Bacterial Growth

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MHOW

Bacterial Growth

- Bacteria divide by binary fission
- Most pathogens grow on artificial culture media
- Some pathogens have never been grown in culture
- Others only grow intracelluarly in tissue culture
- Generation time is determined by: available nutrients; temperature; and pH

MECHANISMS OF BACTERIAL GROWTH

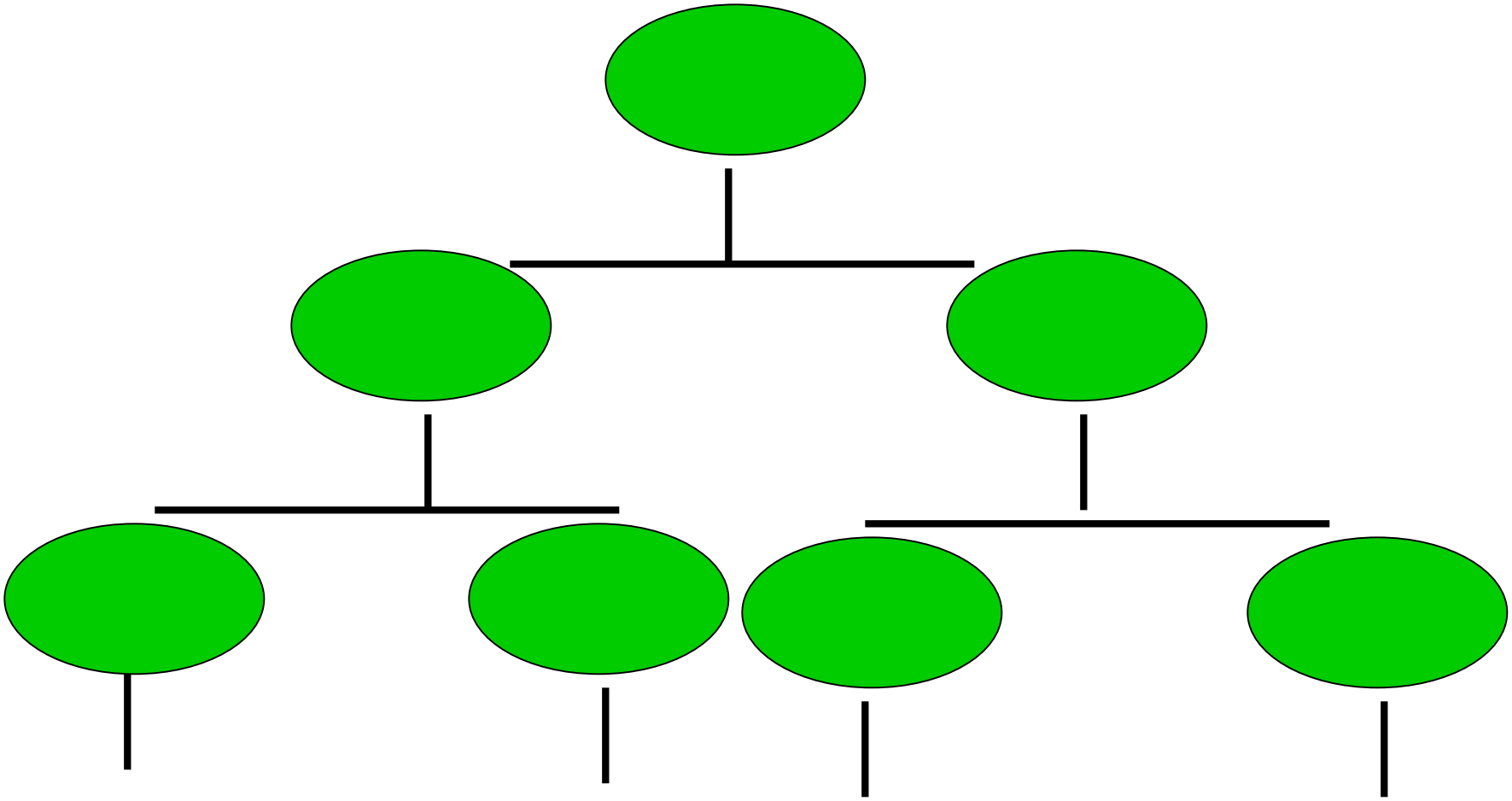
- **Binary fission**
- **Budding**
- **Fragmentation**
- **Formation of sporangiospores & condiospores**

BINARY FISSION

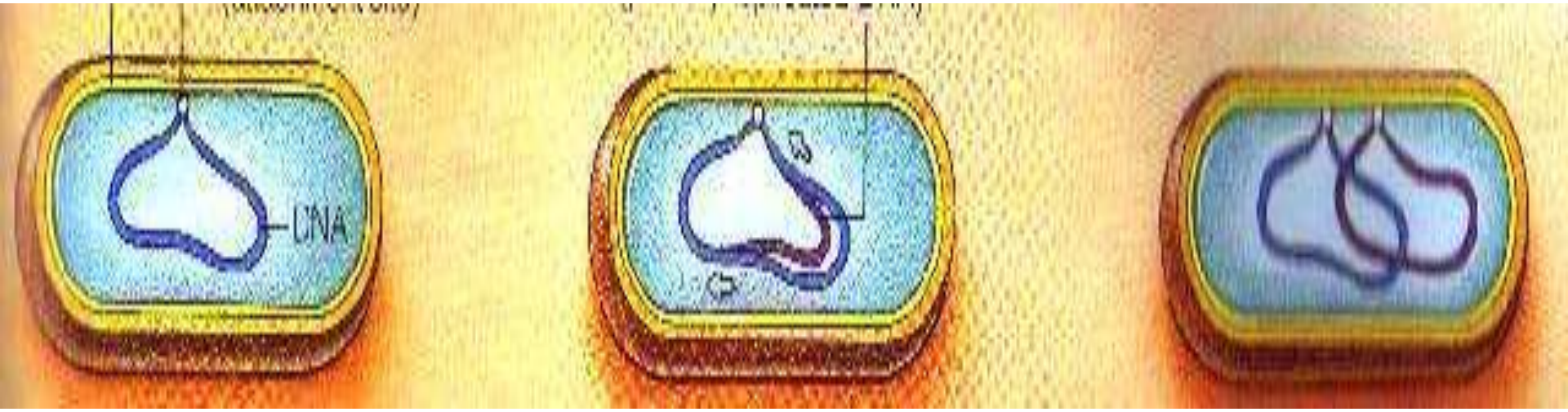
Binary fission

- The normal reproductive method of bacteria is transverse binary fission in which a single cell divides into two identical cells after developing a cross wall (transverse) septum.
- It is an asexual reproductive process.
- Thus, bacteria increase their numbers by geometric progression or exponential growth, i.e. doubling of bacterial population every generation as : 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations).

Bacteria Replicate by Transverse Binary Fission



and so on….



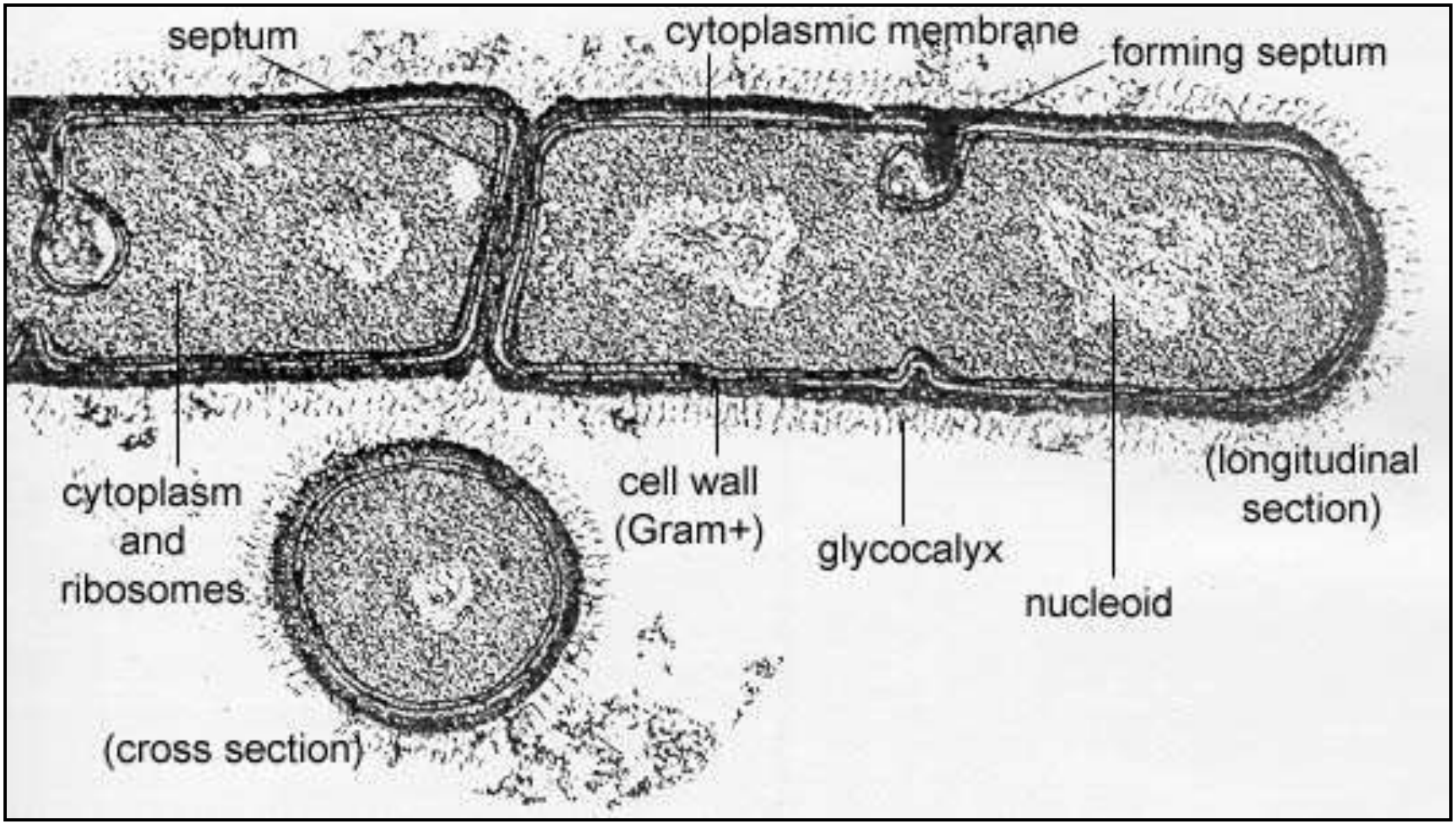
First the chromosomal DNA makes a copy

Then DNA replicates

NEXT THE CYTOPLASM AND CELL DIVIDES BY FORMATION OF TRANSVERSE WALL SEPTUM

The two resulting cells are exactly the same

Binary fission



- **In the center of bacterium, a group of proteins called Fts proteins form a ring at the cell division plane called as divisome.**

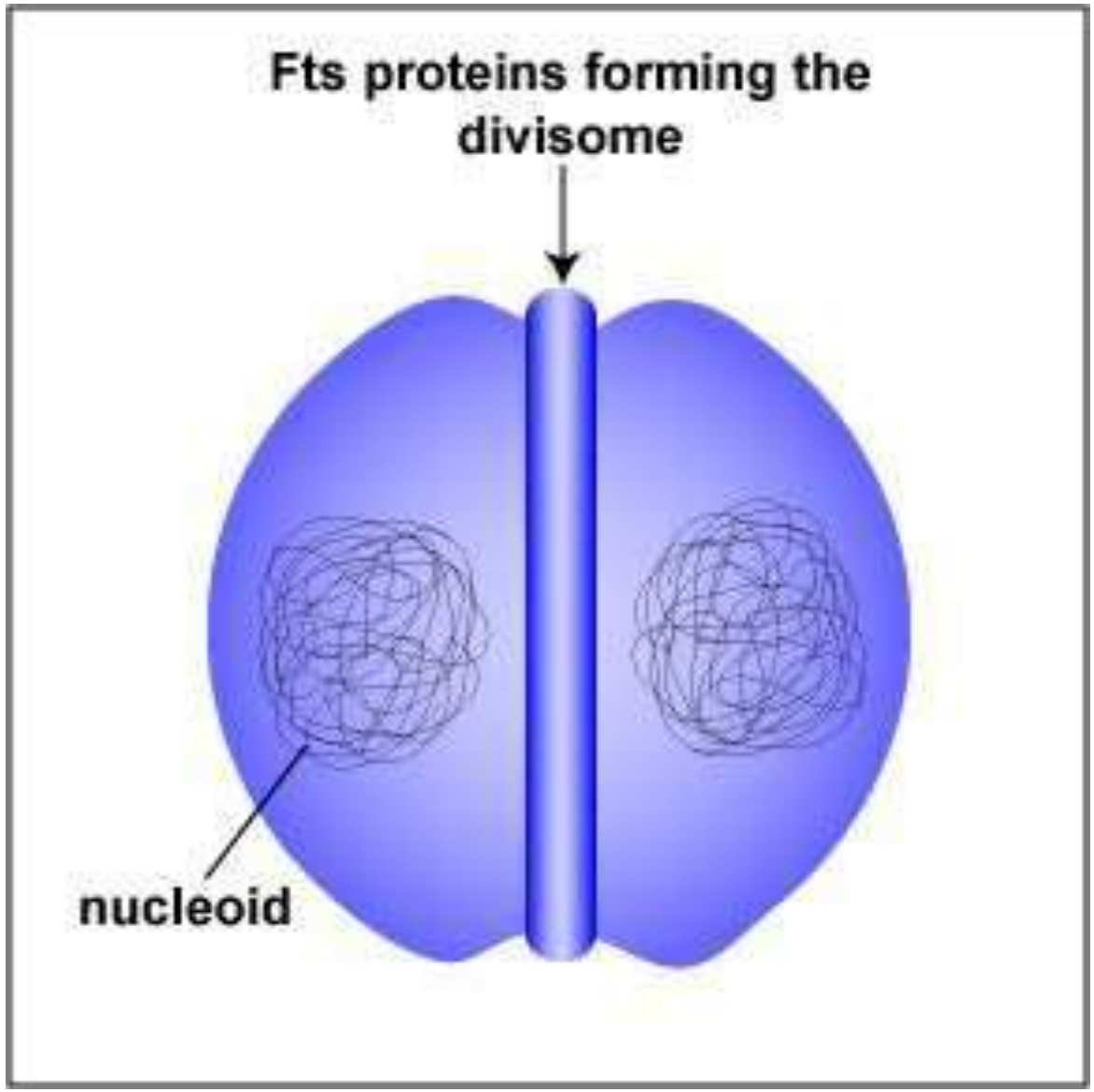
- **During DNA replication, each strand of the replicating bacterial DNA attaches to divisome.**

- **The bacterial cell membrane coordinates the process.**

- **The two daughter DNA molecules remain attached at divisome, side-by-side, while new membrane and cell wall is synthesized as a transverse septum in between the two newly formed chromosomes**

- **When septum formation is complete the cell splits into two progeny cells.**

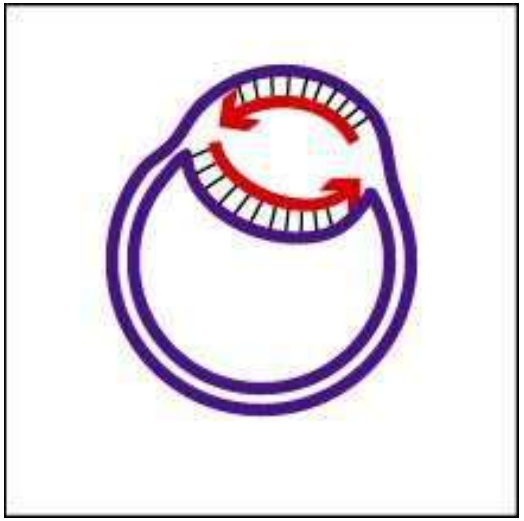
Binary fission



REPLICATION OF D.N.A.

DNA replication in bacteria

- DNA is replicated by uncoiling of the helix, strand separation by breaking of the hydrogen bonds between the complementary strands, and synthesis of two new daughter strands by complementary base pairing.
- Replication begins at a specific site in the DNA called the origin of replication.
- DNA replication is bi-directional from the origin of replication

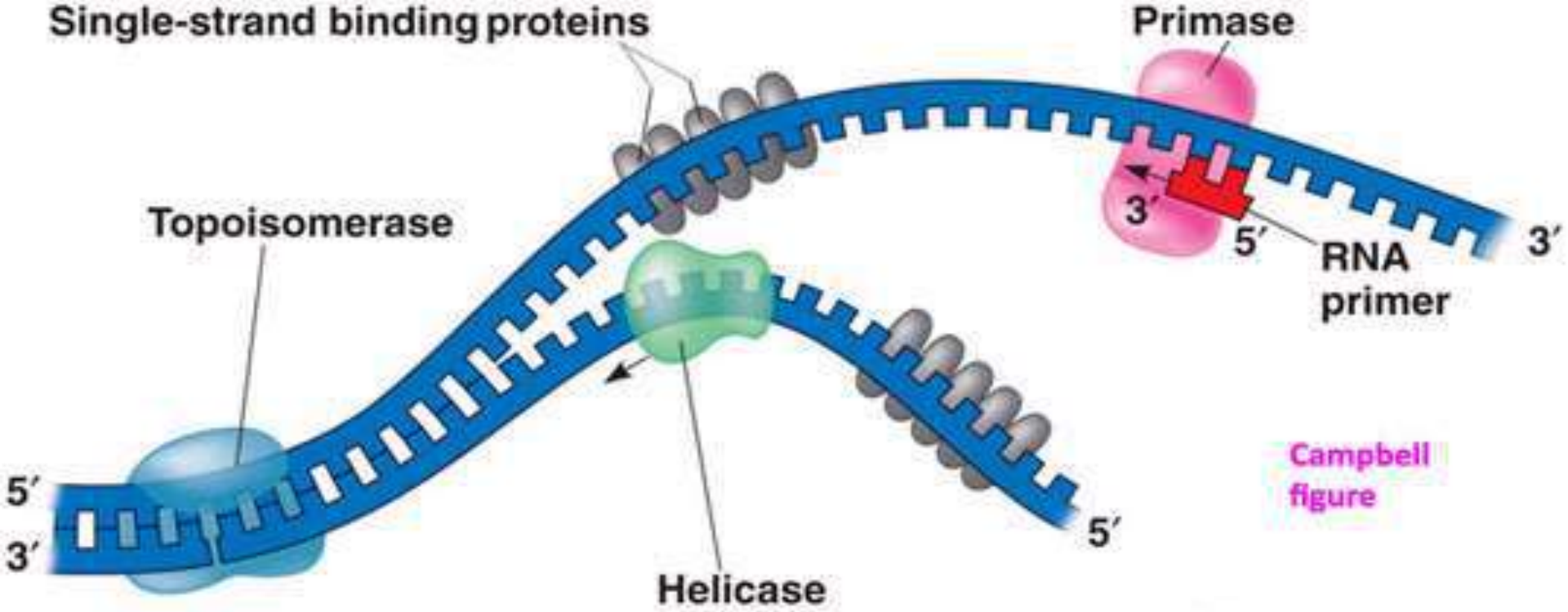


Enzymes involved in bacterial DNA replication

Several enzymes and proteins are involved in the replication of bacterial DNA, which form a Replication Complex (Replisome).

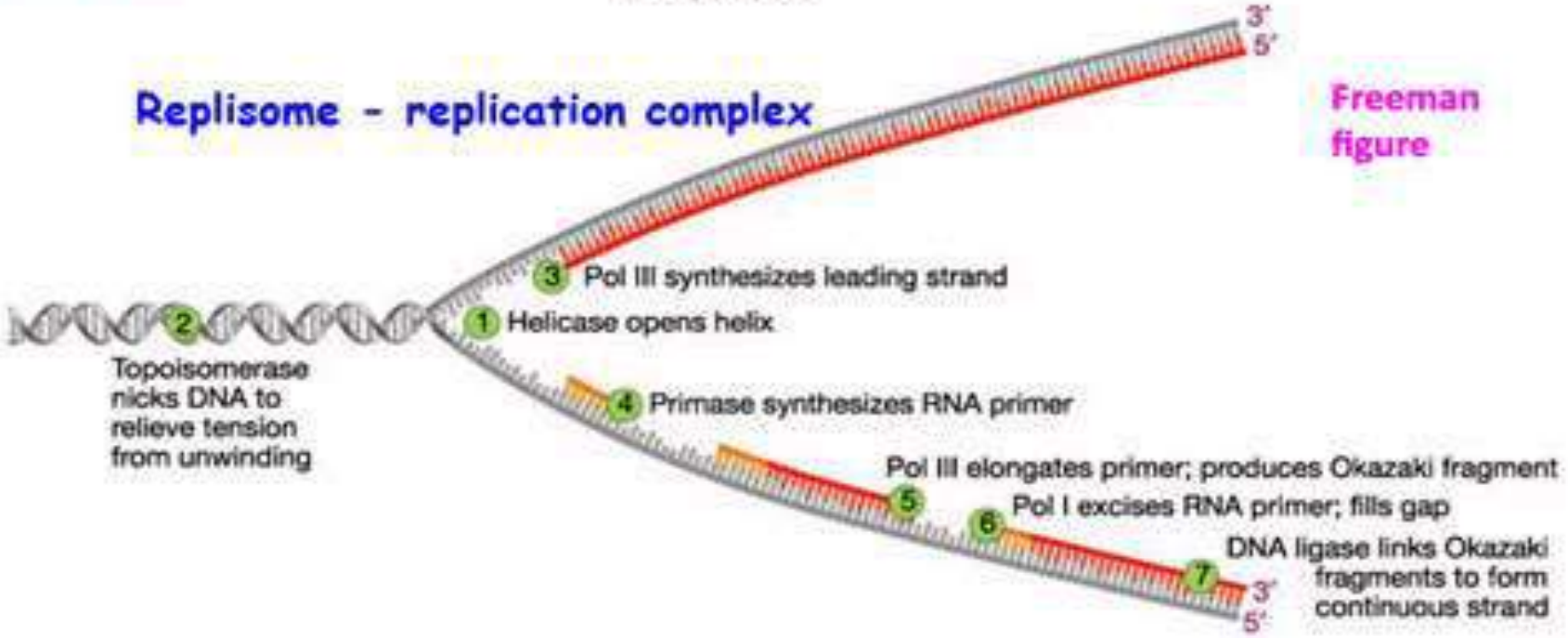
- **Helicase – unwind DNA at *Ori C* to form a Y-shaped replication fork or replication bubble**
- **Topoisomerase [DNA gyrase] - removes supercoils (Fluoroquinolnes inhibit topoisomerases)**
- **Single strand binding proteins - stabilize replication fork**
- **Primase - makes RNA primer**
- **POL III - synthesizes new DNA strands: reads 3' to 5' and synthesizes in 5' to 3' direction; proof reading**

  (Sulphonamides, inhibit the synthesis of new nucleotide bases)
- **DNA polymerase I - removes RNA primer (1 base at a time) and adds DNA bases**
- **Ligase - repairs Okazaki fragments (seals lagging strand 3' open holes)**

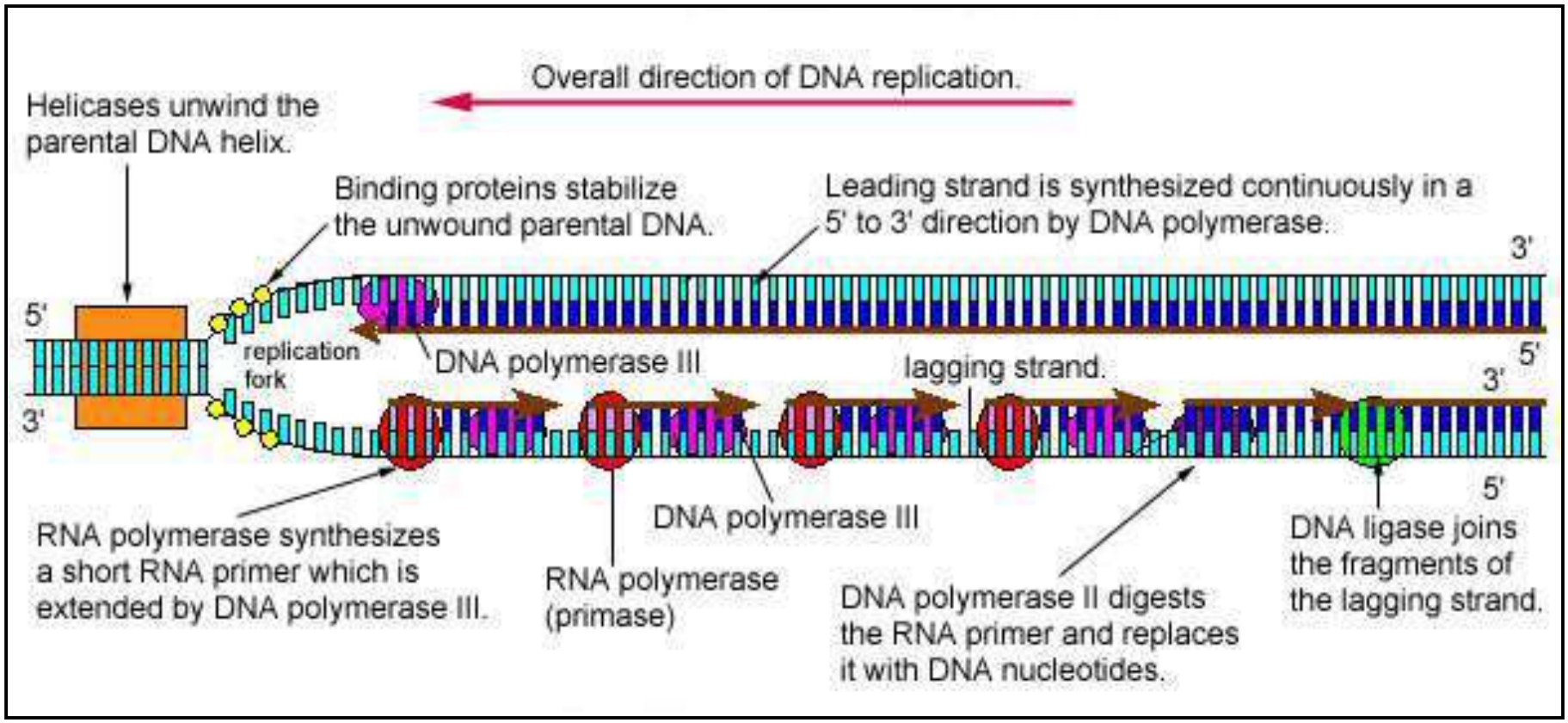


Campbell figure

Replisome - replication complex



Freeman figure


Overall direction of DNA replication.
Helicases unwind the parental DNA helix.
Binding proteins stabilize the unwound parental DNA.
Leading strand is synthesized continuously in a 5' to 3' direction by DNA polymerase.
3'
5'
replication fork
DNA polymerase III
lagging strand.
3'
5'
3'
5'
RNA polymerase synthesizes a short RNA primer which is extended by DNA polymerase III.
RNA polymerase (primase)
DNA polymerase III
DNA polymerase II digests the RNA primer and replaces it with DNA nucleotides.
DNA ligase joins the fragments of the lagging strand.

Replication of DNA – rolling circle mechanism

Another mechanism of DNA synthesis is Rolling Circle, which occurs in plasmids. One strand remains closed while an enzyme nicks the other strand. The nicked strand rolls off the intact circle and synthesizes a complimentary strand. Simultaneously the intact circle revolves 360° and acts as a template for a new complimentary strand.

Other Mechanisms of Bacterial Growth

Budding

Some bacteria reproduce by budding, a process in which a small protuberance (bud) develops at one end of the cell, enlarges and develops into a new cell that later separates from the parent cell, e.g. *Rhodopseudomonas acidophila*

Fragmentation

Bacteria that produces extensive filamentous growth reproduce by fragmentation of the filament into small cells, e.g. *Nocardia*,

Formation of sporangiospores and Condiospores

Some species of *Streptomyces* produce many spores per organism by developing cross wall at the hyphal tip and each spore give rise to a new organism.

KINETICS OF BACTERIAL GROWTH

GROWTH RATE AND GENERATION TIME

- The time required for a bacterial cell to divide, i.e. a population to double, during log-phase, is known as the generation time. For a population, it is often called "Mean Generation Time"
- Under a given set of growth conditions (medium, temperature, pH, etc.) each bacterial species has a genetically determined generation time.
- Typically, generation times range from about 12 minutes to 24 hours. Most bacteria of medical interest have generation times of 15 min. to an hour or so.
- The reciprocal of the Generation Time (1/G) is called the "Growth Rate Constant" (k, [generations/unit time]).

The generation time is given by the formula:

G = t/n

where,
G = generation time
t = time interval in hours or minutes
n = number of generations

Generation time of some bacteria

1. ***Escherichia coli*** **- 17min.**
2. ***Bacillus megaterium*** **– 25min**
3. ***Staphylococcus aureus*** **- 27-30 min**
4. ***Mycobacterium tuberculosis*** **- 792-932 min**

Calculation of Generation Time

The relationship between the number of bacteria in a population at a given time (N_t), the original number of bacterial cells in the population (N_0), and the number of divisions those bacteria have undergone during that time (n) can be expressed by the following equation:

$$N_t = N_0 \times 2^n$$

Solving for 'n': $\log N_t = \log N_0 + n\log 2$

or $n\log 2 = \log N_t - \log N_0$

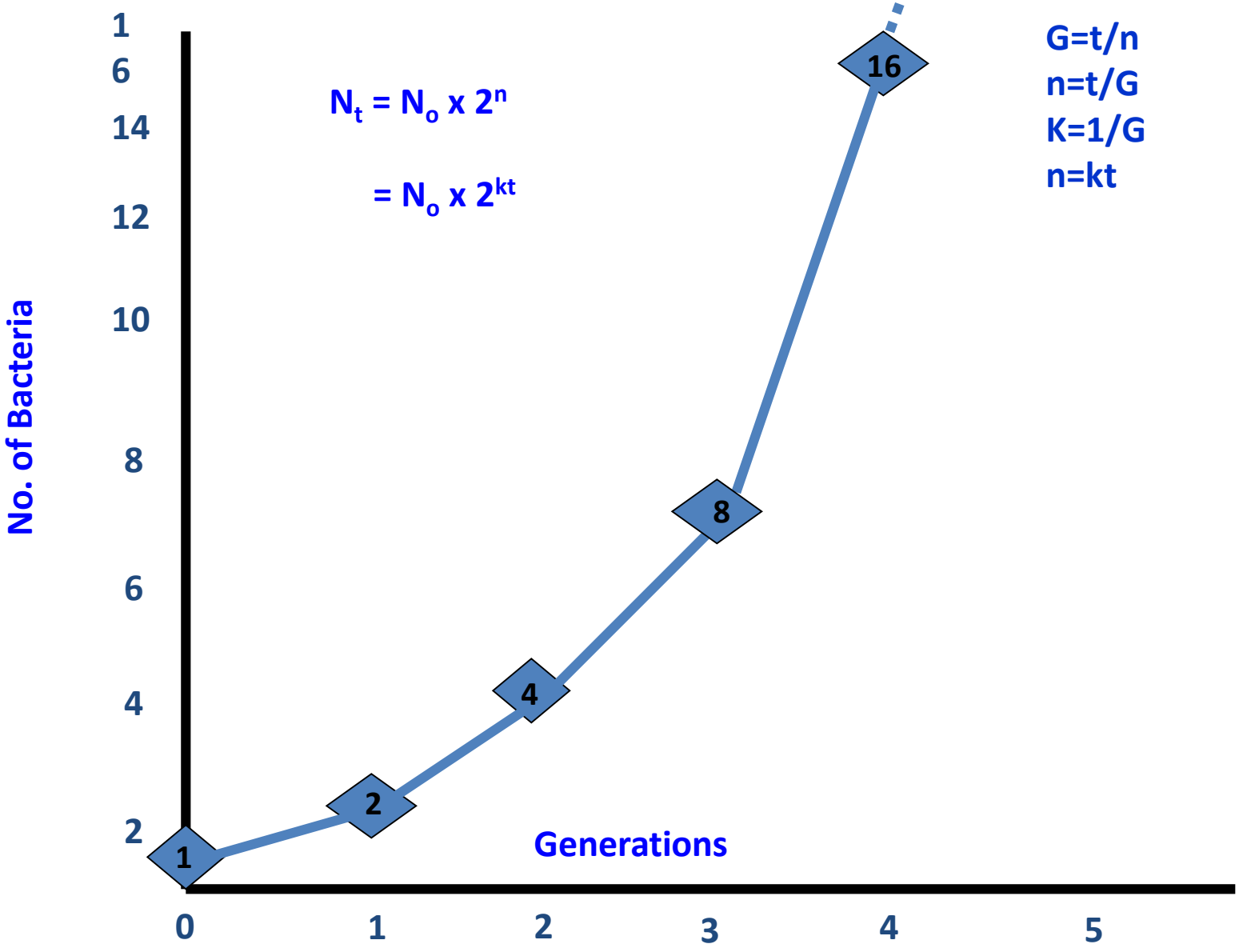
or $n = \log (N_t - N_0) / \log 2 \ (=0.301)$

or $n = 3.3 \log N_t / N_0$

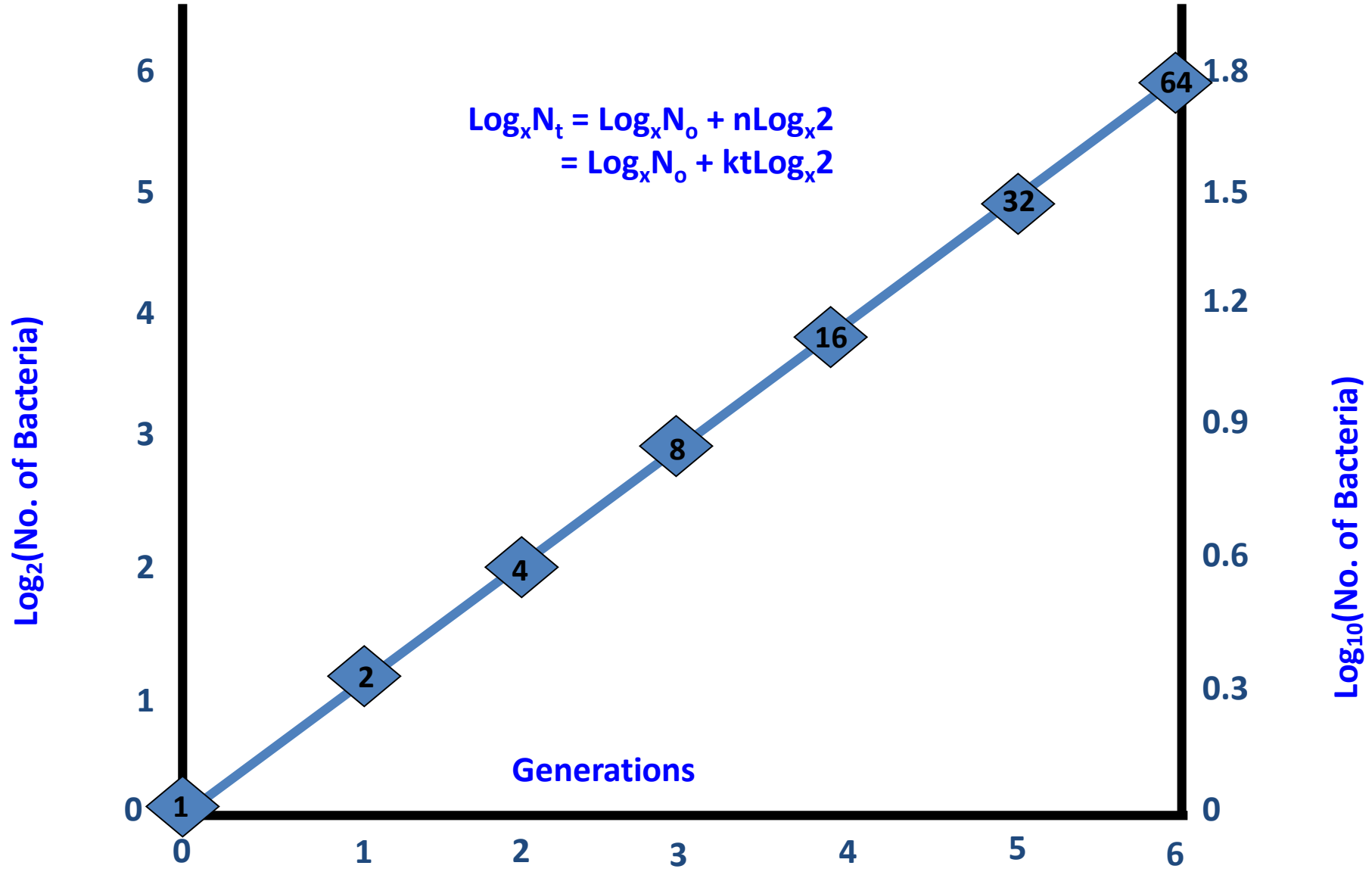
Since $G = t/n$

Therefore $G = t / 3.3 \log N_t / N_0$

Bacterial Growth Curves (Linear Plot)



Bacterial Growth Curves (Logarithmic Plot)

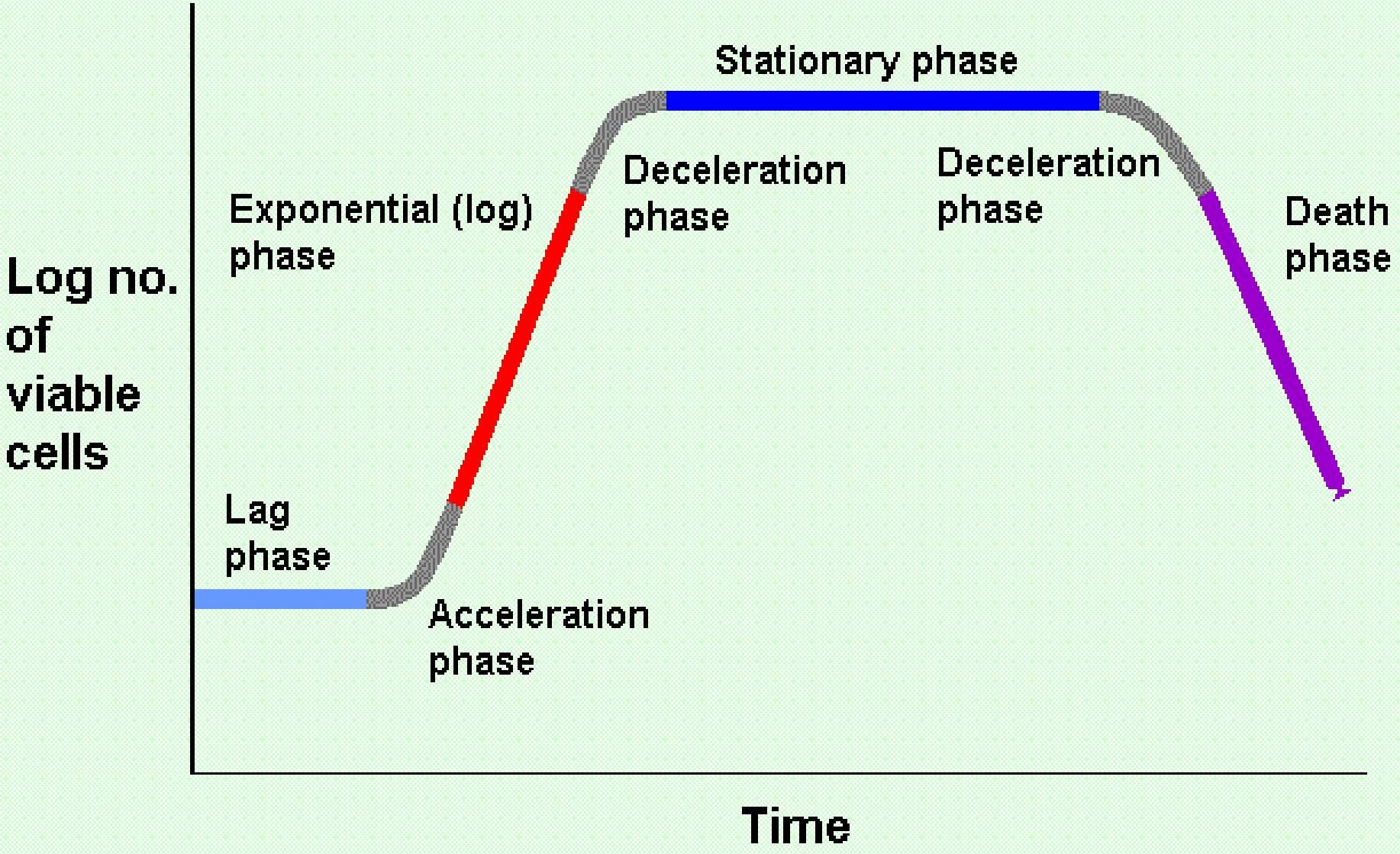


THE BACTERIAL GROWTH CURVE

- **When one measures the growth of a bacterial culture in a closed system such as a flask or a fermenter and plots the logarithm of cell number over time then one obtains a characteristic curve, which is called a bacterial growth curve.**
- **The growth curve consists of four distinct phases, with a transition period in between each phase:**

1. **Lag phase**
2. **Log (logarithmic or exponential) phase**
3. **Stationary phase (in nature, this is where organisms 'spend' most of their time).**
4. **Decline (death) phase**

Bacterial growth curve



What's Happening At Each Step?

- **Lag Phase: Cells are adjusting to new medium and environment – no increase in cell number.**
- **Exponential (Log) Phase: Cells are growing, replicating at the growth rate characteristic for the strain in given conditions. Generation time can be experimentally determined by counting cell numbers at various points along the curve.**
- **Stationary Phase: Environment becoming hostile (depleted nutrients, build-up of waste products, etc.) Cells "replacing" but viable count not increasing. Spores would form at this stage.**
- **Death Phase: Cells dies off exponentially to some low (but non-zero) number.**

The Lag Phase

- Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged; this is referred to as lag phase.
- This lag in division is associated with a physiological adaptation of the cells to the new environment prior to the resumption of division, i.e. bacteria are “acclimatizing” with their surroundings.
- Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and high in metabolic activity.
- The length of the lag phase is apparently dependent on a wide variety of factors such as the size of the inoculum; time necessary to recover from physical damage in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes.
- 1 hour to several days

The Log Phase
(logarithmic phase, exponential phase)

- Lag phase is followed by log phase during which binary fission occurs and the bacteria multiply at the fastest rate possible under the conditions provided (population doubles every generation).
- This phase of growth is called logarithmic or exponential because the rate of increase in cell number is by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations).
- On plotting on a semi-log graph paper, i.e., log cell number versus time, log phase results in a straight line.
- During log phase, the bacterial division is at a constant rate (generation time), but varies with species, temperature and media.
- Microbes are most sensitive to adverse conditions and antibiotics .

The Stationary Phase

- **Stationary phase is a steady-state equilibrium where the rate of cell growth (division) is exactly balanced by the rate of cell death, i.e. death rate = rate of reproduction.**
- **viable count remains same; total cell count increase**
- **Cell death occurs because of one of three factors: (a) exhaustion of available nutrients and water; (b) accumulation of catabolic end products (c) exhaustion of space – "biological space", and (d) change in oxygen concentration and pH.**
- **The stationary phase is not necessarily a period of quiescence. Bacteria produce secondary metabolites, such as antibiotics and the sporulation process starts.**

The Decline Phase
(death phase)

- **Stationary phase is followed by a die-off of cells during which the number of deaths exceeds the number of new cells formed, i.e. Death rate > rate of reproduction.**
- **Cell death in bacteria cultures basically means that the cells are unable to resume division following their transfer to new environments.**
- **During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.**
- **Deaths are due to the factors in stationary phase in addition to lytic enzymes that are released when bacteria lyse.**

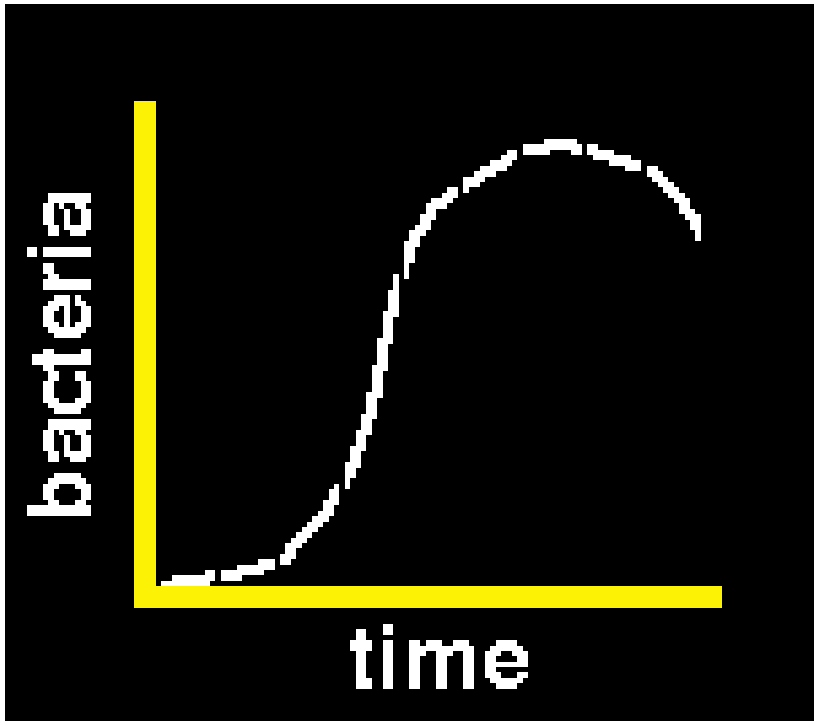
GROWING BACTERIA

BACTERIAL GROWTH IN LIQUID MEDIUM

- **Microorganisms can be cultivated either in liquid or solid growth media.**
- **Liquid media cultivation is done either in small Universals (up to 20 ml), in Erlenmeyer flasks (up to 1 l) and fermenters (from 1 l onwards up to excess of 100000 l).**
- **Methods of liquid cultivation:**
    1. **Batch cultivation**
    2. **Continuous cultivation**

Batch Cultivation

Most cultivations on a laboratory scale are carried out using batch culture techniques. This employs a closed system such as a flask or a fermenter in which neither the nutrient supply is replenished nor spent medium is removed and, therefore, one records a typical growth curve.

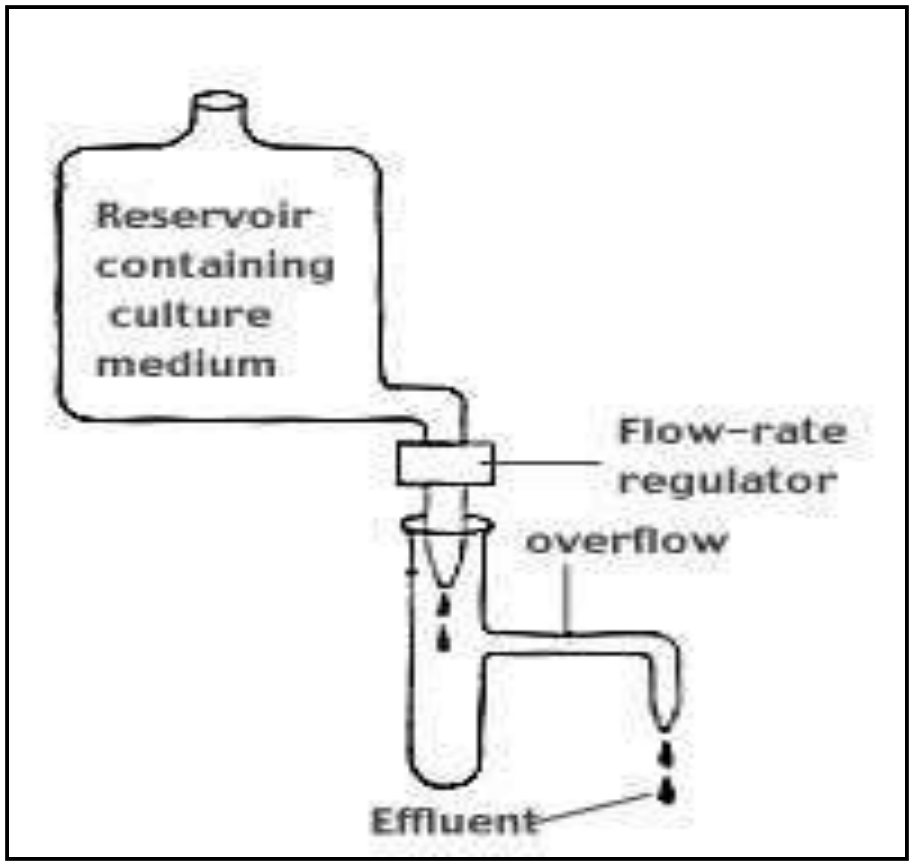


CONTINUOUS CULTURE

- Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of **continuous culture.**
- Continuous culture is carried out in a vessel known as **chemostat.**
- Chemostat is designed to **relieve the conditions that stop exponential growth** in batch cultures. The microbial cells inside are in always maintained in exponential phase and divide at a constant growth rate.
- In this system fresh nutrients are continuously fed into the chemostat and spent broth/biomass is removed at volumetrically equivalent levels; therefore the **culture volume of the vessel remains constant.**

- **Chemostat once set up can be operated for weeks or even months. The microbial cells inside are in exponential phase and divide at a constant growth rate.**

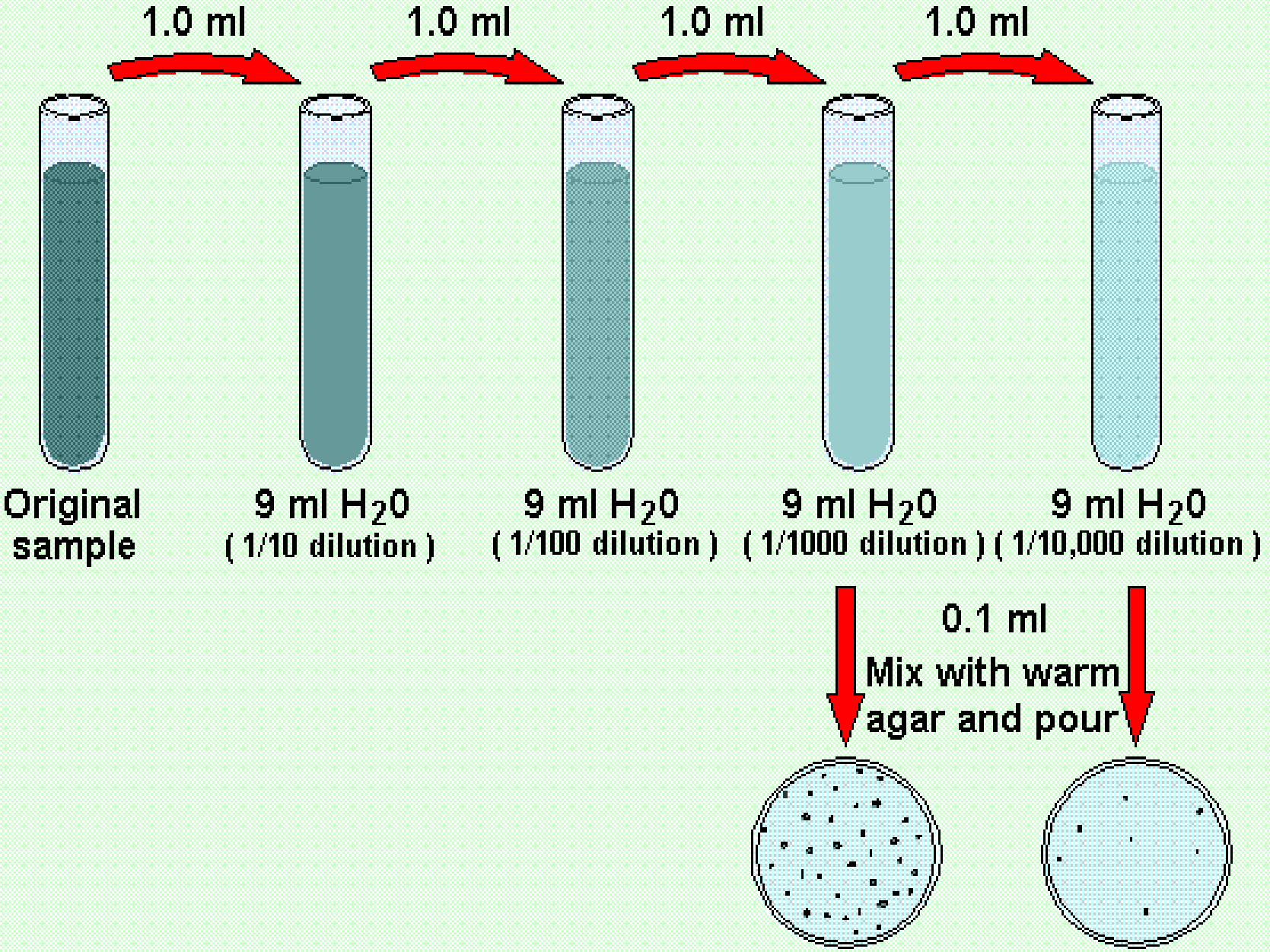
- **Continuous cultivation are used for the production of SCP.**



BACTERIAL GROWTH ON SOLID MEDIUM

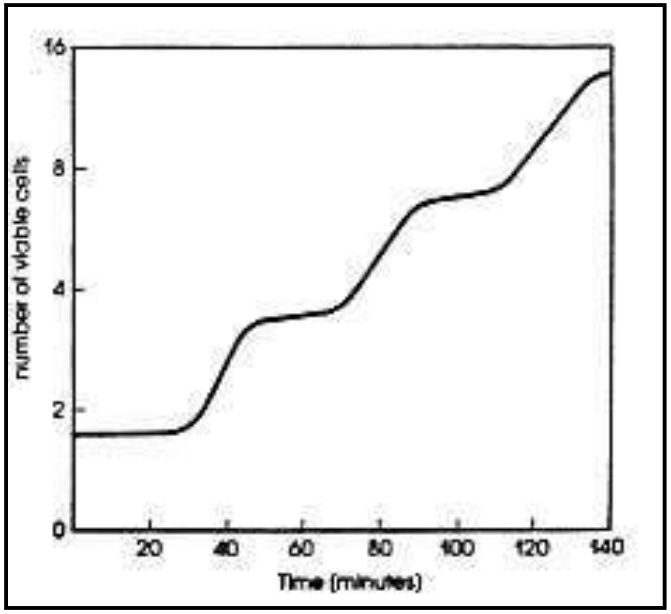
- Growth on solid medium is usually obtained by cultivating microorganisms on agar slopes, plates, or Roux flasks.
- Large scale growth on solid medium is very difficult to achieve for which bacteria are essentially grown in liquid culture.
- Cultivation of cultures on solid support has two main advantages:
  - Cultures can be easily checked for the presence of contamination (purity of culture)
  - A viable cell count can be carried out using spread plate or pour plate techniques .
- The latter approach is done by serial dilution of culture followed by inoculation on agar plates and is based on the assumption that each individual cell gives rise to a single colony.

Serial dilution



SYNCHRONOUS GROWTH OF BACTERIA

- **Synchronized cultures are composed of cells all of which are at the same stage of the bacterial growth cycle.**
- **Information about the growth behavior of individual bacteria can be obtained by the study of synchronous cultures.**
- **A number of techniques have been devised to obtain bacterial populations at the same stage in the cell cycle.**



DOWNSTREAM PROCESSING

- **This expression refers to the techniques, which need to be carried out after growth.**
- **At this stage the biomass has to be separated from the culture medium for further processing.**
- **This is achieved by various techniques such as centrifugation (high g-forces will pellet cells towards the bottom of a container), filtration or flocculation of the cells.**

MEASUREMENT OF BACTERIAL GROWTH

How Growth Is Detected qualitatively

- **Growth can be detected visually on solid medium (agar) by the appearance of colonies – colonial morphology after an appropriate period of incubation.**

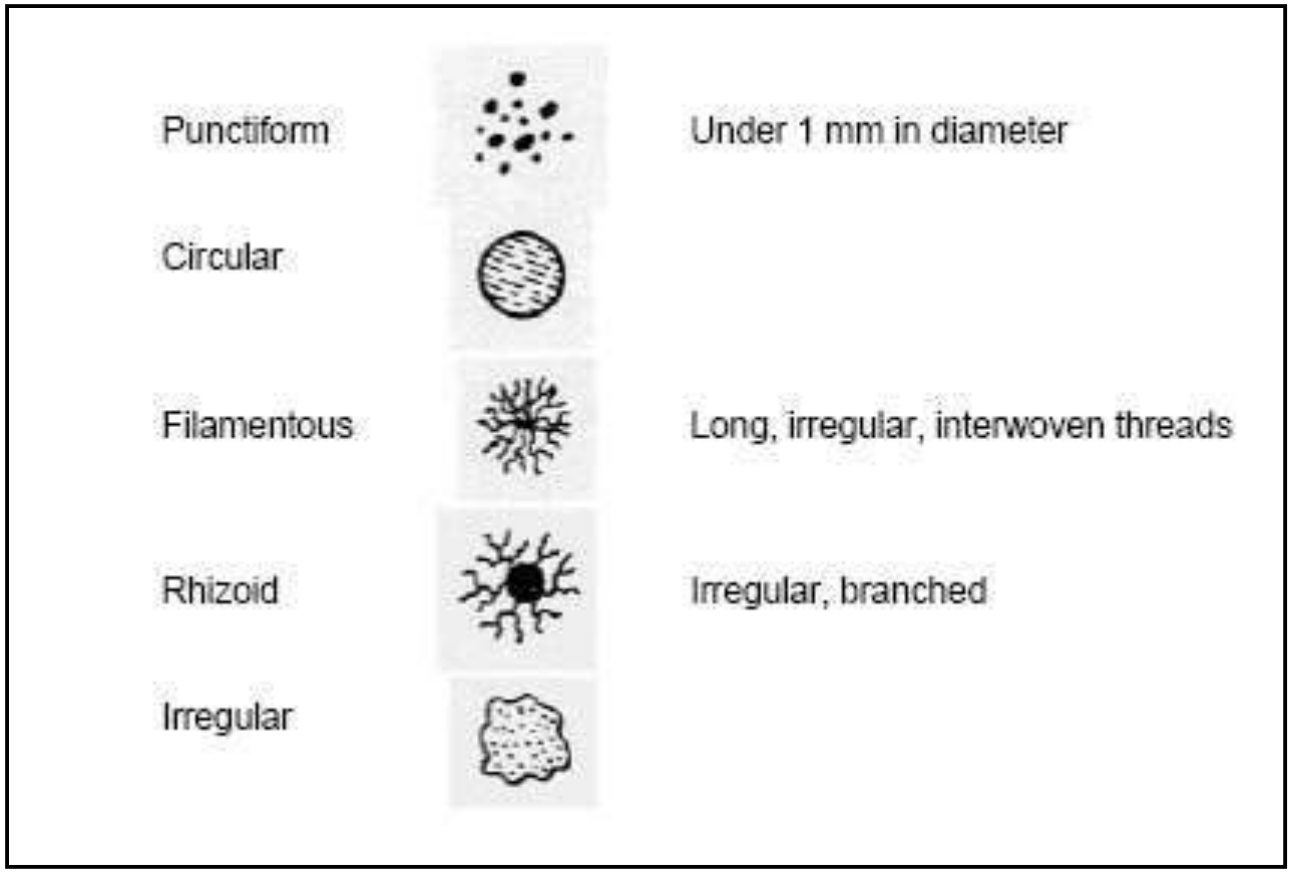
- **Growth can be detected visually in liquid medium (broth) by the appearance of turbidity (cloudiness) of the solution after an appropriate period of incubation.**

"Just barely visible" turbidity implies a bacterial density of approximately 10^6 - 10^7 (= 1 to 10 million) cells/ml.

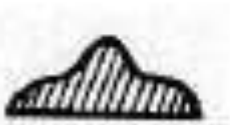
COLONIAL MORPHOLOGY

Single colonies of microorganisms on agar plates can be described using the following terms

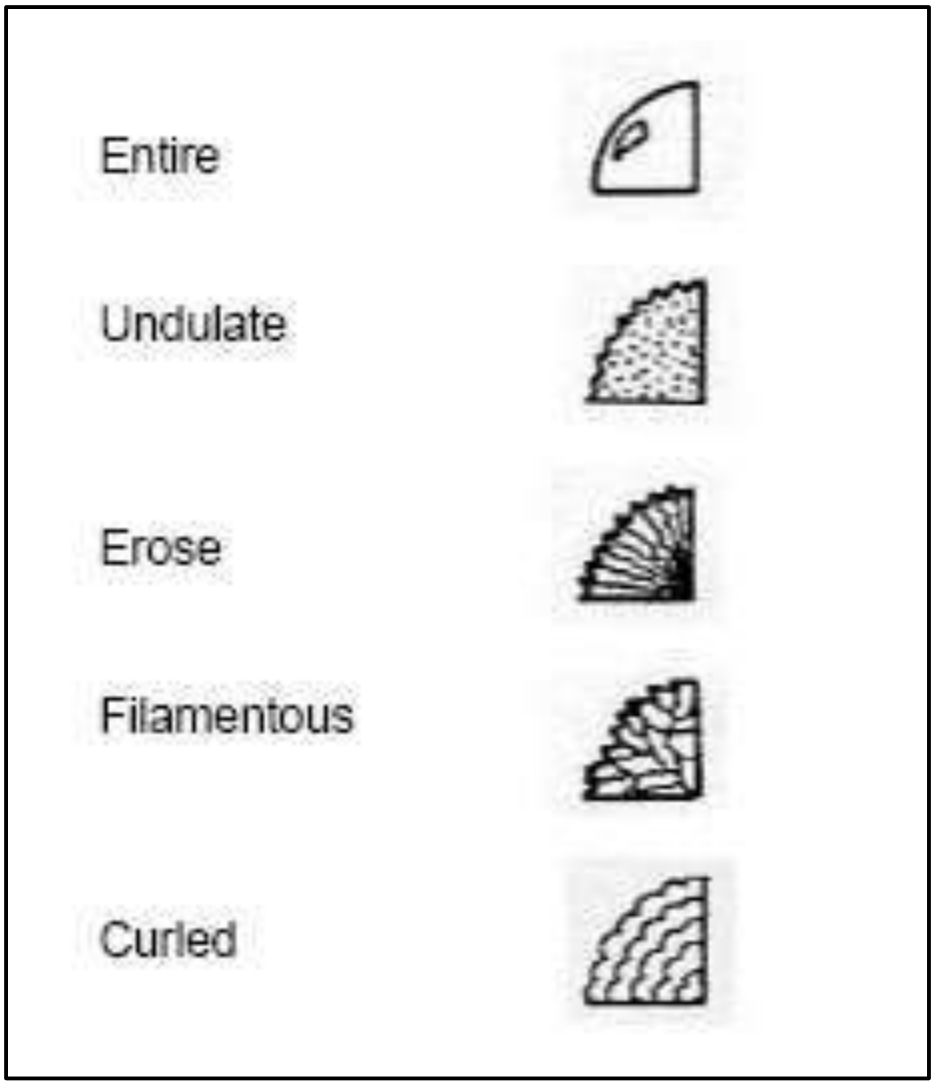
FORM OF COLONY



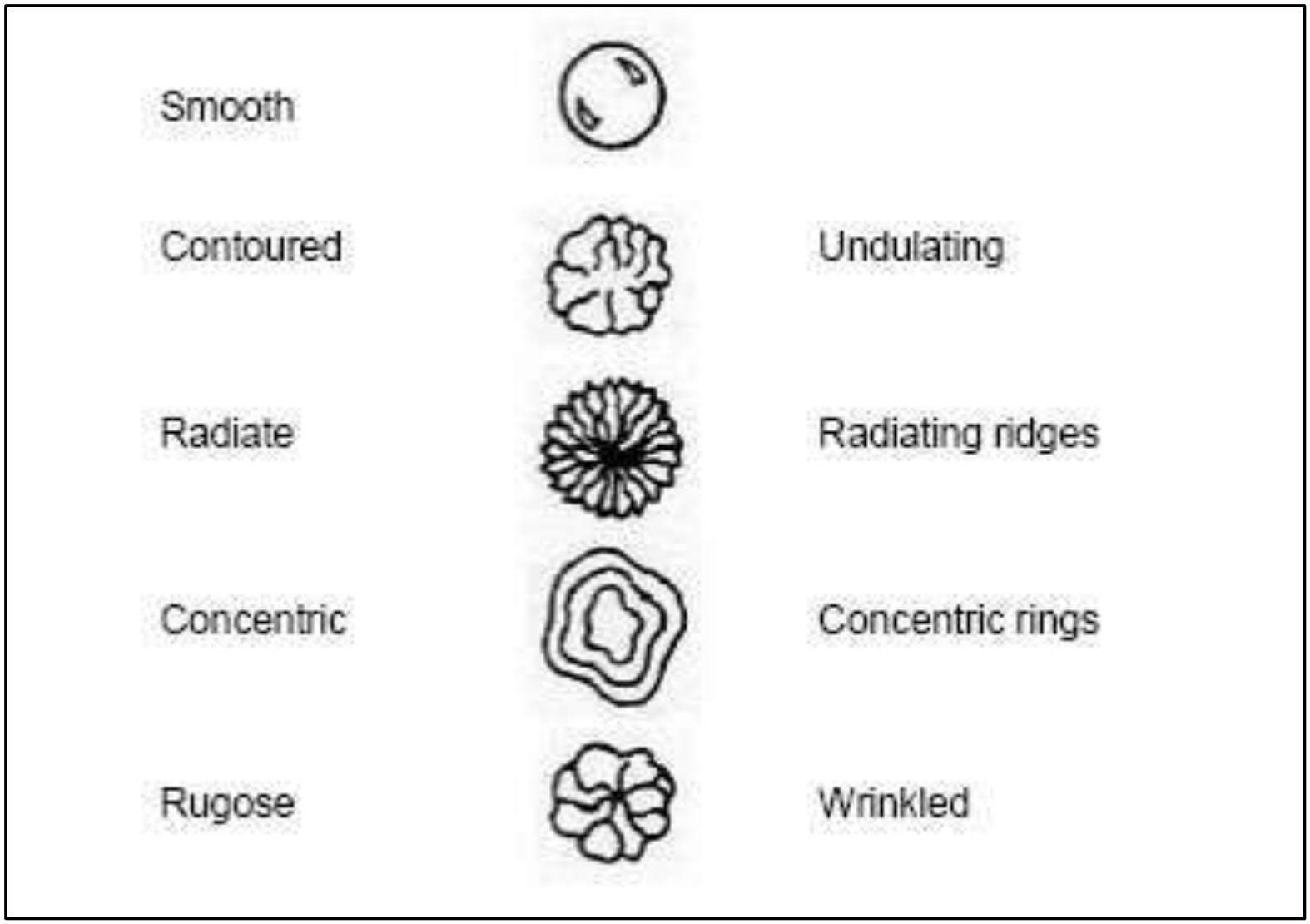
ELEVATION OF COLONY

Effuse		Very thin, spreading
Flat		
Raised		
Convex		
Umbonate		

MARGIN (EDGE) OF COLONY



SURFACE OF COLONY



PIGMENTATION

Water Insoluble and Water-Soluble Pigment in Chromogenic Bacteria

A. Water-insoluble pigment

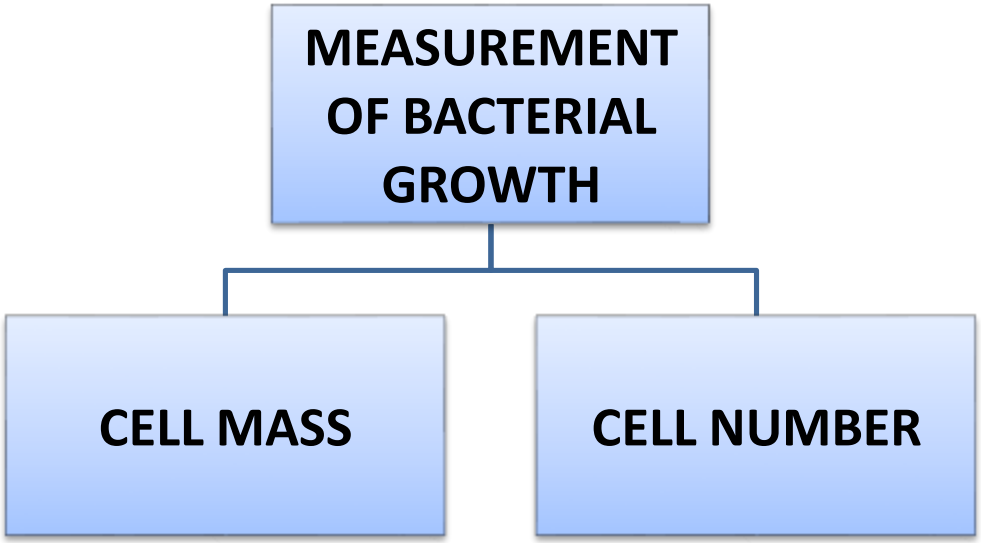
B. Water-soluble pigment

OPTICAL CHARACTERISTICS

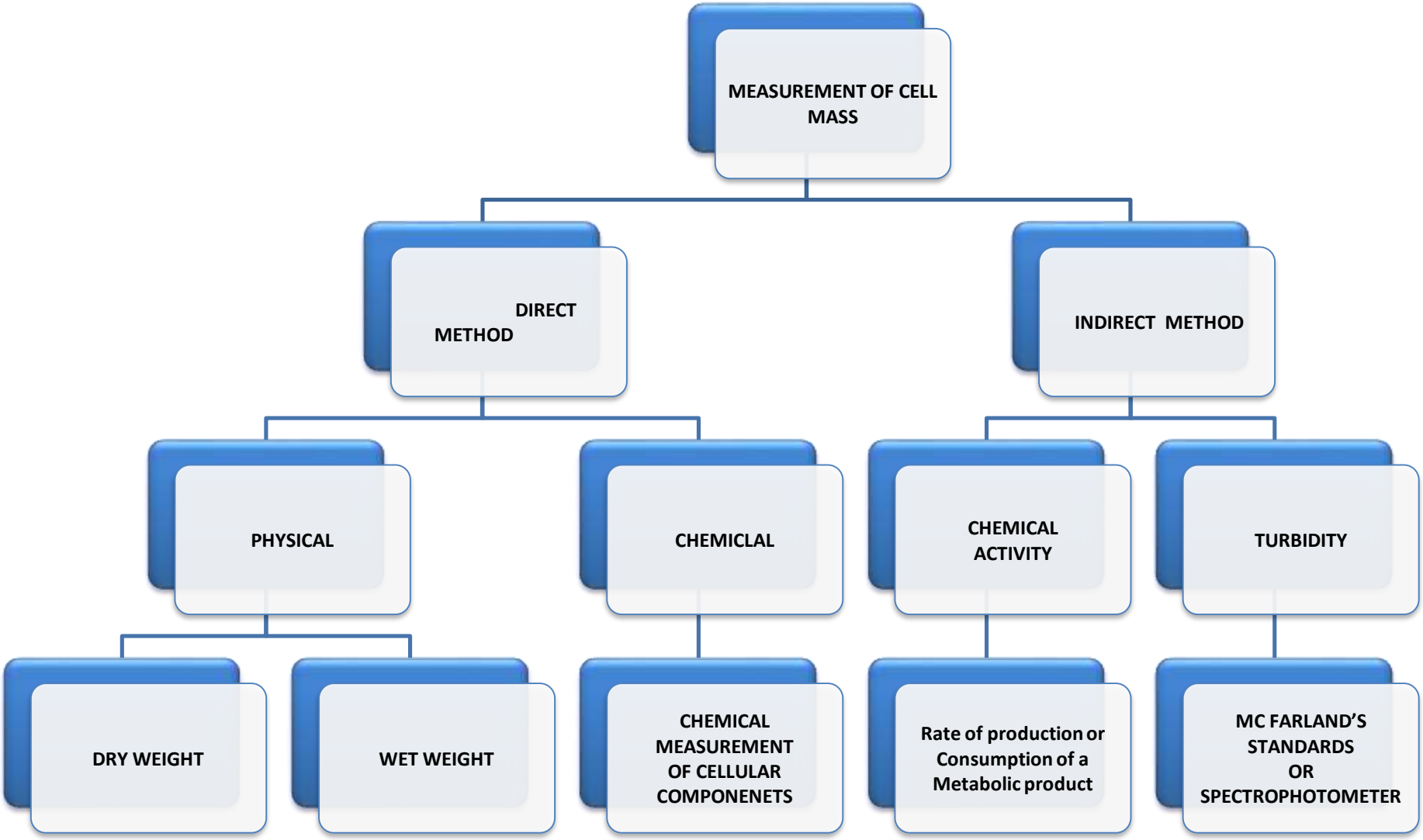
Opaque, translucent, dull, mucoid, etc.

How Growth Is Measured Quantitatively

For unicellular organisms such as the bacteria, growth can be measured in terms of two different parameters: changes in **cell mass** and changes in **cell numbers**



MEASUREMENT OF CELL MASS


MEASUREMENT OF CELL MASS
DIRECT METHOD
INDIRECT METHOD
PHYSICAL
CHEMICLAL
CHEMICAL ACTIVITY
TURBIDITY
DRY WEIGHT
WET WEIGHT
CHEMICAL MEASUREMENT OF CELLULAR COMPONENETS
Rate of production or Consumption of a Metabolic product
MC FARLAND'S STANDARDS OR SPECTROPHOTOMETER

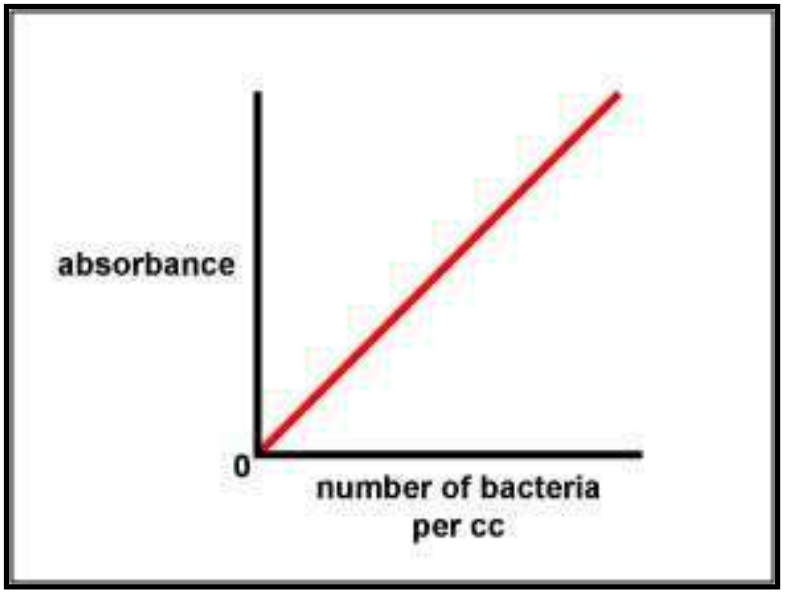
Direct methods

1. Direct physical measurement of dry weight, wet weight, or volume of cells after centrifugation.
2. Direct chemical measurement of some chemical component of the cells such as total N, total protein, or total DNA content.

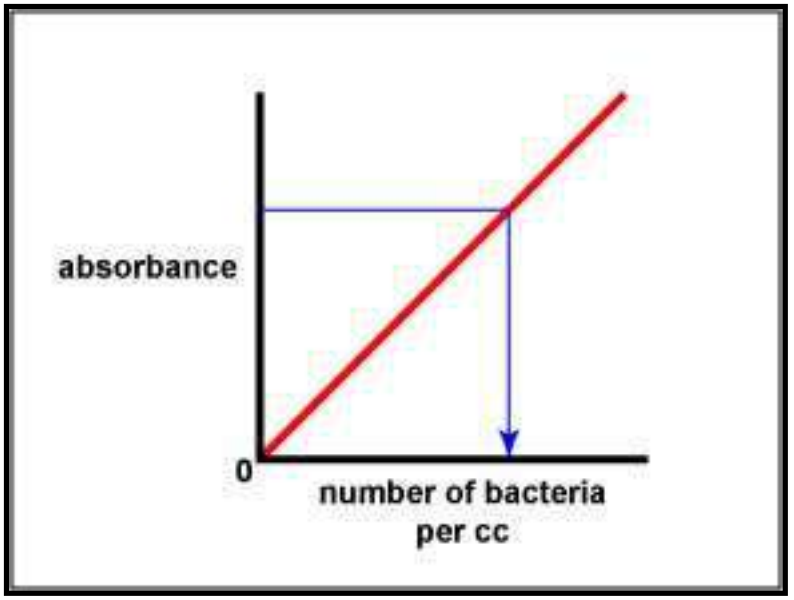
Indirect methods

1. Indirect measurement of chemical activity such as rate of O_2 production or consumption, CO_2 production or consumption, etc.
2. Measurement of cell mass by turbidity -. The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number. The method is simple and non-destructive, but the sensitivity is limited; turbidity is limited to relatively high cell concentrations – approximately 10^7 cells per ml for most bacteria.

A Standard Curve Plotting the Number of Bacteria per cc versus Absorbance



Using a Standard Curve determine the Number of Bacteria per cc in a sample by Measuring the Sample's Absorbance

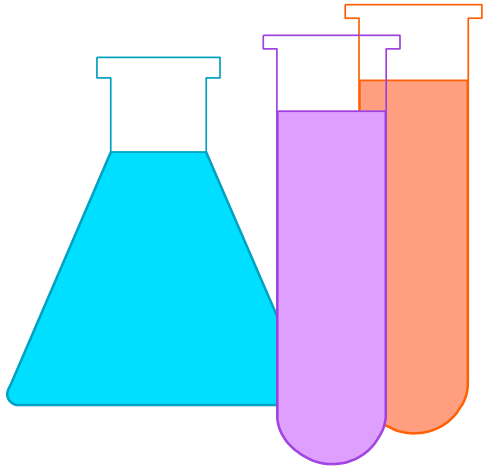


Measuring turbidity (live + dead) in liquid culture

- Turbidity can be measured "by eyeball"using a graded series of turbidity standards for comparison.

- A typical standard series of this type is the "McFarland Standards", a series of water suspensions of Barium Sulfate ranging from '0' (no turbidity) to '10' (turbidity about like milk).

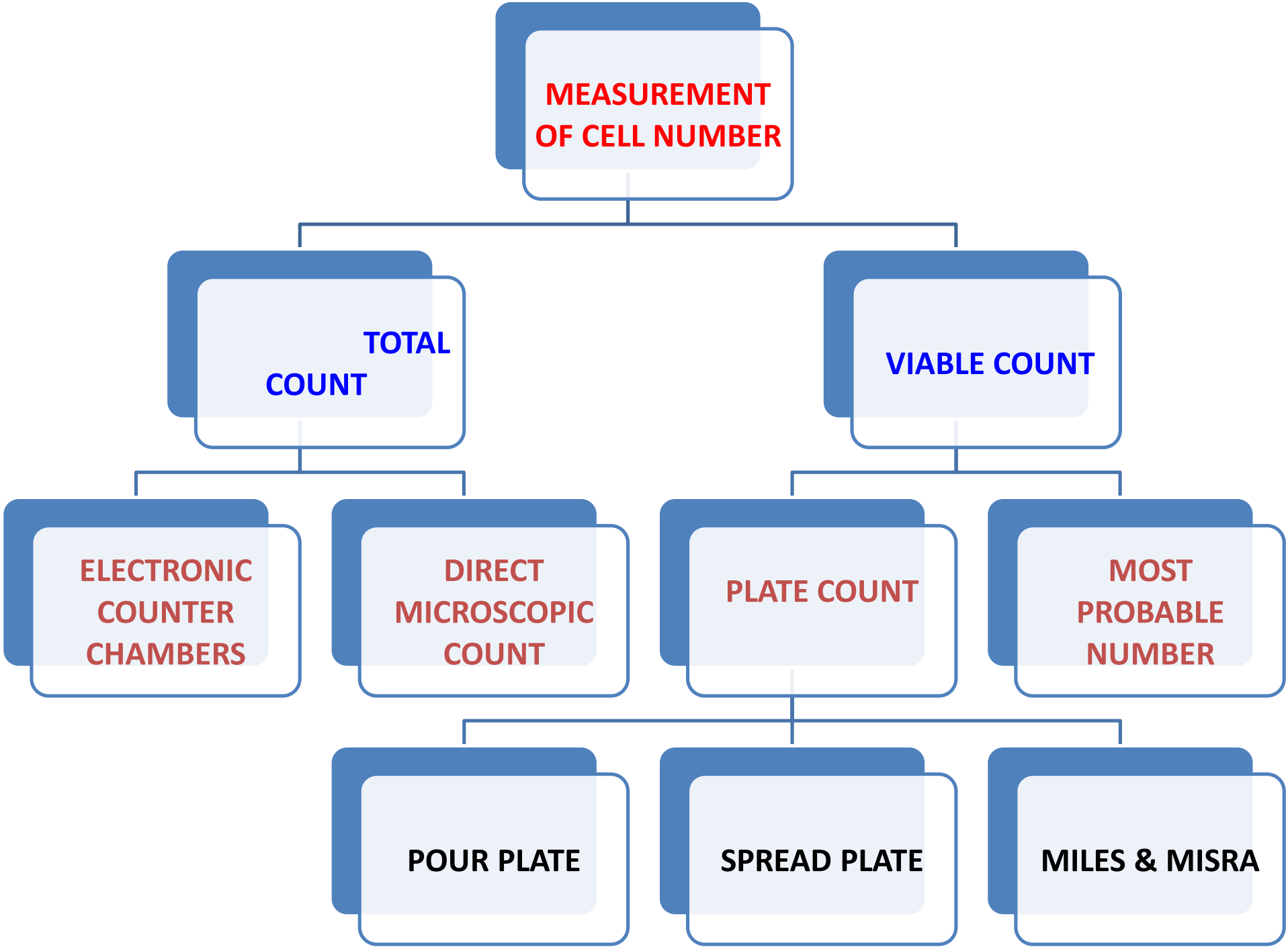
- Turbidity can be measured more precisely optically/electronically using a spectrophotometer.



The McFarland Standard Series

A Spectrophotometer

MEASUREMENT OF CELL NUMBER


MEASUREMENT OF CELL NUMBER
TOTAL COUNT
VIABLE COUNT
ELECTRONIC COUNTER CHAMBERS
DIRECT MICROSCOPIC COUNT
PLATE COUNT
MOST PROBABLE NUMBER
POUR PLATE
SPREAD PLATE
MILES & MISRA

TOTAL versus VIABLE COUNT

Total count

- **A total count is a direct counting method in which all cells are counted, whether dead or alive .**
- **requires the employment of microscopes.**

Viable count

- **A viable count is a direct counting method in which only live cells are counted.**
- **Viable counts can be accomplished by such techniques as:**
  - ***pour plating***
  - ***spread plating***
  - ***most probable number method***

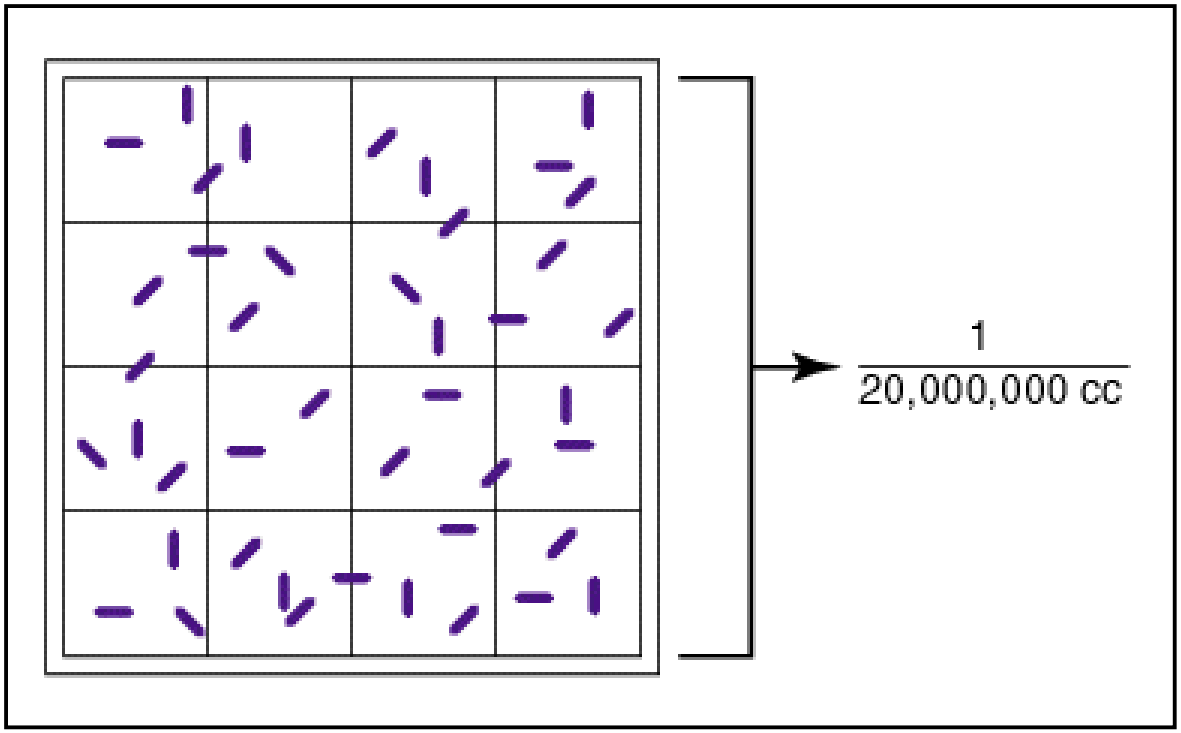
Direct microscopic counts

- **Direct microscopic count is a determination of the number of microorganisms found within a demarcated region of a slide known to hold a certain volume of culture .**
- **Use special slides known as counting chambers, e.g. Petroff-Hausser counting chamber , Neubauer chamber and Helber chamber**
- **Another method is Breed's smear technique.**

Limitations:

1. **Dead cells cannot be distinguished from living ones.**
2. **Only relatively high concentrations of bacteria may be enumerated (>10^7 cells per ml)**
3. **Dilute samples have to concentrated by centrifugation or filtration**

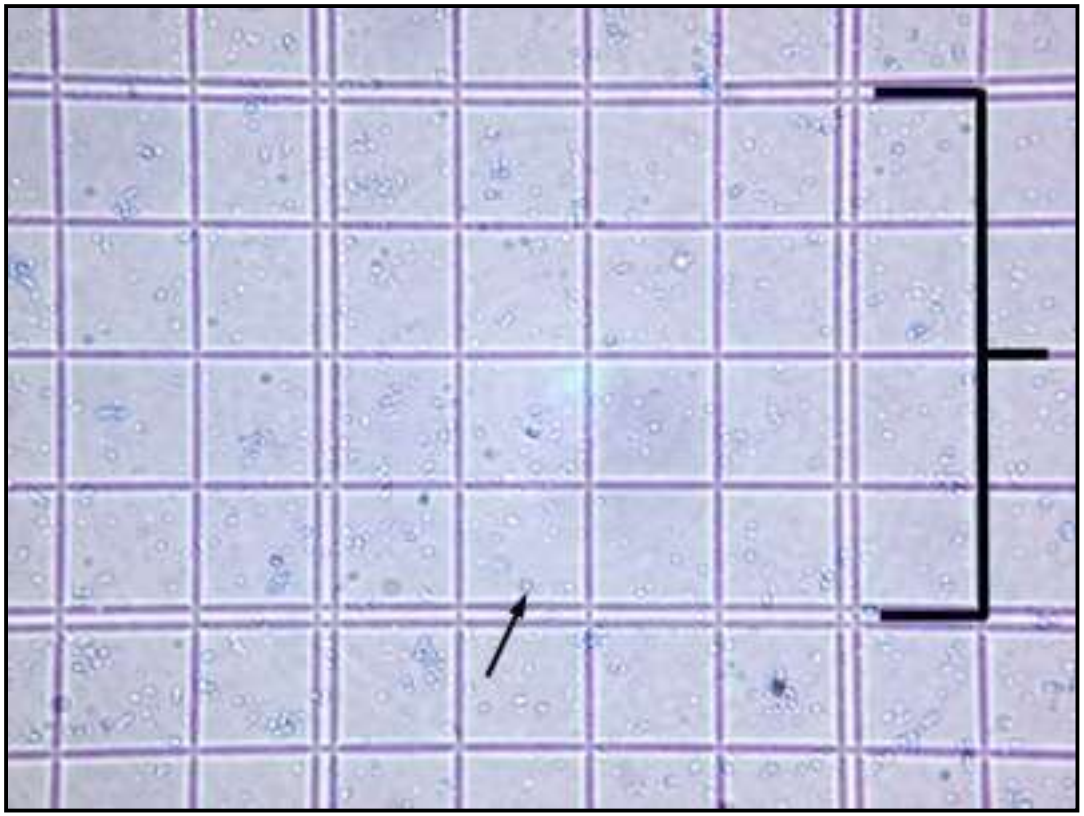
Large Double-Lined Square of a Petroff-Hausser Counter.



Using a microscope, the bacteria in five squares are counted

The number of bacteria per cc = N x 20,000,000

Petroff-Hausser Counter as seen through a Microscope



The double-lined "square" holding 1/20,000,000 cc is shown by the bracket. The arrow shows a bacterium

Breed's direct smear method

- This technique is used commonly for counting of bacteria in milk.
- A grease-free slide is placed over a template of 1 cm × 1 cm and a 0.01 ml of sample is spread over this area.
- The smear is allowed to dry, fixed by heat and stained with methylene blue for one minute.
- After drying the smear is examined under the oil immersion objective and the bacterial cells are counted.
- Bacteria/ml in sample = $N \frac{\text{(Area of smear =100 sq. mm)}}{\text{(Area of one field=3.14 × r}^2\text{)}} \times 100$

where, N= average number of bacteria/field

r= radius of oil immersion field (0.08 mm)

Electronic counting chambers

- **Count numbers, and measure size and distribution of cells.**
- **For cells of the size of bacteria the suspending medium must be very clean.**
- **Such electronic devices are more often used to count eukaryotic cells such as blood cells.**
- **Used to count efficiency of HEPA filters**
- **Example Coulter chamber.**

Viable cell counts

(Plate counts)

- **Plate counts involve spreading a sample of a culture on a nutrient agar surface after diluting the sample or cell suspension in a non-toxic diluent (e.g. water or saline).**

- **If plated on a suitable medium, each viable unit grows and forms a colony.**

- **Each colony that can be counted is called a colony forming unit (cfu) and the number of cfu is related to the number of viable bacteria in the sample.**

- ***Advantages*** **:**
  - **(a) sensitivity (theoretically, a single cell can be detected);**
  - **(b) allows for inspection and presumptive identification of the organism on the basis of colonial morphology**

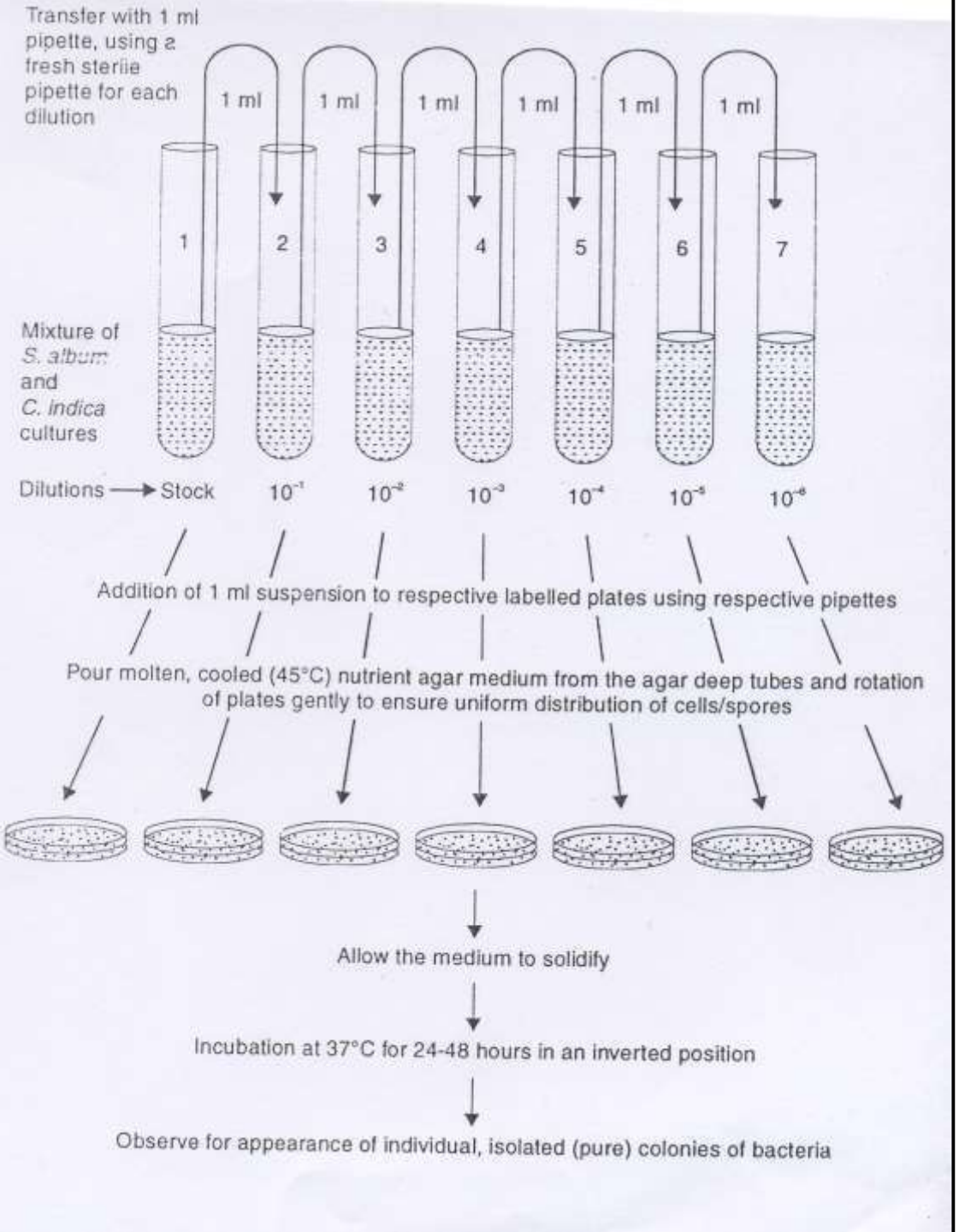
Limitations :

- only living cells develop colonies;
- cell clumping can lead to an undercount of viable cells;
- colonies develop only of those organisms for which the cultural conditions are suitable for growth;
- require lengthy incubation period (minimum-24hrs) for colonies to become visible;
- prevention of crowding often requires serial dilution;
- too few cells requires concentration, e.g., by centrifugation or filtration

Typically a plate with cfus between 30 and 300 is selected for the purpose of counting.

Pour plate

- The pour-plate method is employed for viable cell count, isolation and pure culture
- Ten fold serial dilutions of the test sample or suspension of microorganisms are prepared in molten agar (45-50°C).
- The molten medium is then poured into Petri dish and allowed to solidify; each dilution in a separate plate.
- Colonies appear within, beneath, and on top of the agar after incubation.

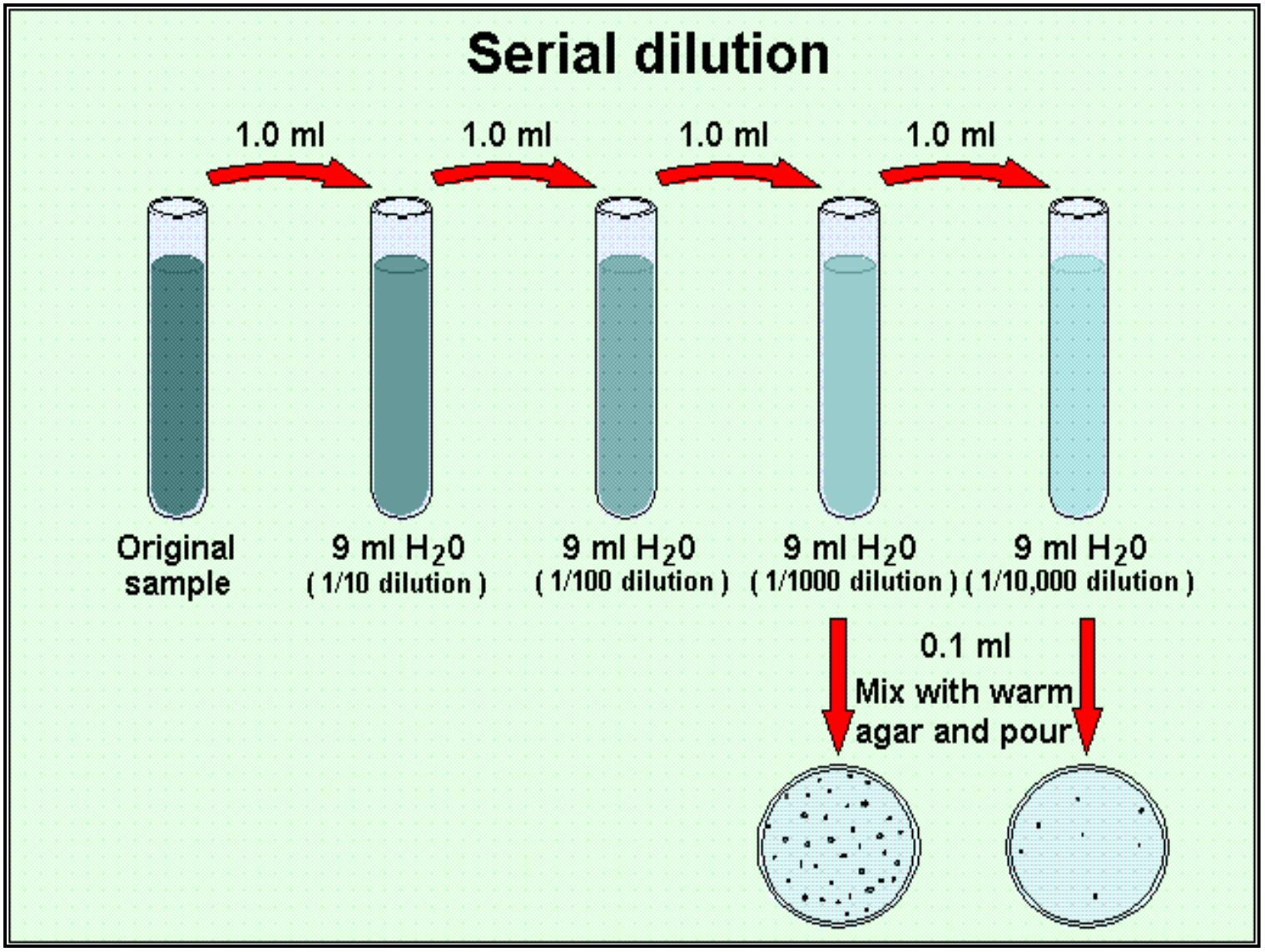

Transfer with 1 ml pipette, using a fresh sterile pipette for each dilution
1 ml
1 ml
1 ml
1 ml
1 ml
1 ml
1
2
3
4
5
6
7
Mixture of S. album and C. indica cultures
Dilutions → Stock
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
Addition of 1 ml suspension to respective labelled plates using respective pipettes
Pour molten, cooled (45°C) nutrient agar medium from the agar deep tubes and rotation of plates gently to ensure uniform distribution of cells/spores
Allow the medium to solidify
Incubation at 37°C for 24-48 hours in an inverted position
Observe for appearance of individual, isolated (pure) colonies of bacteria

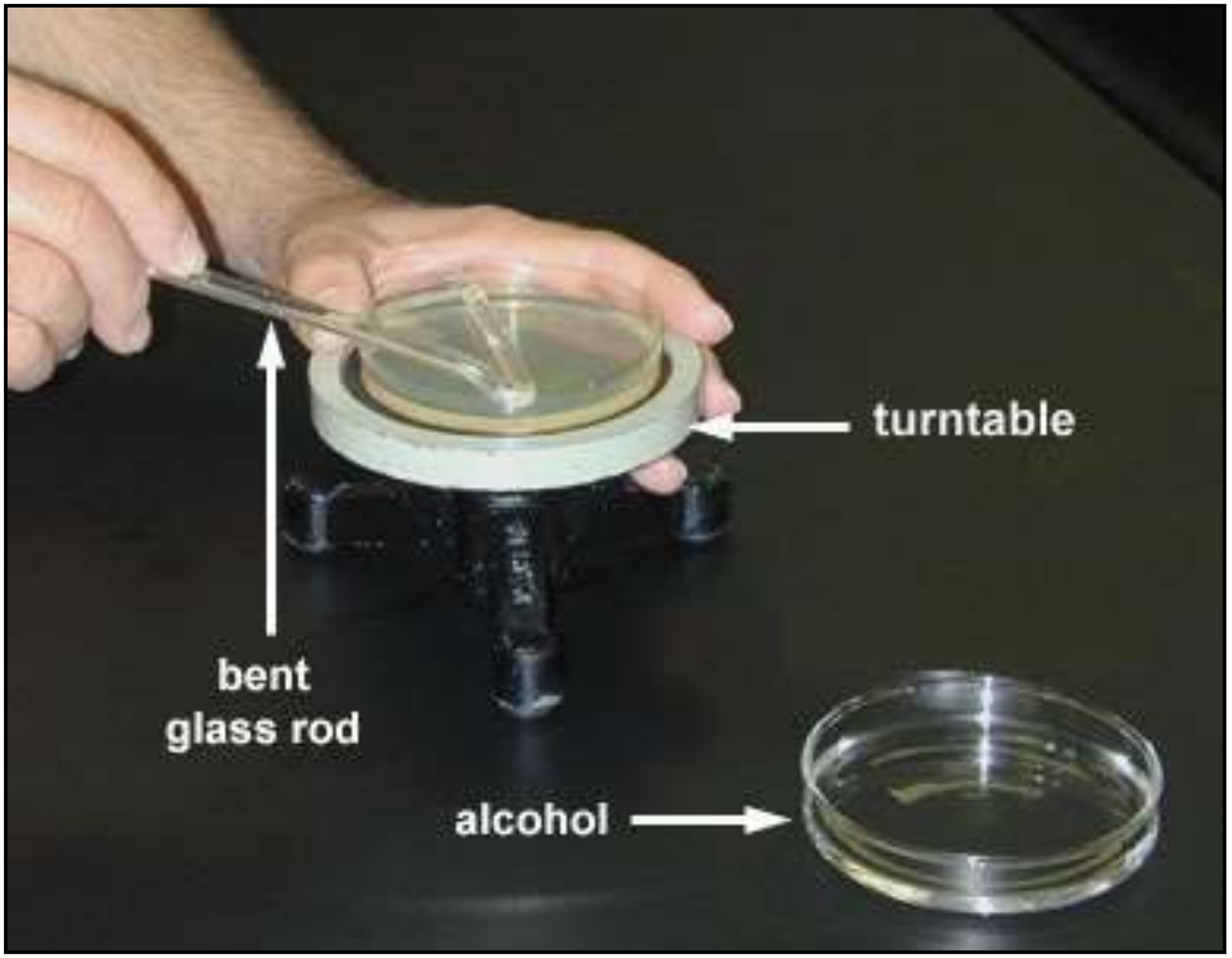
Disadvantages:

a) The cells do not develop typical colonial morphology and are not easily accessible for further testing.
b) It is not useful for temperature sensitive bacteria.
c) It is difficult to count sub-surface colonies.

Spread plate

- The spread plate method is employed for viable cell count, isolation and pure culture.
- Prior to inoculation, ten fold serial dilutions of the test sample or suspension of microorganism are prepared in a suitable diluent.
- A small volume (0.1ml) of each dilution is then inoculated onto the surface of agar that has already solidified in a Petri plate and then spread around the agar surface.
- The plates are incubated for 24 - 48 h at 25 - 37 ºC.
- Colonies will grow solely on the surface of the agar.
- This technique is advantageous particularly when cells are sensitive to relatively high temperatures.


Serial dilution
1.0 ml
1.0 ml
1.0 ml
1.0 ml
Original sample
9 ml H_2O (1/10 dilution)
9 ml H_2O (1/100 dilution)
9 ml H_2O (1/1000 dilution)
9 ml H_2O (1/10,000 dilution)
0.1 ml
Mix with warm agar and pour



Using a Bent Glass Rod and a Turntable to Spread a Bacterial Sample

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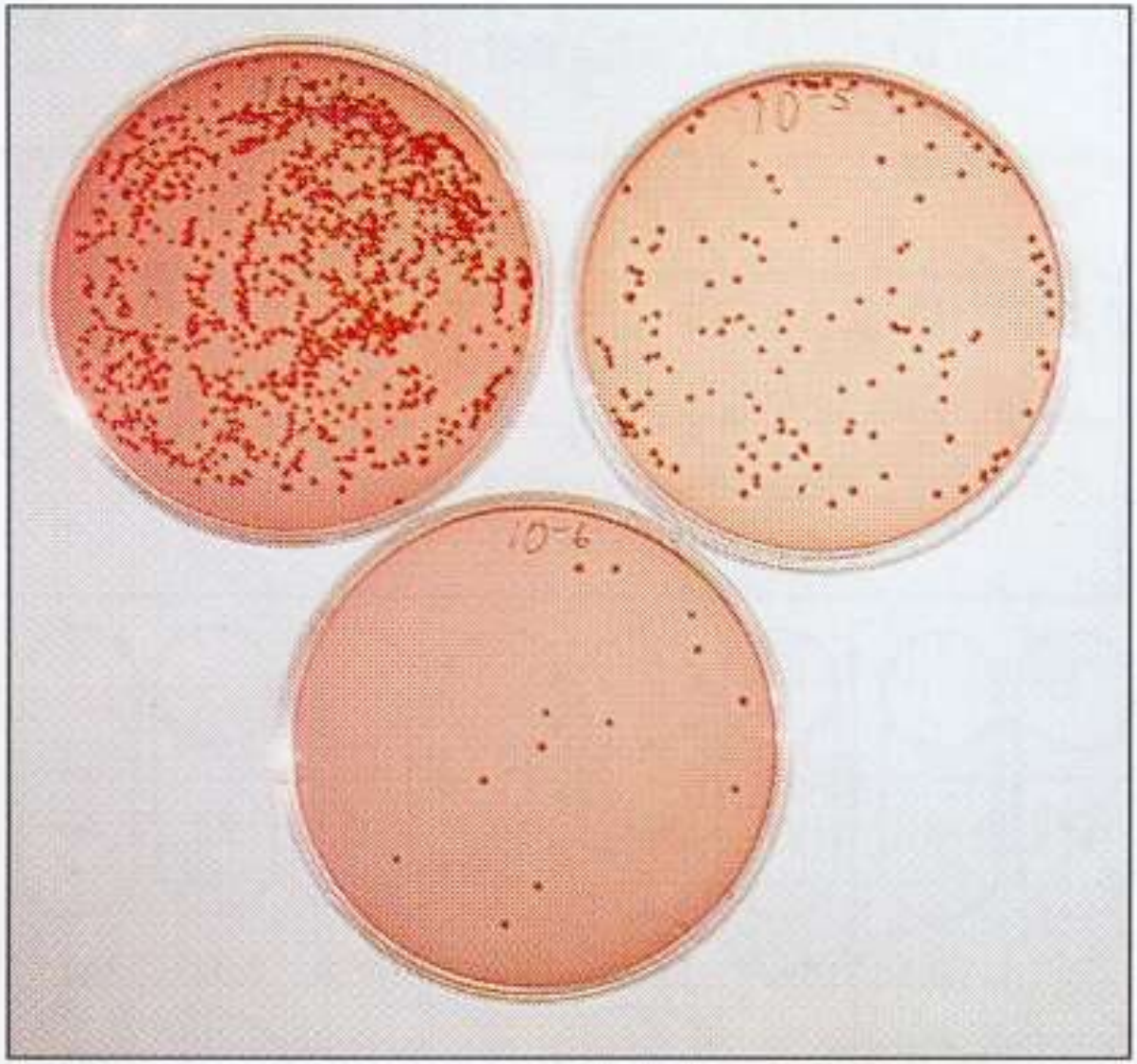
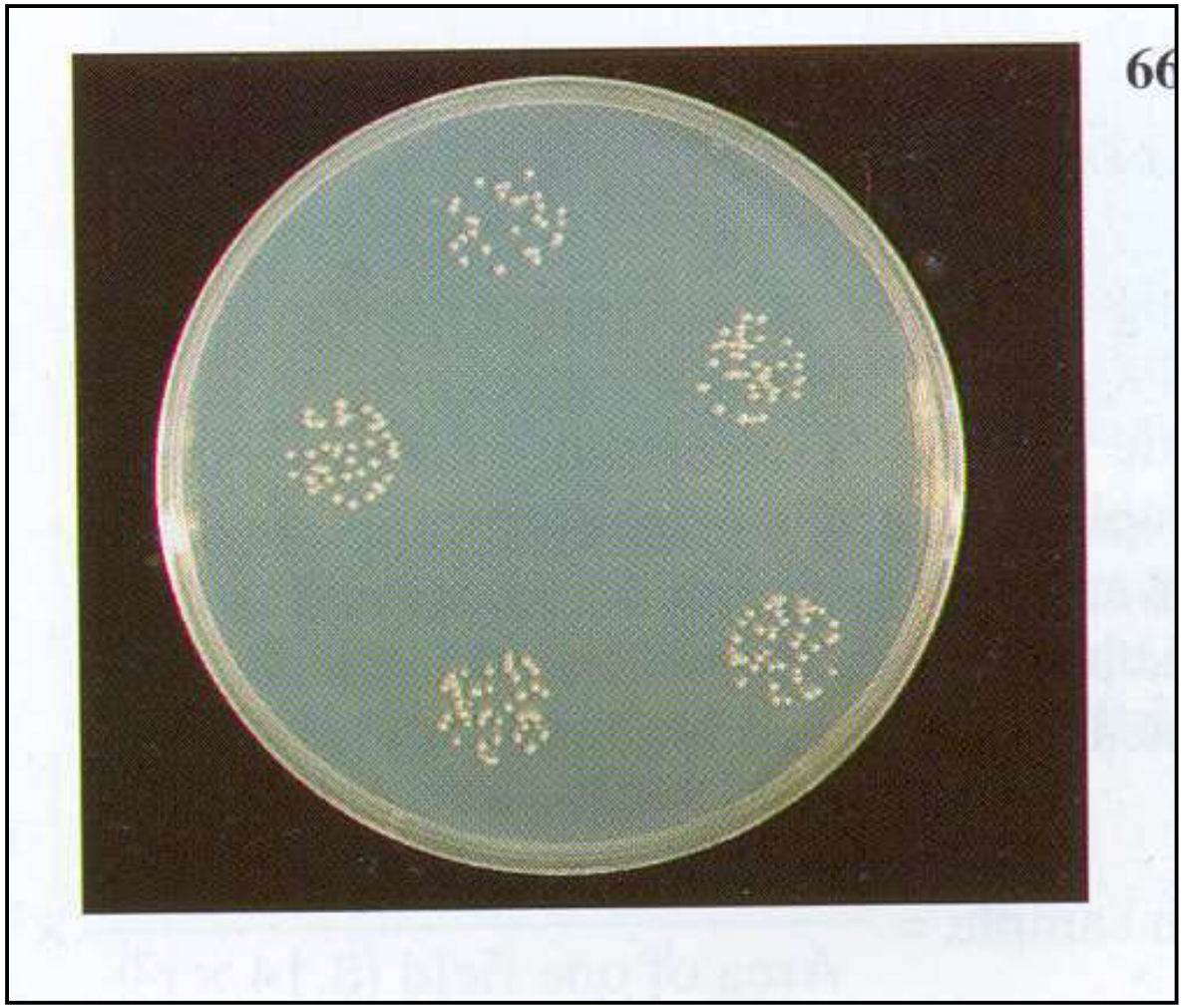


Miles-Misra technique

- This is a modification of spread method in which the agar plate is divided into 8 sectors.
- An inoculum of 0.02 ml is dropped on the surface of agar.
- The drops are allowed to dry and the plates are incubated for 24 - 48 hours at 25 - 37 ºC.
- After incubation, plate inoculated with a sample dilution yielding about 30 colonies is selected for counting using a colony counter.



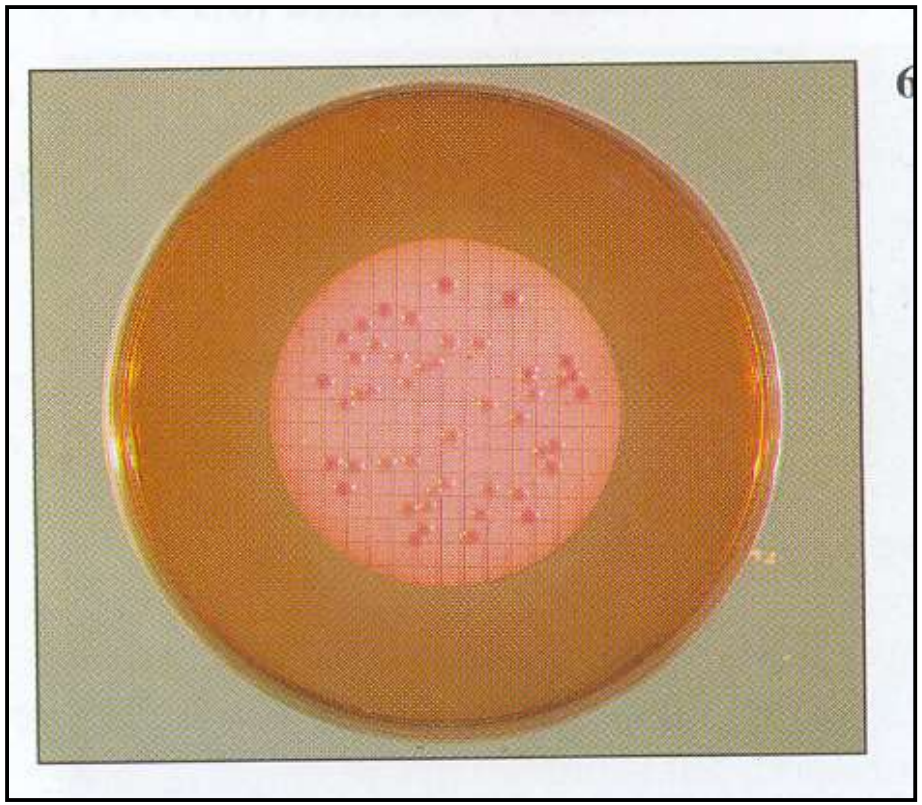
Miles – Misra technique

Filtration

- In filtration, bacteria are retained on the surface of a membrane filter of pore size 0.22 µm and then transferred to a culture medium to grow and subsequently be counted.

- If too few colonies are present then the original culture must be concentrated prior to determining its plate count.

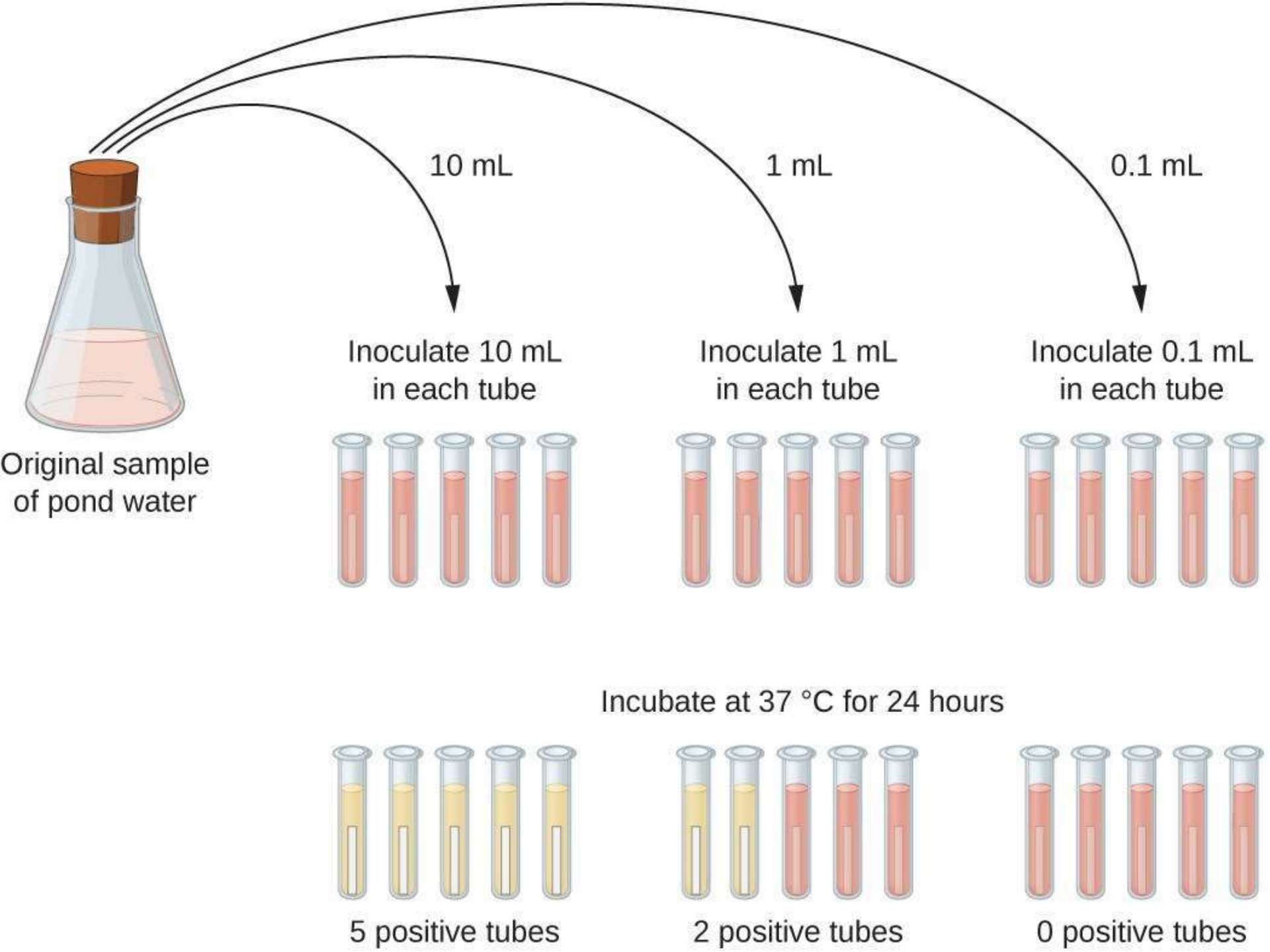
- This is a useful method for determining the number of bacteria in a water sample or other clear fluid where the bacterial number is low.



Filtration method

Most probable number method [MPN]

- Most Probable Number (MPN) is a method used to estimate the concentration of viable microorganisms in a sample using broth of different dilutions
- It is commonly used in estimating microbial populations in soils, waters, agricultural products and is particularly useful with samples that contain particulate material that interferes with **plate count enumeration methods.**
- MPN is most commonly applied for quality testing of water
- A group of bacteria commonly referred as faecal coliforms act as an indicator for faecal contamination of water
- Water to be tested is diluted serially and inoculated in **lactose broth**. Coliforms, if present, in water utilize the lactose present in the medium to produce acid and gas. The presence of acid is indicated by color change of the medium and the presence of gas is detected as gas bubbles collected in the inverted durham tube present in the medium


10 mL
1 mL
0.1 mL
Original sample of pond water
Inoculate 10 mL in each tube
Inoculate 1 mL in each tube
Inoculate 0.1 mL in each tube
Incubate at 37 °C for 24 hours
5 positive tubes
2 positive tubes
0 positive tubes

Some Methods used to measure bacterial growth

Method	Application	Comments
Direct microscopic count	Enumeration of bacteria in milk or cellular vaccines.	Cannot distinguish living from nonliving cells
Viable cell count (colony counts)	Enumeration of bacteria in milk, foods, soil, water, laboratory cultures	Very sensitive if plating conditions are optimal
Turbidity measurement.	Estimations of large numbers of bacteria in clear liquid media and broths	Fast and non-destructive, but cannot detect less than 10^7 cells per ml

Measurement of biochemical activity e.g. O_2 uptake, CO_2 production, ATP production, etc.	Microbiological assays.	Requires a fixed standard to relate chemical activity to cell mass and/or cell numbers
Measurement of total N or protein	Measurement of total cell yield from very dense cultures	only practical application is in the research laboratory
Measurement of dry weight or wet weight of cells or volume of cells after centrifugation.	Measurement of total cell yield in cultures	probably more sensitive than total N or total protein measurements