**Discipline Courses-I**
**Semester-I**
**Paper: Phycology and Microbiology**
**Unit-I**
**Lesson: Bacterial Culturing techniques**
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# Microbial Culturing

"Culturing" microbes means propagating and maintaining micro-organisms. In this section we will study why, how and under what conditions should we culture micro-organisms?

## Why do we culture microbes?

Micro-organisms play vital role in various aspects of our life. Besides being an inherent part of our system (human microflora), micro-organisms find applications in almost every sector ranging from industrial, food, and pharmaceutical to environmental. Micro-organisms aid in the manufacture of industrially important products, food products and recycle of substances etc. The other side of the coin, however, is that a tiny but significant proportion of micro-organisms cause human diseases. Other detrimental effects of microbes include food spoilage, biodeterioration etc.

Both these positive as well as negative implications of microbes are very important and need to be studied in detail. The basic tool for studying microbes is by their 'culturing'. The preliminary step before we can study any aspect of microbial growth, physiology or application, we need to first culture (grow) them, therefore microbial culturing becomes the first and most important technique in microbiology. Microbial cultures are used to determine the type of organism and its abundance.

## How to culture microbes?

In this section we will learn about the requirements and techniques used for culturing microbes. Pre-requisite for the growth of a microbe is a "culture medium" which is a source of nutrients required for the growth and maintenance of the micro-organism.

## Culture media

Types of media

**I. Based on the composition of the medium, it can be of two types:**

1. Complex/ Chemically undefined media

It is referred to as 'complex' because the exact components and their quantity is not defined. It consists of complex raw materials mainly plant and animal hydrolysates such as peptone, soyabean extract, beef extract and meat extract.

Complex media is used for routine cultivation of micro-organisms. Example of the most commonly used complex medium is nutrient broth/ nutrient agar.

**Table :** Composition of complex medium

Source: Author

| **Nutrient Broth** | **Quantity** | **Nutrients supplied** |
|---|---|---|
| Beef extract | 3.0 g | Vitamins, minerals |
| Peptone | 5.0 g | Amino acids, peptides |
| Water | 1 L | |

2. Synthetic/ Chemically defined media

In this medium, the exact chemical composition and quantity of each constituent is known. Synthetic medium is used for determining the nutritional requirements of heterotrophs.

**Table :** Composition of synthetic medium

Source: Author

| **Synthetic broth medium** | **Quantity** | **Nutrients supplied** |
|---|---|---|
| Glucose | 5.0 g | Simple sugar |
| Ammonium phosphate | 1.0 g | Nitrogen, phosphate |
| Sodium chloride | 5.0 g | Sodium and chloride ions |
| Magnesium sulfate | 0.2 g | Magnesium ions, sulfur |
| Potassium phosphate | 1.0 g | Potassium ions, phosphate |
| Water | 1.0 L | |

**II. Based on the physical nature of the medium, it can be of three types:**

**1. Liquid medium**

Liquid media are usually used for propagation of pure culture. In addition to supplying nutrients, liquid medium can assist in the maintenance of pH using various buffers.

**2. Solid medium**

Solid media are generally agar based. Agar is a non-nutritive, solidifying agent added to a medium for its solidification. Agar is a polysaccharide derived from marine red algae. It is dissolved in aqueous solutions and gels when the temperature is reduced below 45$^{o}$C. It is used at a concentration of 1.5-2%. It forms a firm, transparent gel that is not degraded by bacteria. The solidified medium can be used to cultivate many different types of microbes, isolate pure cultures, for determining characteristics of colonies and to measure population growth etc.

**3. Semi-solid medium**

Besides liquid and solid media, semi-solid medium is also prepared by addition of agar at 0.5%. It is used for cultivation of microaerophilic bacteria and for determination of bacterial motility.

**III. Based on the function of the medium, it can be of three types:**

**1. Selective media**

Selective media 'selects-out' a microbe from a mixture of micro-organisms. These media contain ingredients that enhance the growth of a particular type of bacterium while inhibiting the growth of other type of bacteria in a mixture. The basic growth medium may contain bile salts, certain dyes, extra salt (NaCl) or an antibiotic to inhibit the intolerant/ sensitive organism while allowing the growth of those species/ pathogens one wants to isolate. Examples: Mannitol salt agar for *Staphylococcus aureus*, MacConkey (MAC) agar for *E. coli*.

2. **Differential media**

Differential media helps distinguish between different microbes in a mixture based on specific biochemical or physiological properties. Example: Blood agar can differentiate between haemolytic vs. non-hemolytic bacteria i.e. bacteria that can hemolyze (destroy) red blood cells (RBC) and others that do not. Among the hemolytic bacteria too, the ones which cause complete lysis of RBC form a clear zone around the colonies and are referred to as 'β-hemolytic'. The ones which partially hydrolyze RBC form a greenish halo around their colony and are referred to as 'α-hemolytic'.

3. **Enriched media**

Certain fastidious micro-organisms are difficult-to-grow in basic media as they have complex nutritious requirements. For cultivation of such organisms, enriched medium is used. In addition to a complex base, enriched media contains special nutrients such as blood, serum or other highly nutritious substances. Example: Blood agar for *Streptococci*, Chocolate agar for *Neisseria* sp. Blood provides protein, carbohydrates, lipid, iron and a number of growth factors and vitamins necessary for their cultivation.

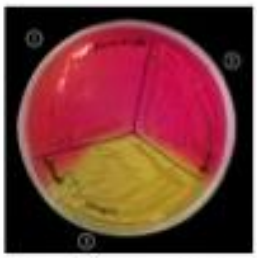
**Table :** List of special type of media

Source: Author

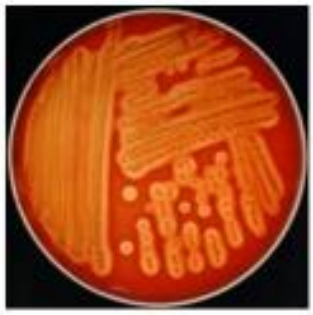
| **Medium** | **Type** | **Function** |
|---|---|---|
| MacConkey agar | Selective and differential | It is a **selective medium** as it contains bile salts and crystal violet which inhibit the growth of gram positive bacteria and thus selects gram negative bacteria.<br>It is a **differential medium** as it contains lactose and neutral red, a pH indicator. Those gram negative bacteria which utilize lactose and produce acidic products can be differentiated from other gram negative bacteria. The colonies of those that release acidic products are red (eg. *E. coli*). |
| Blood agar | Enriched and differential | It is an **enriched medium** as it contains blood that supports the growth of many fastidious bacteria.<br>It is a **differential medium** as it can distinguish between hemolytic vs. non-hemolytic bacteria. Hemolysis appears as a clear zone (β-hemolysis) or greenish halo (α-hemolysis) around the colony. (eg. *Streptococcus pyogenes*) |
| Mannitol salt agar | Selective and differential | It is a **selective medium** as it contains 7.5% NaCl that selects for staphylococci.<br>It is a **differential medium** as it differentiates pathogenic staphylococci based on their ability to utilize mannitol and release of acidic products that cause a pH indicator (phenol red) to turn yellow. |

| | | |
|---|---|---|
| | | (eg. *Staphylococcus aureus*). |
| Eosin methylene blue (EMB) agar | Selective and differential | It is a **selective medium** as it contains two dyes, eosin Y and methylene blue which inhibit the growth of gram positive bacteria and thus selects gram negative bacteria.<br>It is a **differential medium** as it contains lactose/sucrose. Those gram negative bacteria which utilize these and produce acidic products can be differentiated from other gram negative bacteria. The colonies of those that release acidic products are green with metallic sheen (eg. *E. coli*). |
| Hektoen enteric agar | Selective and differential | It is a **selective medium** as it contains inhibitors that prevent the growth of gram positive bacteria.<br>It is a **differential medium** as it contains indicators of lactose fermentation and $H_2S$ production.<br>*Salmonella* produces black colonies, whereas *Shigella* produces translucent green colonies. |

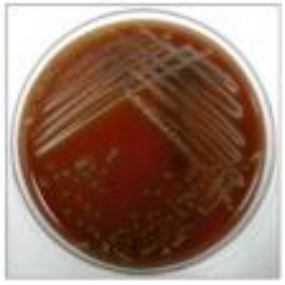
A. Mac Conkey's agar with lactose fermenting *E. coli* colonies



B. An MSA plate with *Micrococcus* sp. (1), *Staphylococcus epidermis* (2) and *S. aureus* colonies (3).



Beta- hemolysis



Alpha-hemolysis

C. Blood agar plates

D. Eosin methylene blue (EMB) agar with *E. coli* colonies

E. Hektoen enteric agar with *Salmonella* (dark colonies) and *Shigella* (light colonies).

**Figure:** Types of media based on function

Source: (a) https://upload.wikimedia.org/wikipedia/commons/thumb/d/d3/E.coli_on_MacConkey_agar.JPG/800px-E.coli_on_MacConkey_agar.JPG

(b http://upload.wikimedia.org/wikipedia/commons/thumb/6/6f/Chapmanes.jpg/220px-Chapmanes.jpg

(c) http://3.bp.blogspot.com/_pz25qMAnLWY/R9rjMIR--lI/AAAAAAAAAJo/Pj5EAehvE_I/s400/Beta+haemolysis.jpg, http://jkoa.org/ArticleImage/0043JKOA/jkoa-47-64-g004-l.jpg

(d) http://upload.wikimedia.org/wikipedia/commons/thumb/8/8d/Coli_levine.JPG/220px-Coli_levine.JPG
(e) https://static.thermoscientific.com/images/F103743~wl.jpg

**Pure cultures**

Micro-organisms usually occur in mixed populations with many species. It is difficult to study a single type of microorganism among a mixture. For this, one needs a **pure culture** (axenic culture), that is, a population of only one species. This is particularly important when trying to identify a pathogen. Pure cultures arise from a single progenitor called **colony forming unit (CFU)**. To obtain pure cultures, all media, vessels, and instruments must be sterile; that is, free of any microbial contaminants. The use of aseptic techniques is critical as well.

Pure cultures can be prepared in several ways. The basic concept is that if cells from a mixture are spatially isolated, each cell will give rise to a separate **colony**. Colony is a visible cluster of micro-organisms in or on a solid medium. Each colony represents a pure culture as each colony arises from a single cell. Few common techniques used for isolation of pure cultures are discussed below:

**1. Streak plate method**

In this method, a dilution gradient of cells is formed across the plate because of which the number of cells decreases with subsequent streaks. This leads to spatial separation of micro-organism; each separate bacterium develops into a visible colony.

Many different streaking patterns can be used to separate individual bacterial cells on the agar surface viz. continuous streak, two- streaks, T-streaks (Three sector-streak), quadrant streak (four streaks) etc. If the initial concentration of microbes is high, the number of streaks to obtain an isolated colony should be more.

**Method of making a streak plate:**

1. An inoculation loop is sterilized by incineration, cooled down and then a loopful of bacterial culture is taken.
2. This loopful of bacterial cells is then streaked across the surface of a sterile solidified nutrient medium.
3. After the initial streak, the inoculating loop is sterilized again, cooled again and the

inoculum for the second sector is obtained from the first sector.

4. The same steps are followed for the subsequent streaks.
5. This is followed by incubation of the plates under favorable conditions.
6. After incubation, i.e. generally 24h, isolated colonies appear.

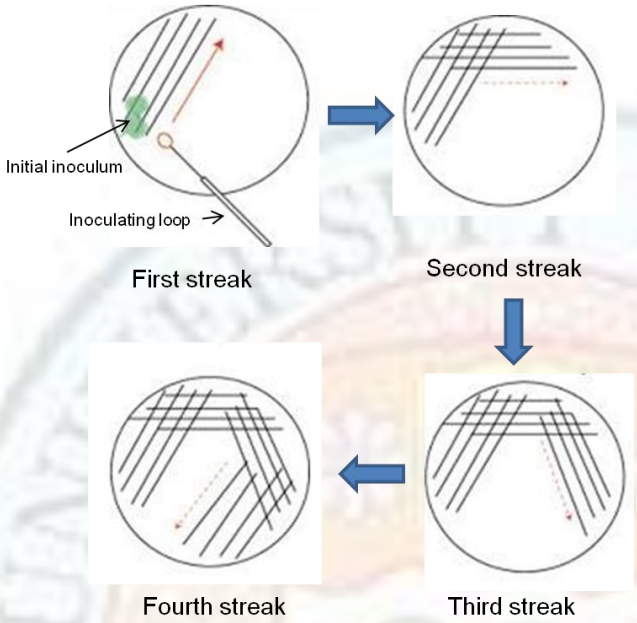



**Figure :** Method of streaking a bacterial culture

Source: Author

**Video**: Movie depicting technique of streaking
Source: author

### 2. Spread plate method

In this method, a mixture of bacterial cells is diluted in sterile saline before separating them spatially. After serial dilutions, 0.1 ml or lesser volume of an appropriately diluted culture is spread over the surface of an agar plate using a sterile glass spreader. The plate is then incubated under appropriate conditions until isolated colonies appear.

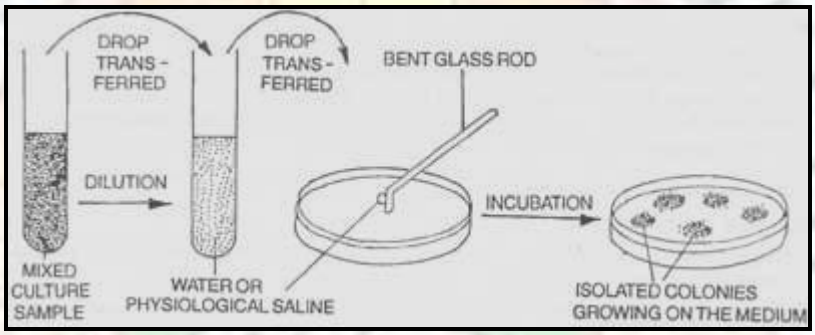



**Figure: Spread plate method**

Source: http://upendrats.blogspot.in/2010_02_01_archive.html

### 3. Pour plate method

In this method also the micro-organisms are diluted before spatial distribution however dilutions are made alongwith melted agar. A known volume of diluted culture is pipetted into a sterile petri-plate; then molten agar is added to it and it is mixed well by gently swirling the plate. An advantage of the pour-plate method is that a larger volume can be used compared to the spread plate method. However, the disadvantage of this method is that the organism must be able to briefly withstand the temperature of melted agar, 45$^{o}$C. Secondly, it is difficult to dig out the sub-surface colonies formed by organisms which are trapped beneath the agar surfaces.

Unlike the streak-plate method, the pour-plate and spread-plate methods can be used to determine the number of bacteria in a specimen.

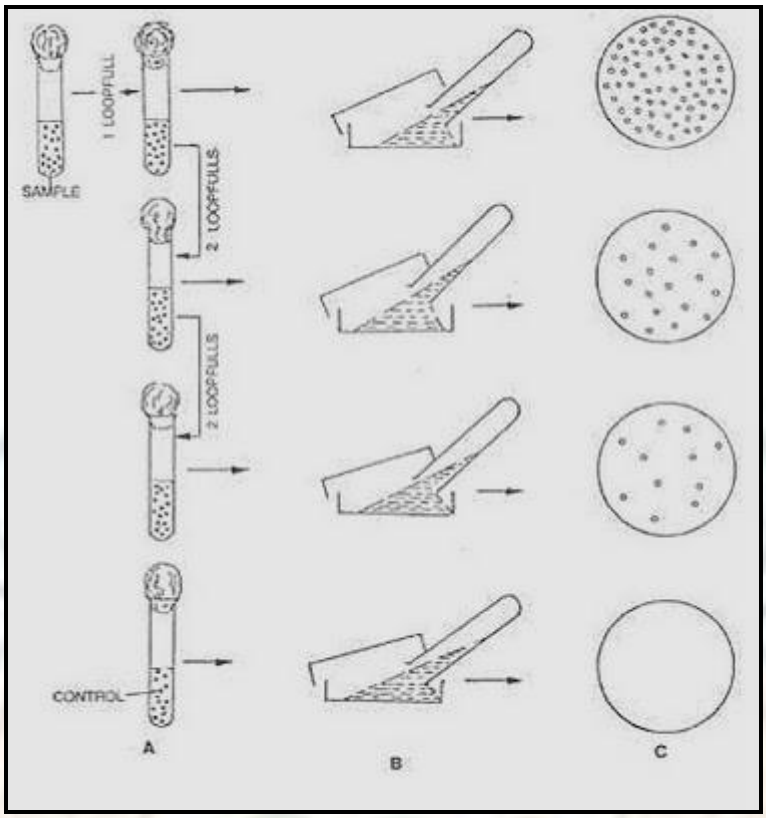



**Figure: Pour plate method**

Source: http://upendrats.blogspot.in/2010_02_01_archive.html

## Culture Maintenance

The main aim of culture maintenance and preservation is to maintain strains alive, uncontaminated and to prevent any change in their characteristics.

**Methods:**

**1. Sub-culturing**

It involves periodic transfer of stock culture to fresh medium to prepare fresh stock culture. The culture medium, temperature and time interval at which transfers are made vary with the species. Usually heterotrophs remain variable for several weeks or months on nutrient agar.

Continuous sub-culturing might result in development of variants/ mutants therefore this method has a disadvantage that it might result in change in characteristics of the cultures.

**2. Lyophilization (Freeze-drying)**

Ordinary drying of most bacteria leads to loss of viability however freeze drying canbe used as an effective method for long term culture preservation.

In this method, water are removed from a frozen product via sublimation. Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase.

The method involves two steps:

a) Pre-freezing of cultures to form frozen structures

A dense cell suspension is placed in small vials (ampules) and frozen at -60 to-78$^{o}$C

b) Drying to remove most water

Drying is performed under vaccum. The vials containing frozen culture are connected to high vaccum. The ice present in them sublimes resulting in dehydrated culture. The vials are finally sealed off under vaccum and stored in a refrigerator.

**Advantages:**

i) Long term storage for more than 30 years
ii) Characteristics of the strain remain unchanged
iii) Minimum storage space is required
iv) Small vials of lyophilized culture can be sent conveniently to other laboratories.
v) Lyophilized cultures are easily revived. Vials are opened and sterile liquid medium is added to it following transfer of rehydrated culture to suitable growth medium.

Overall, lyophilized process results in stable, readily rehydrated, long term preservation of cultures.

A.

B.

**Figure :** Culture preservation by lyophilization A. Lyophilizer B. Lyophilized cultures

Source: A: Author

B: https://ncma.bigelow.org/ncma/images/rotator/lyophilized.jpg

**Cryopreservation**

Freezing a suspension of living cells results in decrease in viability as ice crystals begin to form and the concentration of solutes in the suspension increases.

Addition of cryoprotectant agents, such as glycerol or dimethylsulfoxide (DMSO) aids cryopreservation

**Method:** The cell suspension is sealed into small vials and then frozen at a controlled rate to -150$^{o}$C. The ampules are then stored in liquid nitrogen refrigerator.

The recovery of cryopreserved cells requires the rapid thawing of the bacterial suspension followed by transferring of the entire contents to an appropriate growth medium.

**Advantages:**

1. Long term storage of 30 years or more.
2. Characteristics of the bacteria remain unchanged.

**Disadvantages:**

1. Relatively expensive since the liquid nitrogen needs to be replenished after regular intervals.

Details of various methods of culture preservation can also be studied in detail from ATCC bacterial culture guide (Web link 1.1).

## Culture Collections

Earlier each microbiologist used to keep a personal collection of cultures important to them. However, maintaining and preserving various cultures is a tedious job and in the process many important strains become lost. There are certain strains which are taxonomically important referred to as 'type strain'. In addition there are other strains whose special properties are useful for various purposes. Preserving such stains is very important and thus there was a need of central bodies for culture preservation. In this respect, now a number of central culture collections exist whose main purpose is the acquisition, preservation and distribution of authentic cultures.

A detailed list of various culture collections of bacteria across the world are given at J.P. Euzéby list (Web link 1.2). Out of all, the most widely known are the American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). In India, the major is Microbial Type Culture Collection (MTCC) located in Chandigarh. Microbiologists who report a new species or any novel property of a novel strain are expected to deposit their strains with one or more national culture collections so that it can be preserved and available in the public domain for other researchers to work.

### How to distinguish between microbes morphologically ?

Each bacterial colony has distinct characteristics which gives a clue for bacterial identification. There are number of characteristics of a colony which one should observe viz. size, margin, surface texture, elevation etc. A vast variation in each of the characteristics is observed:

**Size:** Pin-point to large

**Margin:** circular, rounded projections, notches, threadlike, rootlike

**Surface texture:** Smooth, rough, mucoid, wrinkled

**Elevation:** Thin, thick, flat, convexity

**Consistency:** Butyrous, viscous, stringy, rubbery, dry, powdery

**Optical features:** Opaque, translucent

**Pigmentation:** Red, Yellow, Black, Pink, Brown

Characteristics of the same culture may vary from medium to medium so to identify, reference culture should be on the same medium.

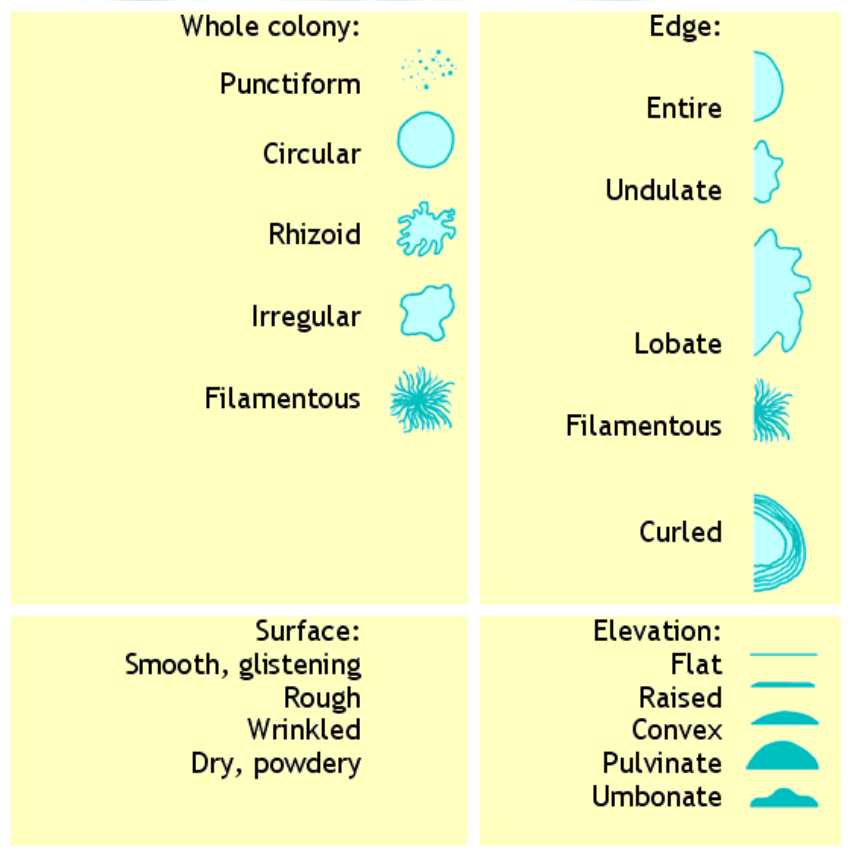



**Figure :** Various colony characteristics

Source: http://upload.wikimedia.org/wikipedia/commons/8/8d/Bacterial_colony_morphology.png

## Are all microbes culturable?

You will be surprised to know that less than 1% of the species in natural water and soil samples can be cultured in laboratory. The rest are non-culturable organism and are thus referred to as **VBNC (Viable but non-culturable).** They are alive but are temporarily

unable to reproduce. VBNC bacteria are identified using direct microscopic examination and amplification of 16S rRNA sequence.

It has been thought that a genetic response triggered by stress or starvation causes an organism to become VBNC. They remain non-culturable as the right conditions/nutritional requirements for their growth in the laboratory have not yet been created. Example: Few Chlamydiae and rickettsiae can only remain viable in animals or cell cultures. They fail to grow in even enriched medium.

The vast diversity of VBNC organisms need to be explored for their untapped potential however, these can have negative implications too. Most of the tests for affirming food and drinking water safety are culture based. As these organisms are VBNC, they could pose serious public health threats. Overall, VBNC organisms open up a vast and unexplored area of research.

# Microbial growth

Till now we have been discussing microbial culturing, its techniques and media. To complete our study, we need to identify the requirements of micro-organisms for growth and also consider the measurements used to evaluate growth.

## Requirements for growth

Microbial growth refers to the increase in the number of cells not the size of cells. All microbes have specific requirements for growth. These can be broadly classified into two categories: nutritional and physical. Nutritional include sources of carbon, nitrogen, sulphur, phosphorus and trace elements. Physical factors include temperature, pH and oxygen concentration.

### Nutritional factors

#### Carbon

Carbon is one of the major structural backbone forming any microbial cell. Based on the source of carbon, micro-organisms can be divided into two categories:

1. **Autotrophs**: Source of carbon is carbon dioxide

2. **Heterotrophs**: Source of carbon is organic compounds such as proteins, carbohydrates and lipids.

**Nitrogen, sulphur and phosphorus**

In addition to carbon, nitrogen and sulphur is also required for protein synthesis. Synthesis of DNA and RNA requires nitrogen and phosphorus. ATP i.e. the energy currency of the cell requires phosphorus. Phosphorus is also essential for synthesis of phospholipids of cell membrane.

Sources of nitrogen can vary from organism to organism. Most bacteria take up amino acids as a source of nitrogen however others use nitrogen from ammonium ions ($NH_4^+$) or nitrate ($NO_3^-$). Some special bacteria like cyanobacteria can use gaseous nitrogen. This process is called **nitrogen fixation**. Natural sources of sulphur are hydrogen sulphide, sulphate ions and sulphur containing amino acids. Important source of phosphorus is the phosphate ion ($PO_4^{3-}$).

**Trace elements**

Certain mineral elements which are required by microbes in very small amounts are referred to as trace elements. Example: Copper, zinc, iron etc. Most of these act as cofactors of certain enzymes.

**Physical factors**

**Temperature**

Temperature is one of the most critical factor governing growth of microbes. Each bacteria has an optimal growth temperature where is exhibits maximum growth. Their maximum and minimum growth temperatures are around 30$^{o}$C apart. On the basis of optimal growth temperature, microbes can be divided into the following three groups:

1. **Psychrophiles (cold-loving microbes)**

   Psychrophiles are microbes that have their optimal growth rates below 15$^{o}$C. They can grow even at 0$^{o}$C and grow till 20$^{o}$C. They are sensitive to higher temperature and cannot even grow at reasonably warm temperatures of 25$^{o}$C. Eg. *Vibrio psychroerythrus*, *Pseudomonas sp., Alcaligenes* etc. These organisms can be responsible for food spoilage at low temperature because they grow well at refrigerator temperatures.

**2. Mesophiles (moderate-temperature-loving microbes)**

Mesophiles are microbes that grow in moderate temperatures with optimum growth temperatures being 25-40°C. Most of the organisms lie in this category. Eg. *Escherichia coli, Enterococcus faecalis, Neisseria gonorrhoeae* etc. Almost all pathogens are mesophiles as human body temperature is 37°C.

**3. Thermophiles (heat-loving microbes)**

Thermophiles are microbes that grow at higher temperatures with optimum growth temperature being 55-65°C . Their minimum growth temperature is around 45°C. Eg. *Thermoplasma acidophilum, Thermus aquaticus*. As thermophiles can survive in high pasteurization temperatures, they generally are contaminants of dairy products.

Hyperthermophiles have growth optima between 85°-113°C. They do not grow below 55°C. Eg. *Pyrococcus abyssi and Pyrodictium occultum* are marine hyperthermophiles.

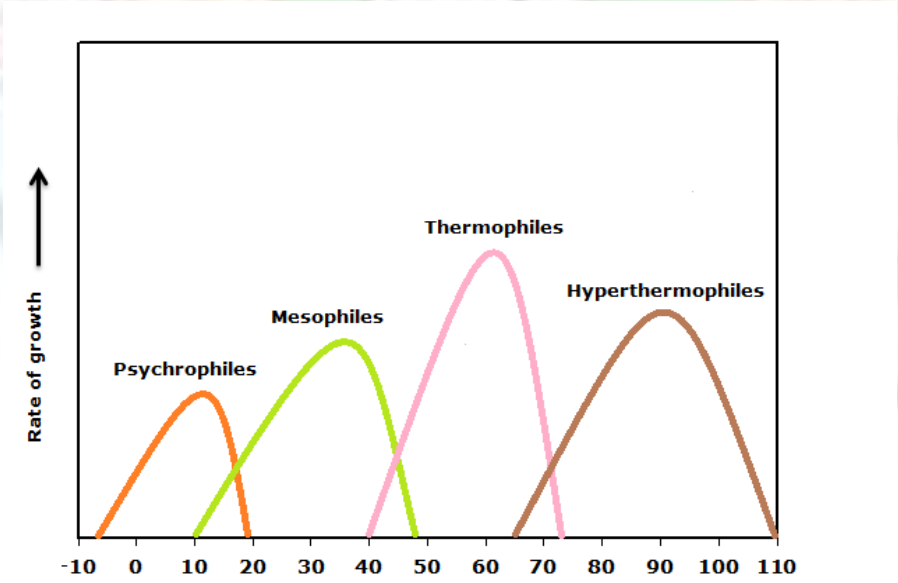



**Figure:** Growth rates of different types of microbes in response to temperature

Source: Author

**pH**

Each microbe has a definite pH growth range in which it can survive. Depending on its pH optima, microbes can be divided into three categories:

1. **Acidophiles**

   They have growth optima between pH 0 and 5.5. Eg. *Thiobacillus thiooxidans, Sulfolobus acidocaldarius.*

2. **Neutrophiles**

   Their growth optimum is between pH 5.5 to 8.0. Eg. *Escherichia coli*, *Staphylococcus aureus*

3. **Alkalophiles**

   Their growth optima is between pH 8.0 to 11.5. Eg. *Bacillus alcalophilus, Microcyctis aeruginosa.*

Most of the bacteria are neutrophiles however molds and fungi generally grow at acidic pH. Acidophilic bacteria are important for the food and dairy industries. Eg. Acid produced by *Lactobacillus* and *Streptococcus* is used to convert milk to buttermilk.

During cultivation of bacteria in a medium, the pH of the medium generally changes. Fermentation of a sugar may result in production of acid which decreases the pH of the medium. Contrarily, utilization of an organic acid will result in increase in pH. These fluctuations of pH might hamper further growth of the organism. To prevent this, buffers can be incorporated into the medium. Buffers can help maintain the pH of the medium during fermentation.

**Oxygen concentration**

Oxygen is essential for existence of most microbes however at the same time it can be poisonous for few others. The requirement for oxygen correlates with its metabolism especially cellular respiration. Based on their oxygen requirements microbes can be classified as follows:

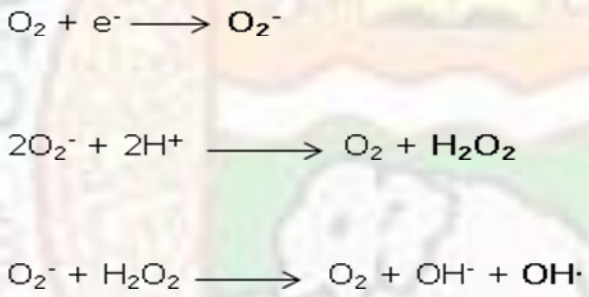
1. Obligate aerobes
2. Obligate anaerobes
3. Facultative anaerobes
4. Microaerophiles
5. Aerotolerant

It can be seen that obligate anaerobes cannot tolerate oxygen at all. They cease to grow even in presence of low concentrations of oxygen. We need to know the reasons for such oxygen toxicity.

The major reasons for oxygen toxicity are the toxic forms of oxygen that are formed. Few cellular reactions which involve molecular oxygen can result in formation of one or more of the following toxic radicals

1. Superoxide free radical ($O_2^-$)
2. Hydrogen peroxide ($H_2O_2$)
3. Hydroxyl radical ($OH^{\cdot}$)

The reactions that lead to the formation of these radicals are as follows:



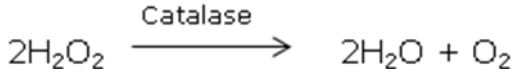
$$O_2 + e^- \longrightarrow \mathbf{O_2^-}$$

$$2O_2^- + 2H^+ \longrightarrow O_2 + \mathbf{H_2O_2}$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + \mathbf{OH\cdot}$$

All these radicals are highly toxic to the cell. Toxicity of superoxide ion is because of its great instability which leads it to steal an electron from neighboring molecule and that of hydrogen peroxide is because it is a powerful oxidizing agent.

Aerobes and facultative organism have developed protective mechanism to protect against these toxic elements. They produce enzymes such as **superoxide dismutase** (SOD), **catalase** and **peroxidases** which neutralize the effect of these toxic radicals. SOD converts superoxide radical to hydrogen peroxide which is in turn converted to water and oxygen by catalase.



$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

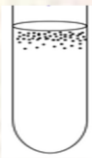
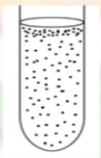
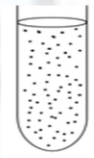
Hydrogen peroxide is also catalysed by peroxidase to water by the following reaction

$$H_2O_2 + \text{reduced substrate} \xrightarrow{\text{Peroxidase}} 2H_2O + \text{oxidised substrate}$$

Catalase and peroxidase prevent the formation of hydroxyl ion thus protecting the organism. Anaerobic bacteria lack both SOD or catalase therefore they are highly sensitive to oxygen.

**Table :** Types of bacteria based on oxygen requirements

Source: Author

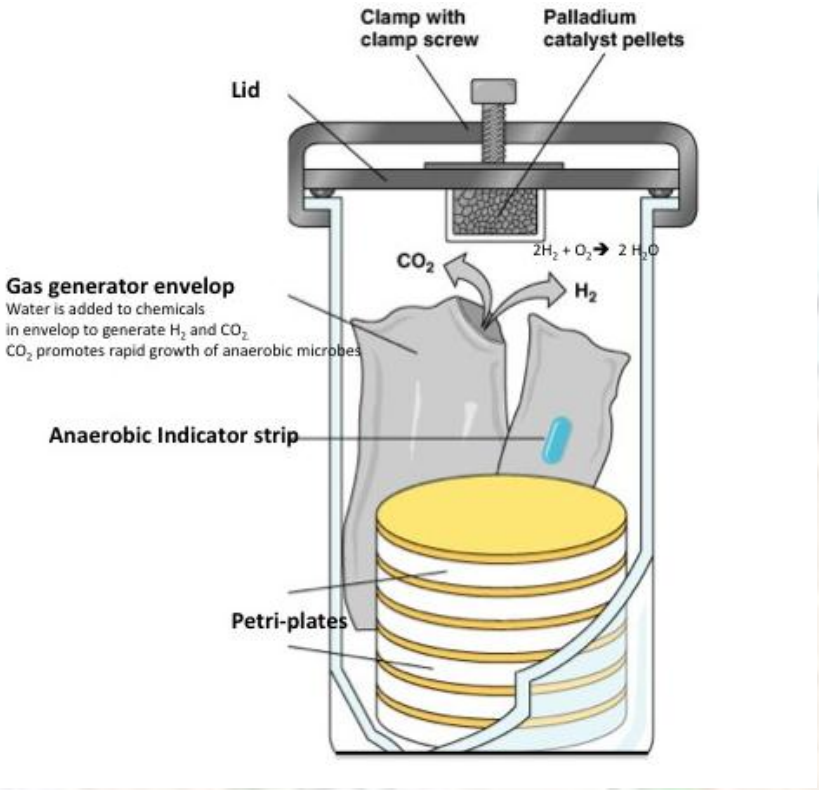
| | Obligate aerobes | Obligate anaerobes | Facultative anaerobes | Microaerophiles | Aerotolerant |
|---|---|---|---|---|---|
| Effect of oxygen on growth | Only aerobic growth. Oxygen required. Dies in absence of oxygen. | Only anaerobic growth. Oxygen not required. Dies in presence of oxygen. | Both aerobic and anaerobic growth. Growth increases in presence of oxygen. | Grows in presence of lower concentration of oxygen only | Anaerobic growth but can tolerate the presence of oxygen. |
| Growth pattern |  |  |  |  |  |
| Explanation of growth patterns | Grow at the top of the test tube in order to absorb maximal amount of oxygen. | Grow at the bottom to avoid oxygen. | Grow mostly at the top, since aerobic respiration is the most beneficial one; but as lack of oxygen does not hurt them, they can be found all along the test tube. | Grow at the upper part of the test tube but not at the top. They require oxygen but at a low concentration. | They not affected at all by oxygen, and they are evenly spread along the test tube |
| Enzymes<br>SOD<br>Catalase | <br>Present<br>Present | <br>Absent<br>Absent | <br>Present<br>Present | <br>Present<br>Low levels | <br>Present<br>Absent |
| Examples | *Mycobacterium* | *Clostridium, Methanococcus* | *Streptococcus, Staphylococcus* | *Campylobacter* | *Enterococcus faecalis* |

**Culturing anaerobic bacteria**

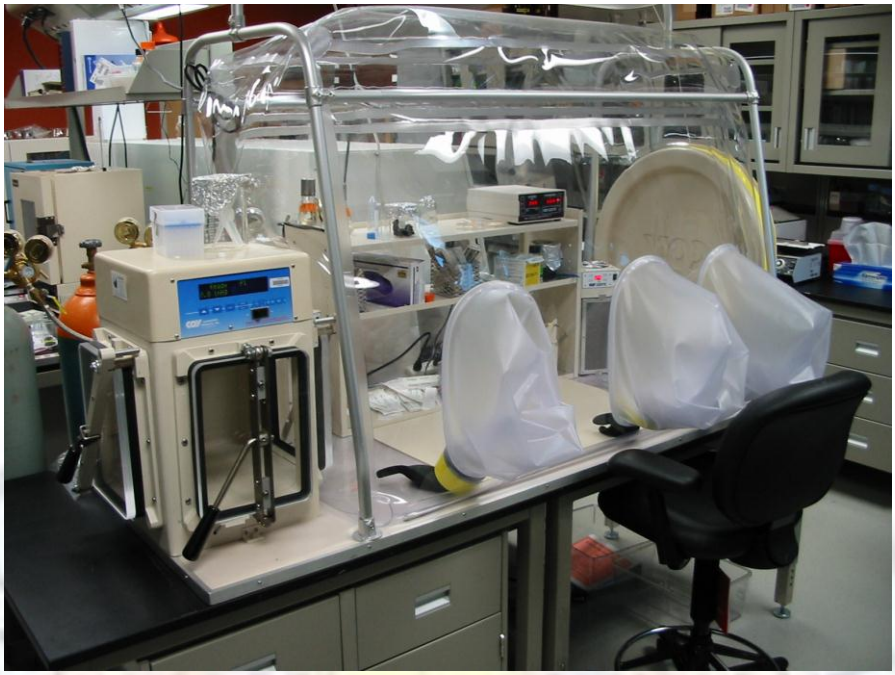
Anaerobic bacteria can only be cultivated in environment which is devoid of oxygen. Reduced medium which has been described earlier is used to grow anaerobic bacteria. Anaerobic bacteria are incubated in **anaerobic jar** (Gas Pak system). Petri-plates are placed in the jar alongwith an $H_2$-$CO_2$ generating system. A palladium catalyst combines the oxygen present within the jar as well as that dissolved in the medium, with the hydrogen and water is formed.

Now, **anaerobic chambers** equipped with air locks and filled with inert gases ($H_2$, $CO_2$ and $N_2$) are also available. Culture media are placed within the chamber and they are

rendered oxygen free by reaction with hydrogen. Media are inoculated within the chamber by means of the glove ports and incubated also within the chamber .




**A.**



**B.**

**Figure :** Anaerobic jar (A) and anaerobic chamber (B)

Source: (A) Author (B)http://www.ornl.gov/sci/ees/bsd/bst/resources/anaerobic%20chamber%202.jpg

**Osmotic and hydrostatic pressure**

Usually micro-organisms cannot tolerate high osmotic pressure. They grow normally in isotonic solution where the osmotic pressure in the cell is equivalent to a solute concentration of 0.85% NaCl. In a hypertonic solution (concentration of solute such as NaCl is higher in surrounding medium than in the cell), growth of the cell is inhibited due to plasmolysis i.e. osmotic loss of water.

However, there are a few organisms which can tolerate high salt concentrations. These are referred to as **halophiles**. Obligate halophiles are those which require high salt concentration for growth. Facultative halophiles are those which do not require high salt concentration but can tolerate higher salt concentrations upto 15%.

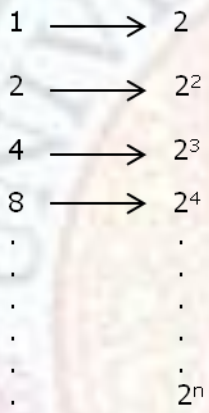
Hydrostatic pressure is referred to as the pressure exerted by the weight of water. Few extremophilic organisms which can tolerate high pressure are referred to as **barophiles**. Such organisms will not be able to survive at normal atmospheric pressures.

## Study of growth

Bacteria generally divide by binary fission. Few bacteria reproduce by budding and fragmentation respectively.

**Generation time**

It is referred to as the time required for a cell to divide that is to double. As binary fission is the most common means of division, we will take it into account. In binary fission, if a single cell divides, it produces two cells.




Where, n is the number of generations.

If we assume there are no cell deaths, then each successive generation doubles the population therefore after a given point of time, the total population, N will be

$$N = N_o \times 2^n$$

where $N_o$ is the initial population inoculated at time zero.

To calculate n, we take log on both sides of the equation

$$\log_{10}N = \log_{10}N_0 + n\log_{10}2$$

$$n = \frac{\log_{10}N - \log_{10}N_0}{\log_{10}2}$$

$$n = \frac{\log_{10}N - \log_{10}N_0}{0.301}$$

$$n = 3.3\,(\log_{10}N - \log_{10}N_0)$$

Therefore, if we know initial population and final population after a given period of time, we can calculate the number of generations.

Further, the generation time (g) can be determined using the following formula,

$$g = \frac{t}{n}$$

$$g = \frac{t}{3.3\,(\log_{10}N - \log_{10}N_0)}$$

All bacteria have different generation time. *E. coli* has a generation time between 15 to 20 min. Among a species also generation time varies depending on various nutritional and physical conditions of growth i.e. medium, pH, temperature etc.

**Growth rate (R)**

It is defined as the number of generations per hour. It is the reciprocal of generation time.

$$R = \frac{1}{g} = \frac{3.3\,(\log_{10}N - \log_{10}N_0)}{t}$$

## Phases of growth

A bacterial growth curve which shows growth of cells over time can be divided into four major phases .

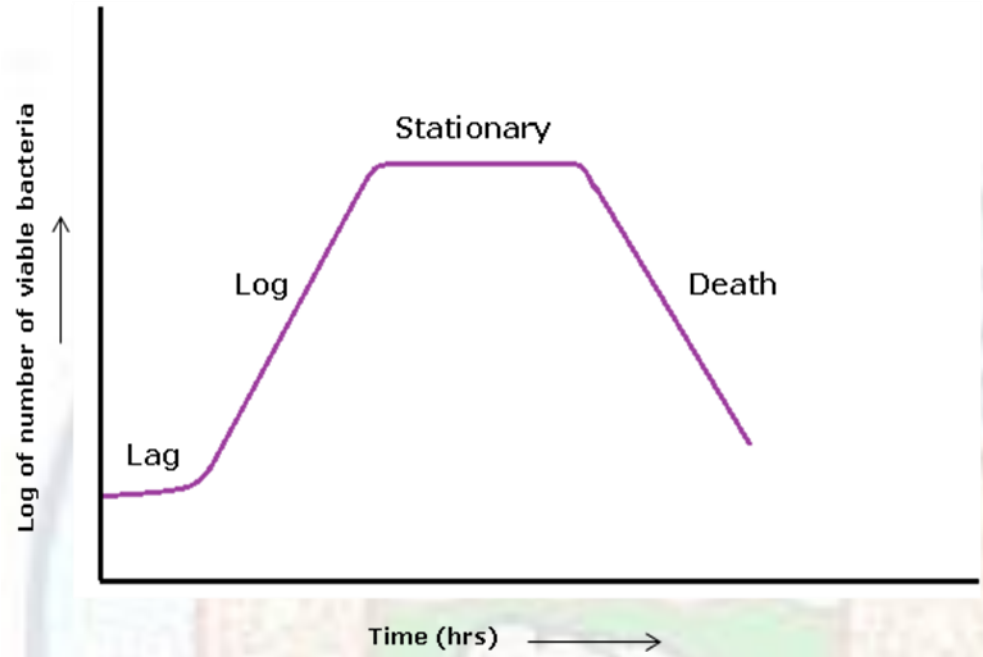



**Figure :** Growth curve depicting various phases of growth
Source: Author

1. **Lag phase**

   The first phase is referred to as the 'Lag phase'. When an inoculum of bacteria is added to a medium, the number of cells does not increase immediately. There is little or no cell division in this phase. During this period, however, the cells are not dormant. They are physiologically very active synthesizing enzymes and various molecules required for growth in the new environment. In the lag phase they are basically adjusting to the new environment and at the end of the lag phase each organism divides.

2. **Log or exponential phase**

   The second phase is the phase of active proliferation. In this, the cells divide steadily at a constant rate. The plot of log of number of cells versus time results in a straight line. In other words, the generation time and growth rate remains constant.

In this phase, the cells are metabolically most active and therefore for most industrial purposes log phase cells are preferred. However, the vulnerability of the cells to radiations or antibiotics is also maximum in this phase as most of these methods effect actively dividing cells.

3. **Stationary phase**

   The third phase is called the 'Stationary phase' as in this phase, the number of cell divisions is balanced by the number of cell deaths and the growth curve reaches a plateau. The metabolic activity of the surviving cells is also slow. The reason for the cells to enter from exponential to stationary phase maybe due to exhaustion of nutrients, accumulation of waste products, changes in pH and dissolved oxygen etc.

4. **Death or Decline phase**

   When the rate of cell death exceeds the rate of cell division, the cells enter the last phase that is the death phase. In this phase the number of viable cells decrease logarithmic therefore it is also referred to as logarithmic decline phase.

**Continuous growth**

As discussed earlier, the log/exponential phase cells are generally preferred for various industrial as well as experimental purposes. In this respect, it is often desired to maintain bacterial cell population at a particular rate in the log phase. This state is referred to as **'steady state'** growth. In this process, the culture volume and cell concentration are both kept constant by adding fresh sterile medium to the vessel and by removing spent medium and cells at the same rate. Chemostat is a commonly employed apparatus used to maintain continuous culture.

## Measurements of growth

Microbial growth as we earlier discussed is the increase in number of cells. It can be quantitated by a number of ways.

**Direct measurement of growth**

Direct measurement of microbial growth can be done by direct microscopic count, by electronic particle counters and plate count method.

**Direct microscopic count**

In this method, a measured volume of unstained bacterial suspension is placed within a specially designed slide called a Petroff Hausser counting chamber. This slide contains a grid of 25 large squares of known area. The total volume of liquid held is $2 \times 10^{-5}$ ml. The numbers of cells are counted in several of the large squares to determine the average number.

Advantage:

- Rapid, simple method
- Morphology of bacteria can also be observed.
- Very dense suspension can be counted after diluting appropriately.

Disadvantage:

- Both live and dead cells are counted in this procedure.

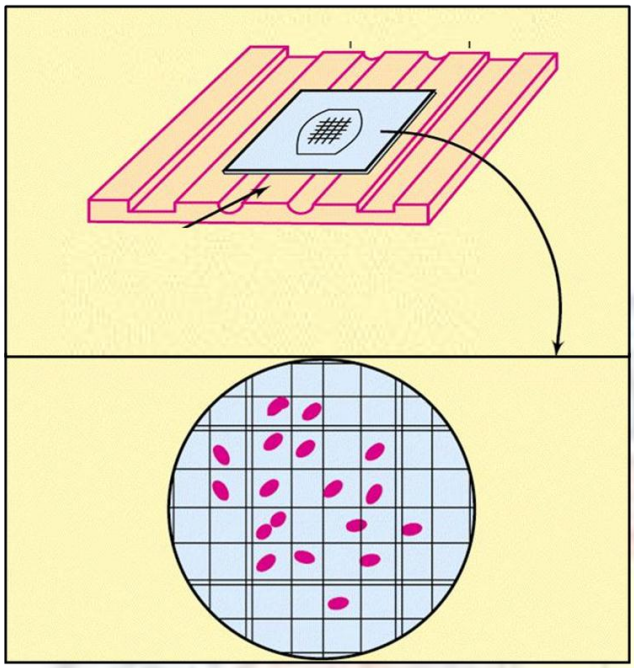


**Figure :** Direct microscopic counting procedure using the Petroff-Hausser counting chamber

Sources: Author

**Electronic particle counters**

In this method, the bacterial suspension is passed through a tiny orifice of an electronic particle counter. As each bacterium passed through the orifice, there is an increase in electric resistance which generates an electric signal which is automatically counted therefore the number of bacteria can be quantitated.

Advantage:

- Rapid

Disadvantage;

- Requires sophisticated electronic equipment
- Orifice tends to become clogged
- Both live and dead cells are counted

**Plate count method**

It is the most commonly used method for measuring growth as it measures the number of viable cells only. This method assumes that each bacterium undergoes multiple rounds of cell division to form a single colony on a agar plate. Each cell is called a colony forming

unit (CFU). In this method, first serial dilutions of the bacterial suspension are prepared in saline.

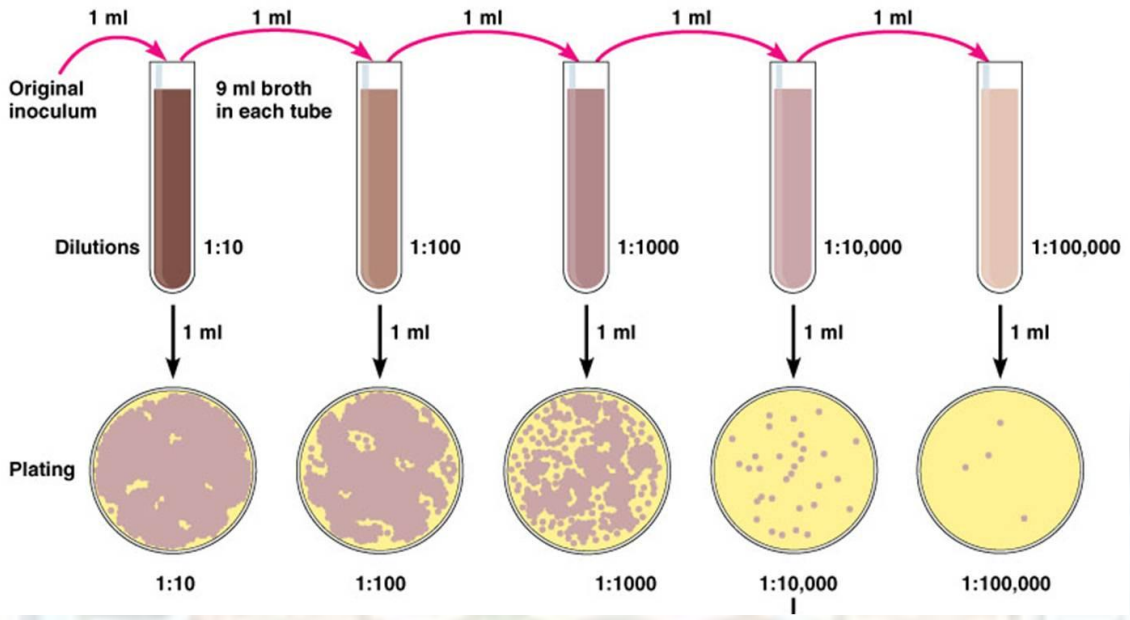



**Figure :** Strategy for preparing serial dilutions

Source: http://faculty.irsc.edu/FACULTY/TFischer/micro/serial%20dilution.jpg

Then appropriate dilutions are plated on solidified medium using either pour or spread plate method as described previously. After incubation under appropriate conditions, the number of CFU are counted.

Advantage

1. Gives viable count of cells
2. Sensitive method as very small number of organisms can be counted

Disadvantage

1. It takes some time generally 24 h for visible colonies to form.

**Indirect measurement**

Indirect measurements can be made by measurements of cell mass by dry weight methods or by turbidity measurements and by cell activity.

**Cell mass**

**Dry weight method**

It is usually used for filamentous bacteria, fungi and molds where the direct plate count method cannot be applied. In this method, mass of cells or mycelium of fungus is removed from the medium, filitered and washed to remove extraneous material and dried to a constant weight in a dessicator. It is then weighed. It can also be used for very dense suspension of cells.

**Turbidity**

As bacterial cells grow in a liquid medium, it becomes turbid. This turbidity is measured as a means of monitoring bacterial growth. The instrument used to measure turbidity is a **spectrophotometer**. In this instrument, a beam of light is passed through a bacterial suspension. Bacteria in a suspension scatter light passing through them. The amount of light scattered (optical density OD) is a function of cell mass therefore more the cells present, higher the OD reading.

Advantage:

It is simple, rapid method

Disadvantage:

Both viable and non-viable cells are measured

**Cell activity**

Another indirect measure of quantifying growth is to measure a population's metabolic activity. This method assumes that the amount of certain metabolite is directly proportional to the number of bacteria present. Example: If a bacterial species ferments glucose to produce acid, then the amount of acid produced can be taken as an indirect measure of measuring growth. Oxygen uptake can also be measured as an indication of metabolic activity and therefore cell number.

# Summary

- **Microbial culturing**
- Culture media can either be complex (exact composition not known) or synthetic (exact chemical composition and amount known). It can also be solidified using agar.
- Culture media can be classified based on function as selective, differential, enriched and reduced media.

- Pure culture is a population of cells that arise from a single cell. It can be obtained by either streak-plate, spread-plate or pour-plate method.
- Microbial cultures can be maintained by sub-culturing, lyophilzation and cryopreservation.
- Huge collections of microbial cultures are preserved in various central culture collection centers.
- Each bacterium has a unique colony characteristic that includes the size, margin, texture, elevation and consistency of the colony.
- A large proportion of micro-organisms are non-culturable and are referred to as VNBC (Viable but non-culturable).

- **Microbial Growth**
- Microbial growth refers to an increase in number of cells.
- Each micro-organism requires various nutritional and physical factors for growth. Nutritional factors include carbon, nitrogen, sulfur, phosphorus and trace elements. Physical factors include optimum temperature, pH and oxygen concentration etc.
- A typical growth curve can be divided into four phases i.e. lag, log, stationary and death phase.
- Measurement of microbial growth can be done by direct and indirect methods. Direct methods include direct microscopic count, electronic plate counter and plate count method whereas indirect method includes determination of cell mass by dry weight method or turbidity and by assessing cell's metabolic activity.

## Exercises

1. What are the four major phases of bacterial growth? Which phase is maintained in a continuous culture and why?
2. A medium is inoculated with six cells of a micro-organism. If its generation time is 60 min., how many cells would be present in the medium after 7 hours of incubation under appropriate conditions?
3. How is media solidified? Where is semi-solid media used?
4. Differentiate between the following:

   Selective media and Differential media

   Complex media and synthetic media

   Beta hemolysis and alpha hemolysis

Microaerophiles and Aerotolerant organisms

Psychrophiles and Thermophiles

5. What are the special requirements for the culturing of anaerobic micro-organisms?
6. Why should buffers be added to a medium used for fermentation of bacteria?
7. Define the following terms: Halophiles, Alkalophiles, Barophiles, Steady state growth, Spectrophotometer
8. What protective mechanisms do aerobic micro-organisms against oxygen toxicity?
9. Expand the following: OD, SOD, CFU, ATCC, MTCC, VBNC
10. Which bacteria are important for food and dairy industry and why?
11. Give the relation between growth rate and generation time.
12. What are the various direct methods of measuring microbial growth? Why is plate count method preferred over other methods?
13. Although psychrophiles and thermophiles do not grow at normal body temperature, they might still be hazardous to humans. Can you think how and why this is possible?
14. If you have been given a mixture of micro-organisms, what techniques will you apply to obtain a pure culture of a certain bacteria from it.

## Glossary

**Aerobe:** An organism that requires oxygen for growth.

**Agar:** Agar is a polysaccharide derived from marine red algae that is used as a non-nutritive, solidifying agent in culture media.

**Anaerobe:** An organism that grows in absence of oxygen, dies in presence of oxygen.

**Autotroph:** An organism that utilizes carbon dioxide as the sole carbon source for synthesizing all cell material.

**Barophile**: A micro-organisms that can tolerate high hydrostatic pressure.

**Chemostat:** A apparatus used to maintain organisms in continuous culture by feeding fresh medium and removing spent medium at the same rate.

**Colony:** A macroscopic, visible mass of cells on a solidified medium arising from a single cell.

**Complex medium:** A chemically undefined culture medium composed of complex sources such as yeast extract, beef extract, meat extract etc.

**Generation time:** It is the time required for a microbial population to double in number.

**Halophile:** A micro-organism that can tolerate high salt concentration.

**Heterotroph:** An organism that uses pre-formed organic compounds as a source of carbon and energy.

**Lag phase:** The first phase of the growth curve just after inoculation of the culture when there is no increase in cell number.

**Logarithmic (log) phase:** The phase of the growth curve where the microbial population increases at an exponential rate.

**Microaerophile:** A micro-organism that grows in presence of lower concentrations of oxygen only.

**Psychrophile:** A cold loving micro-organism that grows optimally at 15$^{o}$C or lower.

**Pure culture:** A population of cells that is identical as they arise from a single cell.

**Stationary phase:** The phase of the growth curve when population growth ceases and plateau is reached.

**Synthetic medium**: A culture medium whose exact chemical composition is known.

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## Suggested Readings

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## Web Links

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