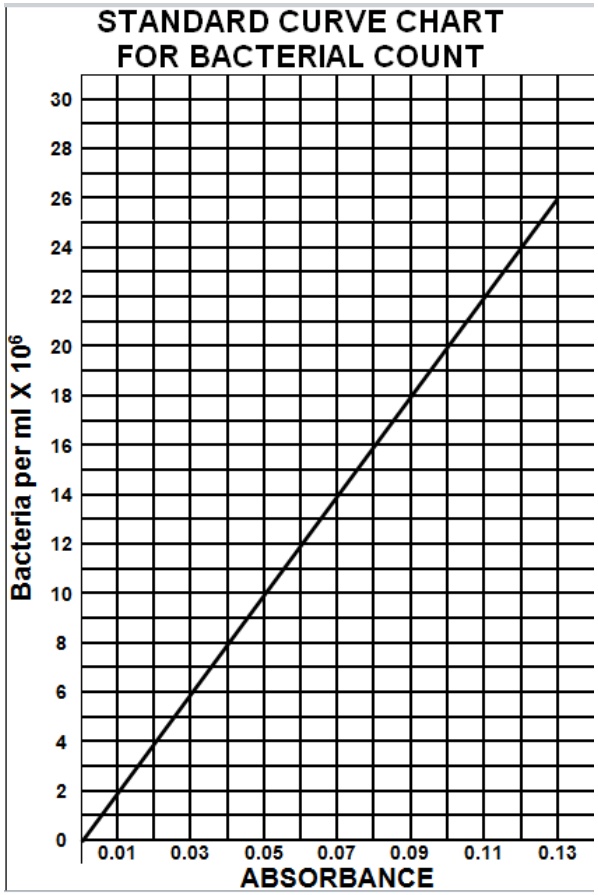


ATA TABLE 3 TURBIDIT

aucet Water

ce	# of Bacteria	Dilution Factor	
		1	1 X
		2	2 X
		4	4 X
		8	8 X
		16	16 X

verage # of Bacterial Cells per ml (Tota

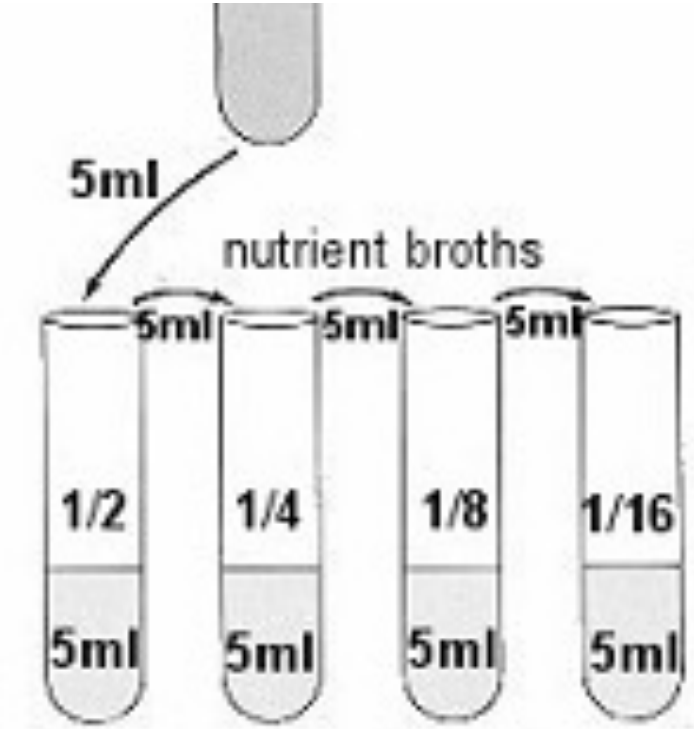

STANDARD CURVE CHART
FOR BACTERIAL COUNT
Bacteria per ml X 10^6
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0.01
0.03
0.05
0.07
0.09
0.11
0.13
ABSORBANCE

PROCEDURE
TURBIDITY COUNT

1) Place the **ORIGINAL** tube of the sample and four tubes of the sterile broth in a test-tube rack. Each tube of broth contains 5 ml of sterile broth.

2) Use four of these tubes (tubes 2 to 5) of broth to make four serial dilutions of the culture.

3) Transfer 5ml of the **ORIGINAL** sample to the first broth tube. Transfer 5ml from that tube to the next tube, and so on until the last of the four tubes has 5ml added to it. These tubes will be 1/2, 1/4, 1/8, and 1/16 dilutions.



- **4)** Set the display mode on the Spectrophotometer to **ABSORBANCE** by pressing the **MODE** control key until the appropriate red LED is lit.
- **5)** Set the wavelength to 520 nm by using the **WAVELENGTH** dial.
- **6)** Standardize the spectrophotometer by using a **BLANK**. The **BLANK** used to standardize the machine is sterile nutrient broth: it is called the **BLANK** because it has a sample concentration equal to zero (# of bacteria = 0).
- **7)** Place the original bacterial specimen into the spectrophotometer.
- **8)** Next insert the 1/2 dilution and read it. Repeat this with the 1/4, 1/8, and 1/16 dilutions. Read to the nearest thousandth (0.001) on the absorbance digital display.

- **9)** Record your values in **TABLE 3** for each of the individual samples, along with the dilutions that they came from.
- **10)** Using the standard curve table given below, calculate the number of bacteria per milliliter for each dilution.

DATA TABLE 3 TURBIDITY COUNT				
SAMPLE NAME: ***Faucet Water***				
Dilutions	**Absorbance**	**# of Bacteria**	**Dilution Factor**	**Dilution factor X Bacteria #**
Original			1	1 X =
1/2			2	2 X =
1/4			4	4 X =
1/8			8	8 X =
1/16			16	16 X =
				Total =
Average # of Bacterial Cells per ml (Total / 5) =				

- **Review the example of absorbance counts acquired and the determinations of # of bacteria for the dilutions using the **STANDARD CURVE CHART** given below. Be sure to keep track of all of the zeros in your calculations of the subsequent calculations for average bacteria per ml.

EXAMPLE

DATA TABLE 3
TURBIDITY COUNT

SAMPLE NAME: EXAMPLE

Dilutions	Absorbance	# of Bacteria	Dilution Factor	Dilution factor X Bacteria #
Original	0.130	26,000,000	1	1 X 26,000,000 = 26,000,000
1/2	0.066	12,900,000	2	2 X 12,900,000 = 25,800,000
1/4	0.034	6,500,000	4	4 X 6,500,000 = 26,000,000
1/8	0.018	3,200,000	8	8 X 3,200,000 = 25,600,000
1/16	0.010	1,750,000	16	16 X 1,750,000 = 28,000,000
Total =				**131,400,000**

Average # of Bacterial Cells per ml (Total / 5) = 26,306,280 bacteria per ml

C) Electronic counters

- Electronic instrument
- As Coulter counter
- Microbial suspension forced through small hole or orifice or capillary tube
- The diameter of this tube is so microscopic that allows only one cell to pass at a time.
- Can count thousands of cells in a few seconds.
- Movement of microbe through orifice/capillary tube impacts electric current that flows through orifice
- **Cannot distinguish living from dead cells**
- **Counts even dust particles.**
- **Suspension must be free of any foreign particles.**
- **Not for prokaryotic cells**
- Quick and easy to use
- Useful for large quantity
- Microorganisms and blood cells.