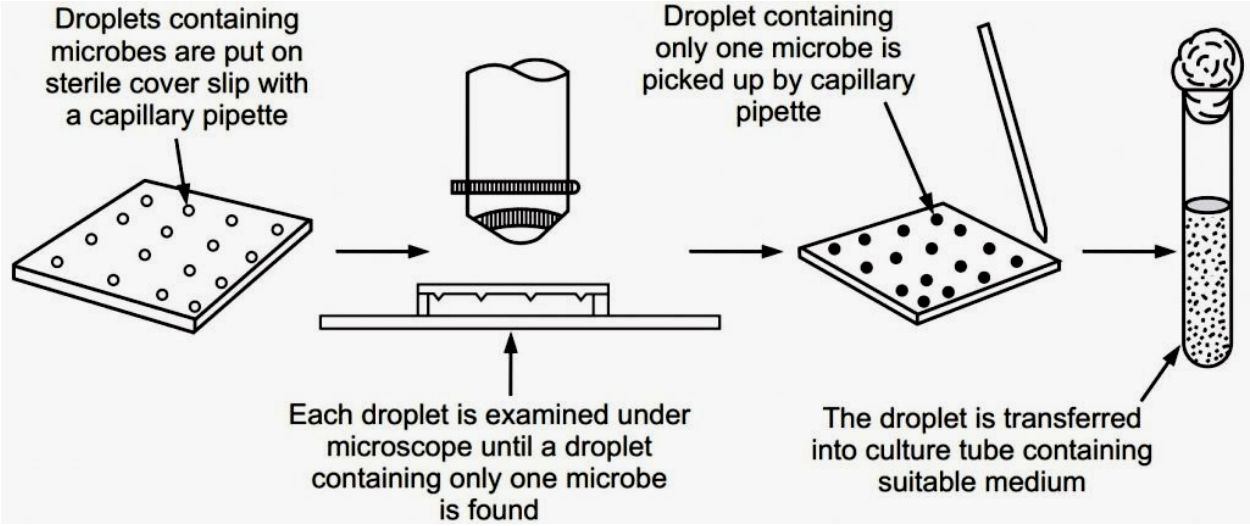
UNIT-III PURE CULTURE TECHNIQUES

Single Cell Isolation Methods

- **Capillary pipette method:**

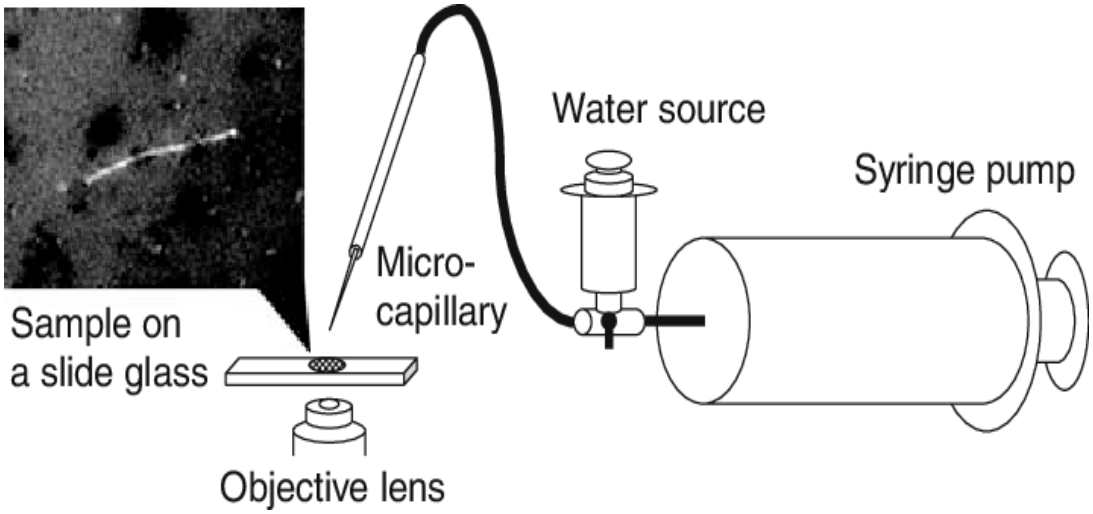
1. Several small drops of a suitably diluted culture medium are put on a sterile glass-coverslip by a sterile pipette drawn to a capillary.
2. Each drop under the microscope is examined until one finds such a drop, which contains only one microorganism.
3. This drop is removed with a sterile capillars pipette to fresh medium. The individual microorganism present in the drop starts multiplying to yield a pure culture.



- **Micromanipulator method: -**

1. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.
2. The micro-manipulator has micrometer adjustments by means of which its micropipette can be moved right and left, forward, and backward, and up and down.
3. A series of hanging drops of a diluted culture are placed on a special sterile coverslip by a micropipette.
4. Now a hanging drop is searched, which contains only a single microorganism cell.

5. This cell is drawn into the micropipette by gentle suction and then transferred to a large drop of sterile medium on another sterile coverslip.
6. When the number of cells increases in that drop as a result of multiplication, the drop is transferred to a culture tube having suitable medium. This yields a pure culture of the required microorganism.



❖ PURE CULTURE TECHNIQUES

Development of pure culture

"A population of organisms cultivated in a medium is called as a culture". While a culture that contain only one species of microorganism is known as a *pure* or *axenic* culture OR A population of cells arising from a single cell is called as a pure culture.

While mixed flora is the rule of nature meaning is that the natural ecosystem like soil, sewage, milk, urine contains mixed population of several species of micro-organisms.

Historical background

Antony Van Leeuwenhoek, 'Father of microbiology[1] in 1863 1^{SI} time observed mixed flora in natural samples like faeces, urine, sewage etc. In the earliest period microbiologists had faced many problems because of contamination, during their research. Later on Joseph Lister, a 'pioneer of aseptic surgery' first time developed a method of isolation of single desired bacteria in pure form by successive dilution of sample using a sterile fluid.

Later on in 1872, a microbiologist 'Schroeter' observed a growth in the form of compact masses of various colors on the slice of decaying potato. While when he observed such masses, microscopically he found that each mass contain single type of organisms. Thus he gave a clue that, it is possible to isolate the organism in pure form by the use of solid media.

Then in 1880 Robert Koch had succeeded in using gelatin as a solidifying agent. Nutrient solution with 5 to 10% gelatin can be used as a solid medium for the growth of organisms. But gelatin has two major drawbacks as -

• In summer days, gelatin melts (at 28^0C), thus can't work as a solidifying agent.
• Being proteinic in nature, it can be utilized (or liquefied) by many micro-organisms as a nutrient.

By that time many scientists had gathered to Koch's lab from all over the world. Dr. Walther Hesse was one of them, who was working with air borne organisms. He was frustrated by gelatin's nature. At that time his wife Fran Fanny Hesse had suggested her husband to use agar-agar as a solidifying agent instead of gelatin. Agar is a polysaccharide derived from sea weed. At that time it was used for stabilizing jellies, thickening of soups. The agar was found to be as a perfect solidifying agent. Since then up to date agar is successfully used as solidifying agent.

Therefore isolation of pure culture is nothing but the gain of isolated colony of desired organism on the surface of agar. Before that one fact should be kept in mind that a single colony is formed from the population of single organism.

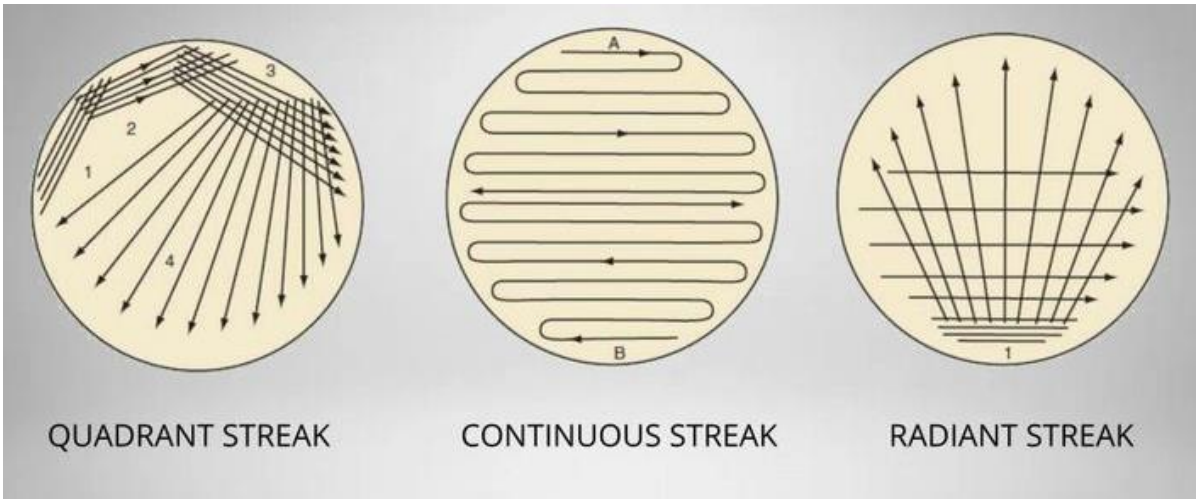
Techniques of pure culture

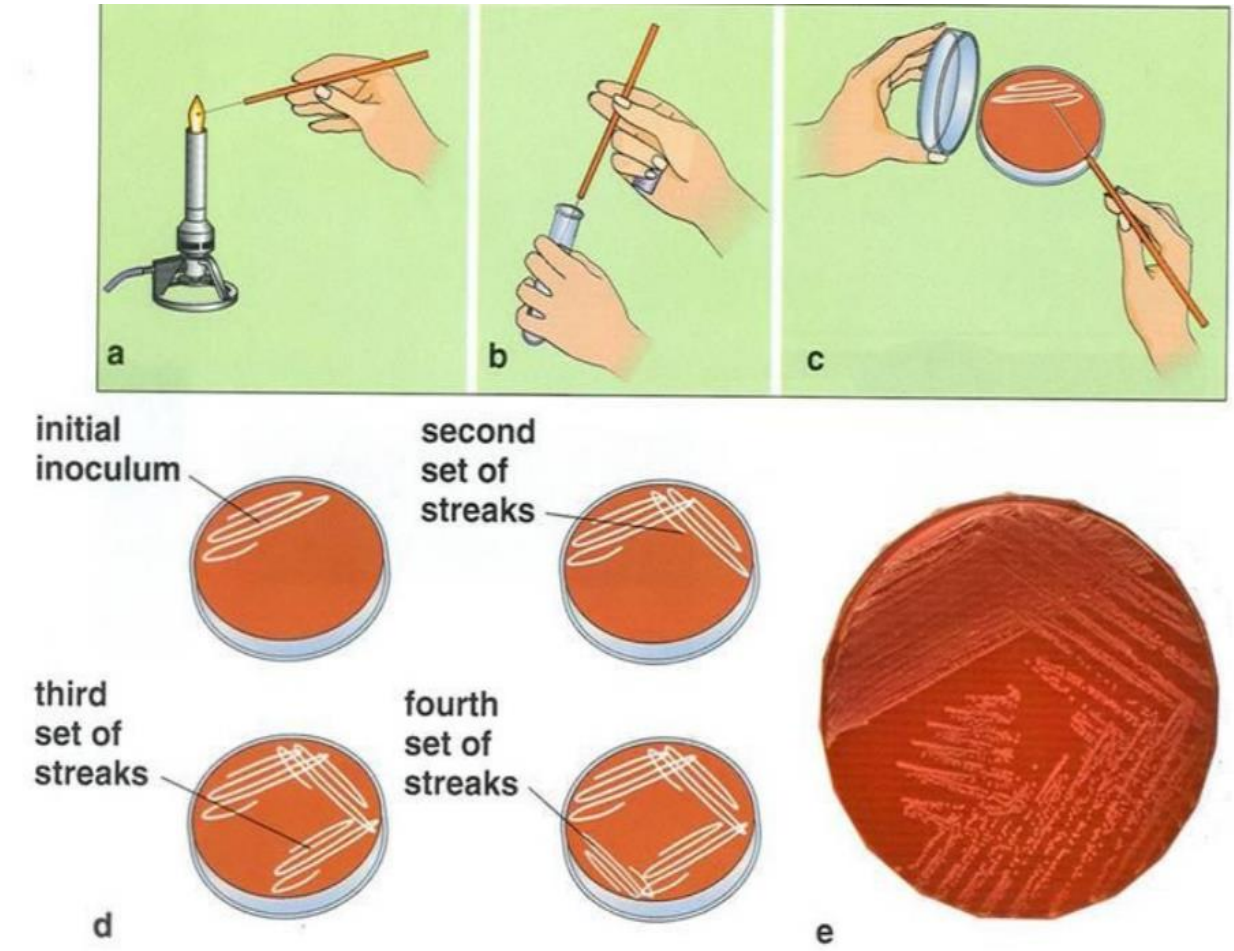
There are four main techniques by which one can get a pure culture.

1. Streak plate technique.
2. Pour plate technique.
3. Spread plate technique.
4. Single cell isolation

1. Streak Plate Method

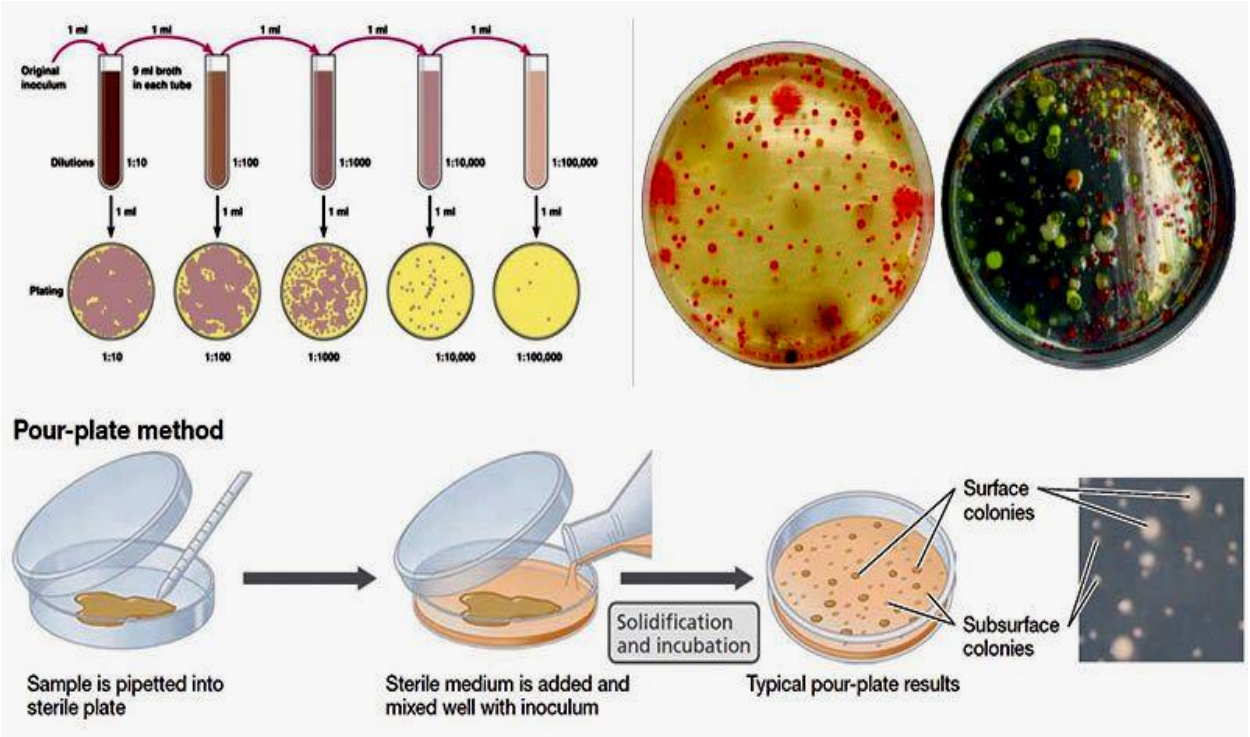
1. The method was developed by Loeffler and Gaffkey in the laboratory of Robert Koch.
2. Streak plate method is the most commonly used method to isolate pure cultures.
3. Principle of streak plate method is continuous dilution of the microbes, resulting in separation of individual cells.
4. The streak plate method depends on spatial separation of single cells.
5. Wire loop called inoculation needle consists of a wooden or glass handle with a nichrome wire, the end which is bend to form a loop, is used to transfer microbes from culture broth.
6. Wire loop is sterilized by holding it in flame until it becomes red hot.
7. The mixed microbial culture is transferred to the edge of an agar plate with the help of sterile nichrome wire loop.
8. Then a series of parallel non-overlapping streaks are made in some specific pattern over the surface of the nutrient medium with the help of sterile inoculating loop as illustrated in Figure.
9. Different patterns can be used for streaking. Common ones are quadrant (four way streaks), continuous streak and radiant streak methods, which are shown in Figure.
10. Then plates are incubated at optimum temperature for suitable time in incubator. These cells form isolated colonies on nutrient agar plate medium.
11. Each colony arises from single cell hence it contains cells of single species called as pure culture.
12. The single colony is picked up with sterile wire loop and suspension is made by adding it in sterile saline solution.


1
2
3
4
A
B
1
QUADRANT STREAK
CONTINUOUS STREAK
RADIANT STREAK


a
b
c
initial inoculum
second set of streaks
third set of streaks
fourth set of streaks
d
e

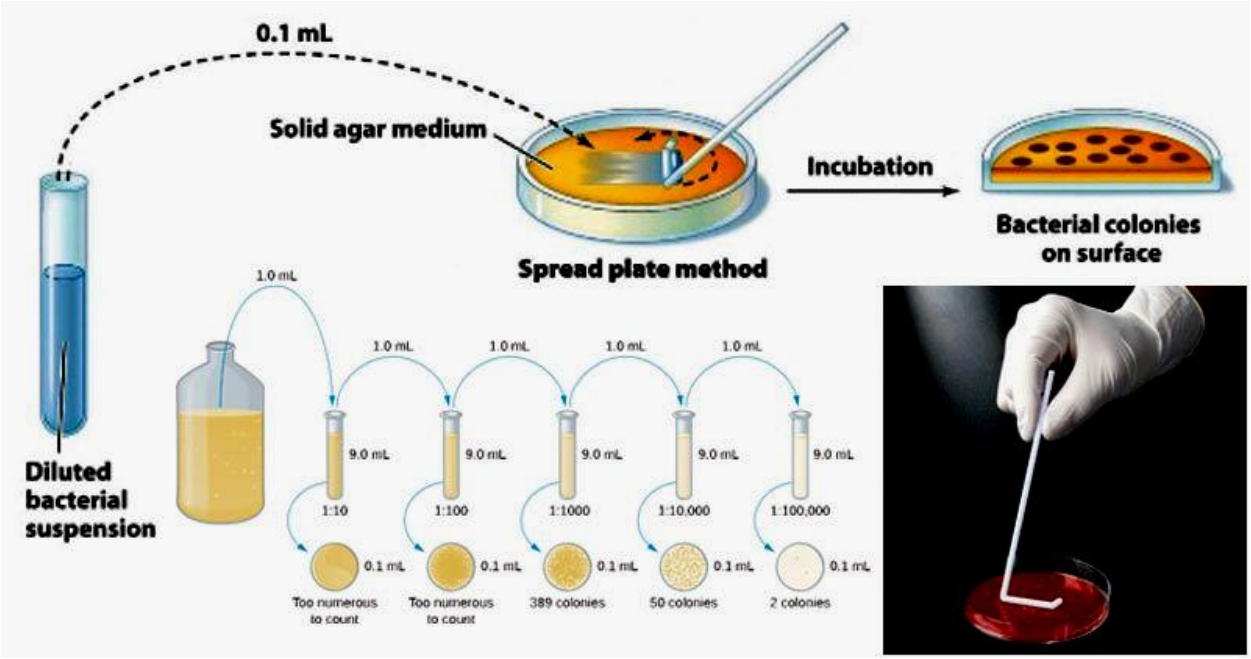
2. Pour Plate Method

1. The method involves mixing of small volume of microbial suspension with molten nutrient agar at 45^{o} C and pouring immediately into sterile petri plate, as shown in Figure.
2. The microbial suspension should be diluted sufficiently to obtain separate colonies on plating.
3. The pour plate method involves adding specified amount (0.1 ml or 1.0 ml) of the dilution to the sterile petri plate.
4. Twenty to twenty five (20-25) ml of nutrient agar medium kept liquefied in a water bath at 45^{o} -50^{o} C is then added to the sterile plate and mixed with the dilution properly by gentle rotation of plate in a circular motion on the table top.
5. This results in uniform distribution of microorganisms. Once the agar has hardened, each cell is fixed in place and forms a distinct colony on incubation.
6. Colonies will appear both within the nutrient agar, as well as, on the surface of agar plate. Look at Figure to see how the colonies look.
7. Colonies obtained at different dilutions are also highlighted in the Figure. Colonies growing on the surface can be used to inoculate fresh medium for pure cultures.



3. Spread Plate Method –

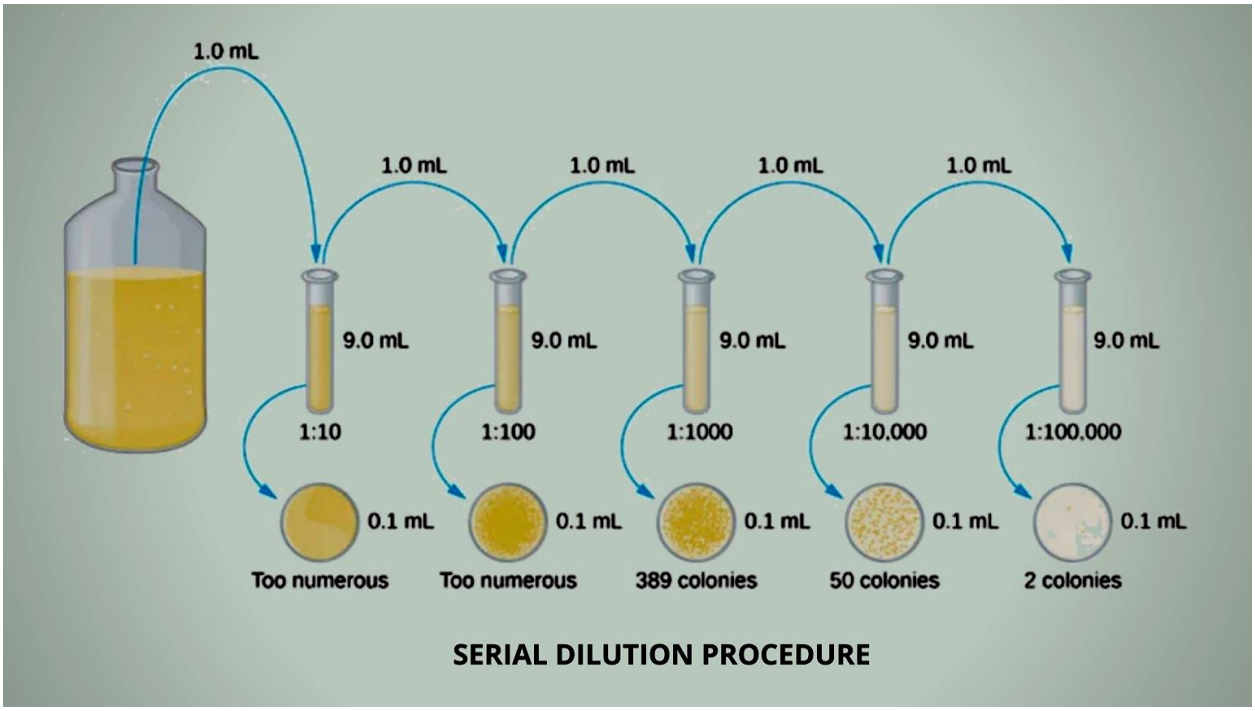
1. In spread plate method, a small volume of the diluted sample (about 0.1 ml) is transferred to the center of a pre-poured solidified agar plate and then spread uniformly over the surface of the medium with a sterile L-shaped glass rod or spreader.
2. After incubation, the dispensed cells form isolated colonies on agar surface, the number of which is used to calculate the amount of microbes in a given sample.
3. Colonies growing on the surface can be used to inoculate fresh medium for pure cultures.



4. Serial Dilution Method

1. Serial dilution is a laboratory technique, in which a stepwise dilution process is performed on a solution with an associated dilution factor. In the laboratory, this method is used to decrease the counts of cells within a culture to simplify the operation.
2. In serial dilution, the cell count or density gradually decreases as the serial number increases in each step. This makes it easier to calculate the cell numbers in the primary solution by calculating the total dilution over the whole series.
3. 5 test tubes containing sterile 9 ml saline in each test tube are taken.

4. With the help of sterile pipette 1 ml sample is taken and added in first test tube and shaken well. The dilution becomes 1: 10
5. Then 1ml sample from the first test tube is added in second test tube and shaken well. The dilution becomes 1: 100
6. These steps are repeated for remaining test tubes. The dilution becomes 1: 1000, 1: 10,000, 1: 100000 etc.
7. Then 1 ml from each test tube is added on sterile Nutrient Agar plate and sample is spread with the help of sterile spreader and plates are incubated at particular temperature for desired time and the plates showing isolated colonies are used to prepare pure cultures.



5. Enrichment Culture Method

1. In this technique conditions for growth are made very favorable for an organism of interest and unfavorable for non-desired organisms.
2. Mixed microbial population is inoculated in a medium with a defined (but limited) chemical composition and allowed to grow under controlled conditions (temperature, air supply, light, pH, etc.).
3. These conditions only suit the growth of a particular type of microorganisms with specific characteristics.

Significance of Pure Culture

Pure culture is needed for -

- Identification of species
- Large scale production of any desired product.
- The study of molecular structure and biochemical characters of desired organism.

HANDLING OF PATHOGENIC MICROORGANISMS

1. Treat all microorganisms as potential pathogens. While the majority of microorganisms are not pathogenic to humans and have never been shown to cause illness, under unusual circumstances a few microorganisms that are not normally pathogenic can act as pathogens. Treat all microorganisms—especially unknown cultures—as if they were pathogenic. A student who has a compromised immune system or has had a recent extended illness should talk with the instructor before working in the microbiology laboratory.
2. Sterilize equipment and materials. All materials, media, tubes, plates, loops, needles, pipettes, and other items used for culturing microorganisms should be sterilized by autoclaving. Otherwise, use commercially sterilized products. Understand the operation and safe use of all equipment and materials needed for the laboratory.
3. Disinfect work areas before and after use. Use a disinfectant, such as a 10% bleach or 70% ethanol solution, to wipe down benches and work areas both before and after working with cultures. Also be aware of the possible dangers of the disinfectant, as 70% ethanol can catch fire around open flame or high heat sources. Bleach, if spilled, can ruin your clothing. Either alcohol or bleach can be dangerous if splashed in the eyes. Students should know where the nearest eyewash station and sink are located.
4. Wash your hands. Use a disinfectant soap to wash your hands before and after working with microorganisms. Non-disinfectant soap will remove surface bacteria and can be used if disinfectant soap is not available. Gloves may be worn as extra protection.
5. Never pipette by mouth. Use pipette bulbs or pipetting devices for the aspiration and dispensing of liquid cultures.

6. Do not eat or drink in the lab, nor store food in areas where microorganisms are stored. Never eat or drink in the laboratory while working with microorganisms. Keep your fingers out of your mouth, and wash your hands before and after the laboratory activity. Cover any cuts on your hands with a bandage. Gloves may be worn as extra protection.
7. Label everything clearly. All cultures, chemicals, disinfectant, and media should be clearly and securely labelled with their names and dates. If they are hazardous, label them with proper warning and hazardous information.
8. Autoclave or disinfect all waste material. All items to be discarded after a class, such as culture tubes, culture plates, swabs, toothpicks, wipes, disposable transfer needles, and gloves, should be placed in a biohazard autoclave bag and autoclaved 30 to 40 minutes at 121° C at 20 pounds of pressure. If no autoclave is available and you are not working with pathogens, the materials can be covered with a 10% bleach solution and allowed to soak for at least 1 to 2 hours.
9. Clean up spills with care. Cover any spills or broken culture tubes with a 70% ethanol or 10% bleach solution; then cover with paper towels. After allowing the spill to sit with the disinfectant for a short time, carefully clean up and place the materials in a biohazard autoclave bag to be autoclaved. Wash the area again with disinfectant. Never pick up glass fragments with your fingers or stick your fingers into the culture itself; instead, use a brush and dustpan. If working with animal or plant pathogens keep the area clear and notify your instructor.

METHODS FOR DISPOSAL OF MICROBIAL WASTE

Biological waste is any material that contains or has been contaminated by a biological agent. Biological waste includes, but is not limited to, Petri dishes, pipettes, tissue culture flasks, syringes, needles, and cell culture media. All biological waste (noninfectious and infectious) generated during laboratory research must be deactivated by autoclaving or chemically treated before disposal.

SHARPS CONTAMINATED WITH BIOLOGICAL WASTE

Sharps are items that are capable of puncturing, cutting or abrading the skin, e.g., needles, scalpel blades, slides and cover slips. Sharps are deactivated by

autoclaving. Place sharps in a container that is red, rigid, puncture resistant, leak-proof and labeled with the biohazard symbol.

- Autoclave your sharps container for a minimum of 30 minutes at 121°C and 15psi
- Log the autoclave run duration, quantity of processed waste, date, and operator
- Label the sharps container with the words “autoclaved”
- Deface any biohazard symbols

SOLID WASTE

Solid biological waste, e.g., pipettes, tissue culture flasks, and multiple well plates, is typically deactivated by autoclaving

1. Collect solid biological waste directly into autoclavable bags
2. Tie a knot using the upper third of the bag and affix heat sensitive indicator tape near the knot
3. Use a secondary container for all autoclave bags until disposal
4. Ensure the autoclave operates for 30 minutes at 121°C and 15psi
5. Log the autoclave run duration, quantity of processed waste, date, and operator
6. Deposit the bag in the red-lidded totes designated for laboratory waste

LIQUID WASTE

Liquid wastes, e.g., cell culture media and serum, are deactivated either by autoclaving or chemical disinfection. Most liquid wastes can be deactivated with bleach.

1. Chemically disinfect with a 1:10 final dilution (vol/vol) of household bleach
2. Swirl flask contents and allow a contact time of 30 minutes
3. Pour down a sink drain connected to the campus sewage system and flush the plumbing with an excess of water
4. Alternatively, liquid waste may be autoclaved for 30 minutes at 121°C and 15psi

METHODS FOR DISPOSAL OF MICROBIAL WASTE

1. Autoclaving

The process of autoclaving involves steam sterilization. Instead of incineration, which can be expensive, autoclaving simply introduces very hot steam for a determined amount of time. At the end of the process, microorganisms have been completely destroyed. This process is particularly effective because it costs much less than other methods, and doesn't present any personal health risks. While some biomedical waste isn't able to be disposed of via autoclaving, around 90% of materials are sanitized this way before being sent on to a landfill.

2. Incineration

The major benefits of incineration are that it is quick, easy, and simple. It effectively removes the waste entirely, and safely removes any microorganisms. However, when burning hazardous materials, emissions can be particularly dangerous. Some states prefer for waste disposal companies to look towards incineration as their first choice, but materials must be reviewed and determined as safe to burn.

3. Chemical disinfection

When it comes to liquid waste, a common biomedical waste disposal method can be chemical disinfection. Chlorine is a regular choice for this process, and is introduced to the liquid waste in order to kill microorganisms and pathogens. Chemical disposal can also be used for solid wastes, but it is recommended that they be grinded first to ensure maximum decontamination. Liquid waste, once decontaminated, is then disposed into the sewer system.

4. Microwaving

During this process, waste is shredded, mixed with water, and then internally heated to kill microorganisms and other harmful elements. One of the main benefits of this process is the shredding aspect; it lowers the volume of biomedical waste, and it is reportedly more energy efficient to use this method

than to incinerate. While it can't be used for all biomedical wastes, it can be utilized for a good 90% of it, just like autoclaving.

❖ MEASUREMENT OF MICROBIAL GROWTH

Microbial growth to determine growth rates and generation times can be measured by different methods. Since growth leads to increase both the number and the mass of the populations, either of the two may be followed. It is necessary to make it clear that no single technique is always best; the most appropriate approach depends upon the experimental situation. It is done by—

A) Measurement of Cell Numbers

B) **Measurement of Cell Mass**

A) Measurement of Cell Numbers

1. **Breed Method (Direct Microscopic Count) DMC**
2. **Counting Chamber Technique**
3. **Viable Count**
4. **Coulter Counter**
5. **Membrane-Filter Technique**

1. Breed Method (Direct Microscopic Count) DMC

A known volume of microbial cell suspension (0.01 ml) is spread uniformly over a glass slide covering a specific area (1 sq. cm). The smear is then fixed by heating, stained and examined under oil immersion lens, and the cells are counted. Customarily, cells in a few microscopic fields are counted because it is not possible to scan the entire area of smear. The counting of total number of cells is determined by calculating the total number of microscopic fields per one square cm. area of the smear. The total number of cells can be counted with the help of following calculations:

a) Area of microscopic field = πr^2

r (radius oil immersion lens) = 0.08 mm.

Area of the microscopic field under the oil immersion lens

$= \pi r^2 = 3.14 \times (0.08 \text{ mm})^2 = 0.02$ sq. mm.

b) Area of the smear one sq. cm. = 100 sq. mm.

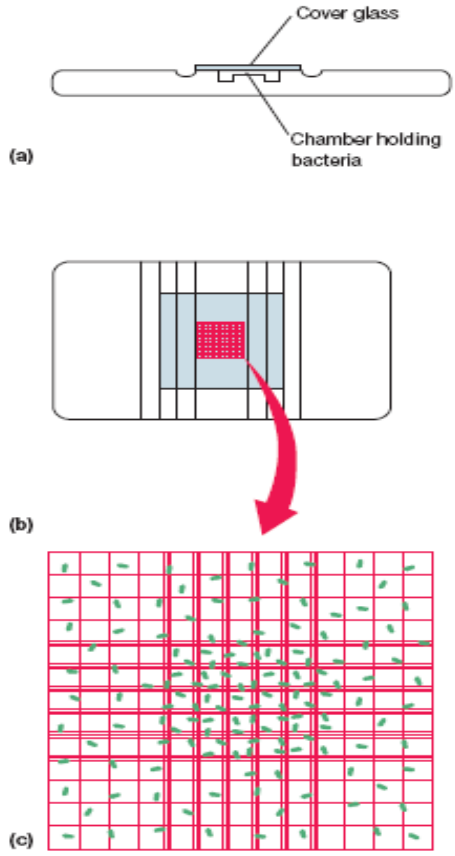
Then, the no. of microscopic fields = 100 / 0.02= 5000

c) No. of cells 1 sq. cm. (or per 0.01 ml microbial cell suspension) = Average no. of microbes per microscopic field x 5000

The direct microscopic method is easy, inexpensive and relatively quick to count microbial cell number. However, using this method, dead cells are not distinguished from living cells and also very small cells are usually missed.

2. Counting Chamber Technique

The number of cells in a population can be measured by taking direct microscopic count using **Petroff-Hausser counting chamber** (for prokaryotic microorganisms) or **hemocytometers** (to larger eukaryotic microorganisms). Prokaryotic microorganisms are more easily counted if they are stained or if phase contrast of florescence microscope is employed. These are specially designed slides that have chambers of known depth with an etched grid on the chamber bottom. Each square on the grid has definite depth and volume. Total number of microorganisms in a sample can be 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).



The Petroff-Hausser Counting Chamber.

a) Side view of the chamber showing the cover glass and the space beneath it that holds a bacterial suspension.

b) A top view of the chamber. The grid is located in the center of the slide.

c) An enlarged view of the grid. The bacteria in several of the central squares are counted, usually at x400 to x500 magnifications.

The average number of bacteria in these squares is used to calculate the concentration of cells in the original sample. Since there are 25 squares covering an area of 1 mm^2, the total number of bacteria in 1 mm^2 of the chamber is (number/square) x (25 squares). The chamber is 0.02 mm deep and therefore,
Bacteria / mm3 = (bacteria/square) x (25 squares) x (50). {1/0.02 = 100/2 = 50}
The number of bacteria per cm^3 is 10^3 times this value. For example, suppose the average count per square is 28 bacteria:
bacteria / cm3 = (28 bacteria)(25 squares)(50)(10^3) = 3.5×10^7.

3. Viable Count / Standard Plate Count (SPC)

The bacterial culture need not contain all living cells. There might be few dead as well. Only living cells will form colony when grown in proper solid medium and under standard set or growth conditions. This fact is used to estimate number of living or dead bacterial cells (viable count) in the given culture. Estimates thus obtained are expressed as a colony forming unit (CFU).

Viable count technique is very much useful in the dairy industry and the food industry for quantitative analysis of milk and spoilage of food products. For convenience, to obtain a colony count for bacteria in milk, 1 ml of well mixed milk is placed in 99 ml of sterile dilute solution (may be water or nutrient broth or saline solution).

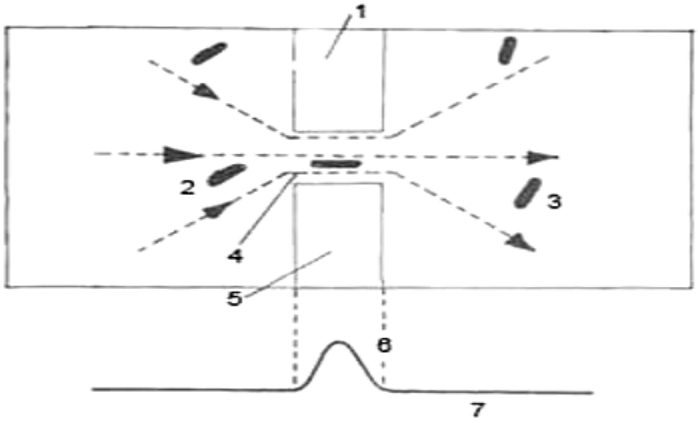
This results in dilution of 1: 100 or 1×10^{-2}. To the petri dish containing pre solidified medium 1 ml of 1: 100 dilution is transferred and incubated at desired is repeated for the preparation of further dilution as 1 : 1000 or 1 : 10, 0000 of bacteria per ml in original sample can be found by multiplying bacterial colony count by the reciprocal of the dilution and of the volume used.

For Example, CFU = 50 for 1: 10, 000 if volume used is 1 ml then,
CFU = $50 \times 10, 000 \times 1$
CFU = 5×10^5

4. Coulter Counter

Coulter counter is an electronic device used to count number of microbes preferably protozoa microalgae and yeasts. In This method, the sample of microbes is forced through a small orifice (small hole). On the both sides of the orifice, electrodes are present measure the electric resistance or conductivity when electric current is passed through the orifice. Every time a microorganism passes through the orifice, electrical resistance increases or the conductivity drops and the cell is counted. The Coulter counter gives accurate results with larger cells. The precaution to be taken in this method is that the suspension of samples should be free of any cell debris or other extraneous matter.

Coulter Counter



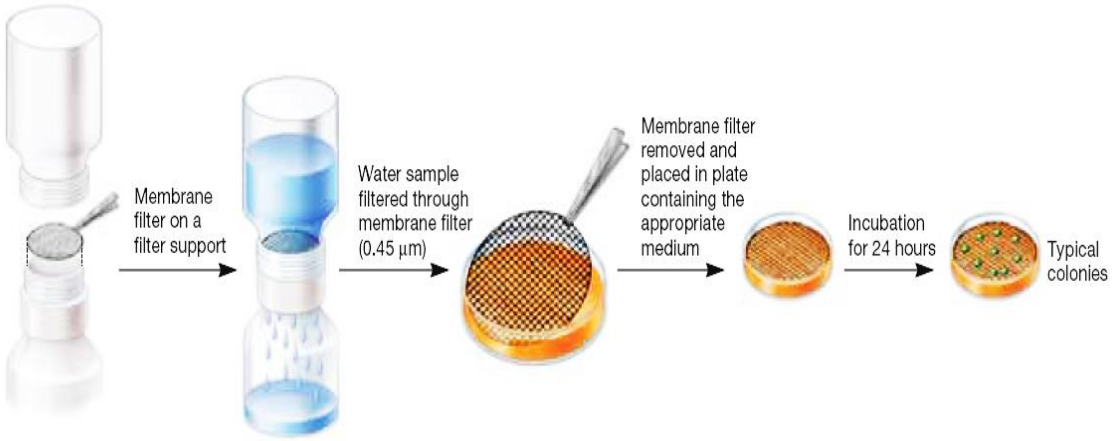
1. Electrode **2**. Flow of Suspending Fluid **3**. Bacterial Cell **4.** Orifice

5. Electrode **6.** Particle Location **7.** Measurement of Voltage

1. **Membrane-Filter Technique**

Microbial cell numbers are frequently determined using special membrane filters possessing millipores small enough to trap bacteria. In this technique a water sample containing microbial cells passed through the filter. The filter is then placed on solid agar medium or on a pad soaked with nutrient broth (liquid medium) and incubated until each cell develops into a separate colony. Membranes with different pore sizes are used to trap different microorganisms. Incubation times for membranes also vary with medium and the microorganism. A colony count gives the number of microorganisms in the filtered sample, and specific media can be used to select for specific microorganisms. This technique is especially useful in analyzing aquatic samples.

Steps of Membrane Filter Technique



B) Measurement of Cell Mass

1. Dry Weight Technique

2. Measurement of nitrogen content

3. Measurement of Turbidity (Turbidometry)

4. McFarland standards (Barium chloride)

1. Dry Weight Technique

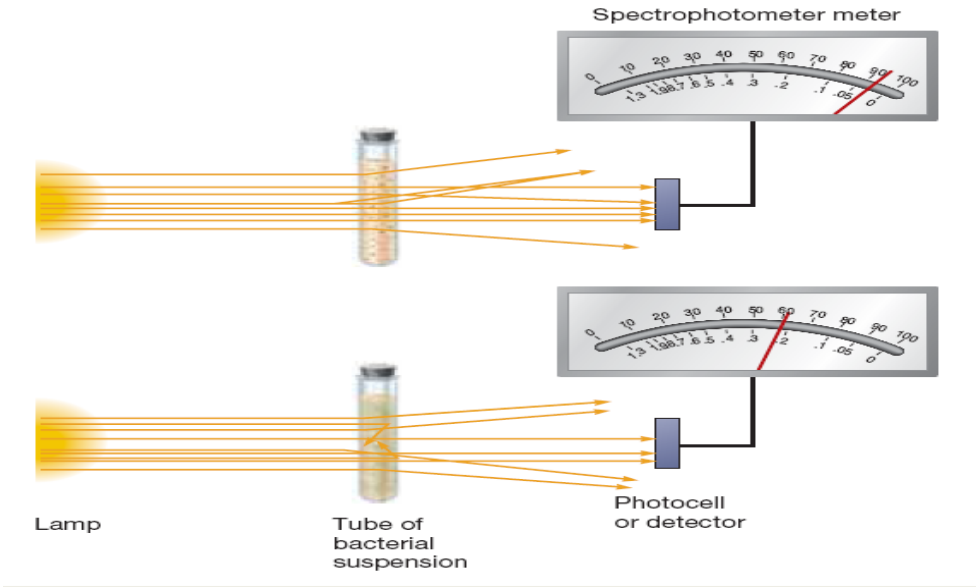
The cell mass of a very dense cell suspension can be determined by this technique. In this technique, the microorganisms are removed from the medium by filtration and the microorganisms on filters are washed to remove all extraneous matter, and dried in desiccator by putting in weighing bottle (previously weighted). The dried microbial content is then weighted accurately. This technique is especially useful for measuring the growth of micro fungi. It is time consuming and not very sensitive. Since bacteria weigh so little, it becomes necessary to centrifuge several hundred millions of culture to find out a sufficient quantity to weigh.

2. **Measurement of nitrogen content**

As the microbes (bacteria) grow, there is an increase in the protein concentration (i.e. nitrogen concentration) in the cell. Thus, cell mass can be subjected to quantitative chemical analysis methods to determine total nitrogen that can be correlated with growth. This method is useful in determining the effect of nutrients or antimetabolites upon the protein synthesis of growing culture.

3. **Measurement of Turbidity (Turbidometry)**

Rapid cell mass determination is possible using turbidometry method. Turbidometry is based on the fact that microbial cells scatter light striking them. Since the microbial cells in a population are of roughly constant size, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. One visible characteristic of growing bacterial culture is the increase in cloudiness of the medium (turbidity). When the concentration of bacteria reaches about 10 million cells (10^7) per ml, the medium appears slightly cloudy or turbid. Further increase in concentration results in greater turbidity. When a beam of light is passed through a turbid culture, the amount of light transmitted is measured, Greater the turbidity, lesser would be the transmission of light through medium. Thus, light will be transmitted in inverse proportion to the number of bacteria. Turbidity can be measured using instruments like spectrophotometer and nephlometer.



4. **McFarland standards**

In microbiology, **McFarland standards** are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range.

Original McFarland standards were made by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 ml of 1% sulfuric acid (H_2SO_4).

Now there are McFarland standards prepared from suspensions of latex particles, which lengthens the shelf life and stability of the suspensions.

The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension is too turbid, it can be diluted with more diluent. If the suspension is not turbid enough, more bacteria can be added.

McFarland Nephelometer Standards:

McFarland Standard No.	**0.5**	**1**	**2**	**3**	**4**
1.0% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density ($1X10^8$ CFU/ml)	1.5	3.0	6.0	9.0	12.0
% Transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.132	0.257	0.451	0.582	0.669

*at wavelength of 600 nm

❖ Cultivation of microorganisms

About 100 years ago, the technique was developed to grow microbes in pure form in laboratory by removing them from their natural sources. It was a milestone in the journey of microbiology science. Because of this achievement it became easy to do close examination of a microbe and its morphological, physiological and genetical study.

For successful cultivation of organisms in lab, it is necessary to supply all required nutrients. 'Any nutrient preparation employed to grow micro-organism in lab is called as culture medium'. In the construction of a culture medium, primary goal is to provide a balanced mixture of required nutrients for good growth of desired organism. Culture media have a great significance. Following are the different purposes for which the culture media are used.

a) For primary isolation of micro-organisms from their natural sources.
b) To determine the biochemical characters of any organism.
c) To determine the growth characters of desired organism.
d) To maintain the culture in laboratory.
e) The transport media are used to preserve the specimen during the time period between sample collection and clinical analysis.
f) The assay media are used to test the effectiveness of antimicrobial drugs.
g) The enumeration media are used to count number of microbes in milk, soil, food, water etc. and numerous others.

i) An ideal or satisfactory culture medium should fulfill following requirements (Properties of good culture medium)

- It should possess all necessary nutrients including growth factors and vitamins.
- It should have a correct desired pH and should have a provision for maintenance of pH for ex. use of *buffer*.
- It should have proper moisture content.
- It should be sterile in microbiological sense.
- It should have desired physical properties like solid or liquid state, clarity etc.

There is a great diversity in the nutritional requirements of microorganisms. It may be as simple as requiring only a few inorganic compounds or may be as complex as requiring a list of inorganic and organic compounds. Nowadays in modem era, the culture media are commercially available in an instant, dehydrated form. But it was a challenging and difficult task in determining the nutritional requirement of organisms and designing of medium suitable for organism.

Common components of media and their functions:

For preparation of culture media different components are added in different combination. Each component performs specific functions in the culture media. Following are a few commonly used components.

1. Peptone

It is a trypsinised or hydrolysed proteins from animals like meat or casein or proteins from vegetables like soyabean or cottonseeds. Proteins from above sources are hydrolysed by proteolytic enzymes like pepsin, trypsin, papain. Due to hydrolysis, the large complex proteins are broken down to proteoses, peptones, peptides and amino acids. Along with this proteinic part, peptone also possesses carbohydrates, many inorganic micronutrients. Therefore it is the most important ingradient of almost all culture media (synthetic and semisynthetic). Peptone performs many important functions when it is in the culture media as

i) It supplies most available form of nitrogen to bacterial cell.
ii) It also supplies energy source to micro-organisms,
iii) It supplies assimilable form of phosphorus, sulphur and other essential elements,
iv) As amino acids are amphoteric in nature, peptone also works as an excellent buffer prevents drastic changes in pH.

2. **Yeast extract:**

Brewer's yeasts are the major raw material for manufacture of yeast extracts, while the Brewer's yeasts are obtained as a byproduct of brewing

industry. Yeast extracts are produced by autolysis of yeast cells. Autolysis is done by keeping yeast cells in distilled water where the pH of distilled water is adjusted to 6.5. Then the distilled water with yeast cells are heated at 45°C for 14 hours, with intermittent stirring. The autolysis is done under controlled condition to avoid destruction of vitamin of B complex group. Yeast extracts also performs following functions. It is the best growth factor of bacteria. It is a rich source of B vitamin. It is used in culture media by replacing meat extracts.

3. Vitamins

Vitamins are most commonly needed growth factor. Vitamins are defined as an organic compound required in very small amount and cannot be synthesized by same cells. First discovered growth factor is the vitamin. Vitamins are required in very minute amount and they show stimulatory effect on the growth of micro-organisms. They are not an energy source or building blocks of macromolecules. But vitamins play different functions. Most of them are working as a 'co-enzymes' of enzymes. Strict autotrophs can synthesize all vitamins but heterotrophs can't synthesize many of them, thus the growth of heterotrophs depends on the availability of vitamins. Vitamins which are needed must be incorporated in the medium. The culture media can be supplemented with vitamins by the use of yeast extracts, meat extracts, and even peptone.

4. NaCl

NaCl is generally added to culture media, but it is not required for the growth of microorganisms. It also does not work as buffer in the culture medium. NaCl is added to a culture medium to maintain the isotonicity of the medium. Therefore the NaCl is added to medium in the concentration which will be isotonic with the cytoplasm of cells. Such isotonic condition is not only needed to prevent plasmolysis or plasmoptysis but also for transport of nutrients into the cell.

5. Agar agar

It is a dried mucilagenous substance derived from aquatic algae belonging to the genus *Gelidium corneum* and related species. These weeds are found

growing in the water of the coasts of Japan, Srilanka, Malaya, and Southern California. It is an acidic polysaccharide, polymer of galactose with one sulphate per ten to fifty residues of galactose. Though it is a polysaccharide, it is fully indigestable and has no nutritive value. Solid media are found to be more useful in obtaining a pure culture than liquid media. Agar agar is an ideal solidifying agent as it possess following important properties.

- It is nontoxic for bacteria.
- Its fibrous structure is fine enough to prevent motility of bacteria within it.
- At the same time it is coarse enough to permit diffusion of even macromolecules.
- It is transparent and colorless.
- It is insoluble in cold water but it melts at the temperature of boiling and forms a viscous gel, remains liquid until temperature decreases up to body temperature, but once it is cooled to body temperature, then it settles in a solid form, make it suitable for the growth of micro-organisms in the form of compact masses called as colonies.
- It is attacked by only a few bacterial species like *Agrobacterium*, *Vibrio, Cytophoga, Pseudomonas* or otherwise, it is unutilizable form, therefore works as a solidifying agent.
- It is easily sterilizable.
- In the culture media 2 to 3% agar-agar powder is satisfactory.

ii) Types of culture media

Depending upon the nature of ingredients, the culture media are of two types.

1. Living media
2. Nonliving media

1 Living media: There are some microorganisms like Viruses, Rickettsia, Chlamydia etc. which are obligatory intracellular parasites. They cannot be cultivated in lab on synthetic media. They require live cells for their growth. Thus, the media which contain living cells are called as living /media. The living media are mainly of three types -

a. Bird embryos
b. Tissue culture
c. Live animals.

2. Non-living media: These are the media which totally lack living cells, but contain non-living material like any pure organic or inorganic chemical or any natural component like milk, blood etc. Depending upon the nature of ingredient, the non-living media are classified into three sub-types –

a) Natural media
b) Synthetic media
c) Semi-synthetic media.

a. Natural media:

Natural media are also called as impirical media. Natural media are prepared using the ingredients which are available in nature like milk, urine, diluted blood, carrot juice, coconut milk, vegetable juice, etc. Such media were used in early stages of development of microbiology. These media are prepared on the basis of previous experience and not on the basis of knowledge about their exact composition. These media are inexpensive and convenient to use, however, as the exact composition of chemical nature of ingredient is not known, they are not reproducible. These media may not be suitable for cultivation of many important organisms.

b. Synthetic media:

These are the media which contain all ingredients of known chemical composition (nature). Each component of the media is highly pure and the exact amount incorporated in the medium is known. Thus such media are reproducible. Such media are designed according to the knowledge about the exact nutritional requirement of the desired organism. Such standardized and reproducible media are most useful in research and in industries and for cell culture, where exact nutritional need of test organism is known. Synthetic media are of two types –

(i) Inorganic synthetic media,
(ii) Organic synthetic media.

(i) Inorganic synthetic media:

Inorganic synthetic media are the media where all components are in inorganic form. They are used generally for autotrophic organisms. For example - The medium used for the isolation of *Thiobacillus thioxidans.* The chemical composition of the medium is given below -

$(NH_4)_2S0_4$	0.2gm.
$MgSO_4$, $7H_2O$	0.5 gm.
KH_2PO_4	3.0gm.
CaCl,	0.25 gm.
powdered sulphur	10.00 gm.
Distilled water	1000 ml.

It is a typical inorganic synthetic medium. Chemical nature and exact amount of each component of the medium is exactly known. The medium provides all necessary nutrients and energy source for the organism, where CO_2 is the carbon source, $(NH_4)_2SO_4$ is the nitrogen source, powdered sulphur is the energy source, KH_2PO_4 is not only the source of, potassium and phosphate but also works as a buffer. $MgSO_4$, $7H_2O$, $CaCl_2$ supplies necessary inorganic elements needed for growth. *Thiobacillus* synthesizes all components of living cells using these ingradients.

Winogradsky's medium is another example of inorganic synthetic media. It is used for the cultivation of *Nitrosomonas* and *Nitrobacter.*

(ii) Organic synthetic media: These are the media which contain all ingredients in organic form. These media are useful for the cultivation of heterotrophic organisms. Example of this medium is the medium used for the growth of fastidious pathogenic organism - *Corynebacterium diphtherias.* The medium contains 21 different chemically pure organic ingredients and they are in accurately weighed amounts as shown below -

Amino acids - eight
Vitamines - three
Carbohydrates - several
Esters - several
salts of Ca, Mg, Cu, K, P, S, etc. where carbohydrates and esters serve as carbon source.

c. Semi-synthetic media:

The media which contain both natural ingredients and pure chemicals (organic or inorganic) are called as Semi-synthetic media. Natural ingredients include meat extracts, peptone, yeast extracts, blood, casein hydrolysate etc. Such media are also called as complex media, non-synthetic media or undefined media. Examples of semi-synthetic media are nutrient agar, peptone water, blood agar, MacConkey's agar, etc.

Nutrient agar is the most common medium used for the cultivation and enumeration of wide variety of organisms. Peptone water is the most suitable and economical and used for the cultivation of many non-fastidious organisms. It is also used to study the ability of organism to ferment different sugars.

The exact chemical composition of all ingredients of semi-synthetic media is not clearly known. Thus, the media are not exactly reproducible, but they provide rich mixture of nutrients. Therefore, these media are widely used. They allow excellent growth as they provide complex nutritional need of different organisms.

General purpose media are designed to grow a broad spectrum of micro-organisms. They contain mixture of nutrients and support the growth of pathogens and non-pathogens alike. But there are some media which are designed especially for special purpose.

I) ***Enriched media:***

If any general purpose medium or basic medium is added with complex nutritionally rich organic substance like blood, serum, haemoglobin, extracts of plants or growth factors (vitamins, amino acids) etc., then such media are called as enriched media. Such media now become suitable for many fastidious organisms. Fastidious organisms are those which require growth factors and complex nutrients for their growth. Fastidious organisms can't grow on basic medium. Because of addition of nutritionally rich component in the basic medium, it is called as an enriched medium.For example

1) **Blood-agar -** It is prepared by addition of sterile rabbit, sheep or horse blood to a sterile nutrient base. It is suitable for the growth of fastidious organisms like *Streptococcuspyogenes.*

2) **Chocholate agar-** It is best for the growth of pathogenic fastidious organism *Neisseria gonorrhea.* (Red blood cells that have been lysed by slowly heating to 80°C)

II) *Enrichment media:*

When anyone wants to isolate a single desired organism from a natural sample with a large microbial population, where number of desired organism is very less, and then the sample is added to a special medium which stimulates or favors the growth of only desired organism and preventing the growth of other unwanted organisms. Then such medium is called as enrichment medium. The technique was first time used by Beijerinck and Winogradsky. They used a salt solution with $NaNO_2$ at pH 8.5 as a nutrient medium; it is then added with soil sample and incubated at room temperature. It resulted in the enrichment of a *Nitrobacter* species from soil.

Enrichment media are generally liquid media. Example –

(1) Tetrathionate broth - It is enrichment medium for *Salmonella* group of organisms. The faeces from the patient suffering from typhoid, if inoculated in tetrathionate broth, then it results in the enrichment of *Salmonella* group of organism, simultaneously preventing the growth of other intestinal organisms.

(2) Medium lacking organic or inorganic nitrogen that is nitrogen free mannitol salt agar is best for nitrogen fixing *Azotobacter* organism from soil.

III) Selective media:

Selective media contains one or more agents (chemicals), that inhibit the growth of different micro-organisms (say A, B, C) and do not inhibit the growth of anyone desired organism (say 'D'). Thus, this medium favours or selects or encourages the growth of only organism 'D'. Selective media are very important in primary isolation of specific organism from natural samples (faeces, urine, saliva, skin, water and soil) containing a dozens of different species.

The isolation is done by suppressing the growth of unwanted background organisms and favoring the growth of only desired organism. Examples –

1) Mannitol salt agar - It contains 7.5% NaCl, which is inhibitory for the most human pathogens except *Staphylococcus aureus.* Therefore it is a selective medium for *Staphylococcus aureus.*

2) MacConkey's agar - It contains bile salt, which is a constituent of human intestine and faeces. It inhibits the growth of most of the Gram positive and non-intestinal organism, simultaneously allowing the growth of only intestinal organisms. Therefore MacConkey's agar is a selective medium for intestinal organisms like *Escherichia coli, Salmonella typhi, Shigella dysentrac-etc.*

Table shows some examples of selevtive media.

Name of medium	**Inhibitory agent**	**Inhibiting the growth of**	**Selective for**
l. Azide blood agar	Azide	Gram negative	*Staphylococcus Streptococcus*
2. MacConkey's agar	bile salt (Sodium taurocholate)	non intestinal	Intestinal
3. Wilson and Blair's medium	Bismuth Sulphite, Brilliant green	Coliforms	Salmonella species

IV) **Differential media:**

The media which allow the growth of different groups of organisms with visible differences in the growth patterns, with the help of which organisms can be differentiated into different groups are called as differential media. Differentiation is observed as differences or variations in the size and colour of colony, in the pattern of colour changes, in the formation of precipitate etc. The variation is achieved because of incorporation of certain chemicals to the medium and also due to the ways by which microbes react to that chemical.

The medium sometime may contain certain particular nutrient and two different types of organisms produce two different types of colonies depending upon the ability of organism to use that specific nutrient. Sometimes dyes or pH

indicator dye is added to the medium as a differentiating agent. It results in the production of different coloured colonies depending upon the product produced for ex. acid or base.

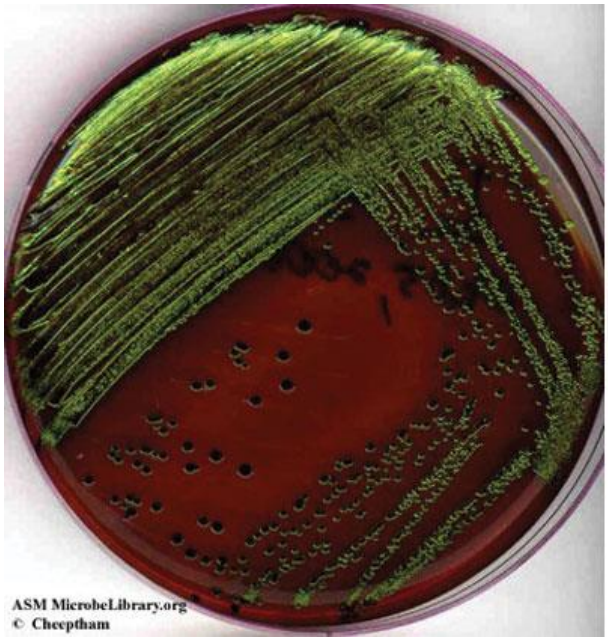
Examples –

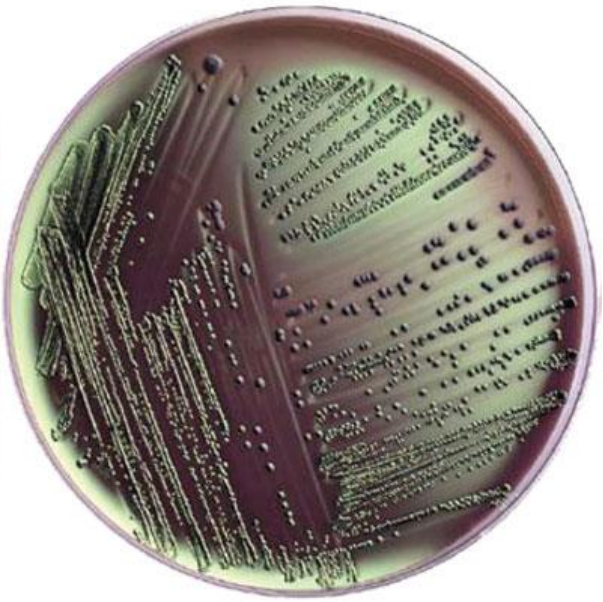
1. MacConkey's agar: The medium helps to differentiate the organisms into two groups as lactose fermenting or lactose non-fermenting. The medium contain lactose as a sugar and neutral red as a pH indicator dye. Neutral red gives pink or red colour in acidic condition and yellow when neutral. If the organism in lactose fermenting, then when ii will ferment lactose, to form organic acids, then pH of medium changes to acidic, which results in production of pink or red coloured colony. On the other hand, if organism unable to ferment lactose, then there is no acid production, no pH change and colonies will remain colourless. *E. coli* can ferment lactose to produce acid, thus colour of colony will be pink. But *Salmonella* can't ferment lactose no colour change, colonies will be off white.

2. Blood agar: It helps to differentiate the organisms into two groups as haemolytic and nonhaemolytic. Because of RBCs in the blood, the medium is red and opaque. If the organisms are haemolytic, there will be lysis of RBCs around the colony; there will be clear zone around the colony. But if organisms are nonhaemolytic, then there will be no lysis of RBCs and no zone of clearance around the colony. *Streptococcus haemolyticus*, which is haemolytic, forms a clear zone around the colony while *Staphylococcus epidermidis* which is non-haemolytic don't form clear zone around the colony.

2. EMB agar (Eosin methylene blue agar)

It helps to differentiates coliforms from noncoliforms. Coliform organisms form a typical that is nucleated black centered colonies with metalic sheen while noncoliforms form atypical that is pink non nucleated, nucoid colonies without metallic sheen. *E.coli* forms typical colonies and *Enterobacteraemgenes* forms atypical colonies.





Role of buffers in culture media

A buffer is an aqueous solution consisting of a mixture of a weak acid and its salt (acidic buffer) or a weak base and its salt (basic buffer). Its pH changes very little when a small amount of strong acid or base is added to it and

thus it is used to prevent changes in the pH of a solution. One example of a buffer solution found in nature is blood. The normal pH of human blood is 7.4.

Acidic buffer solutions

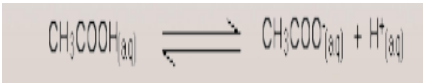
An acidic buffer solution is simply one which has a pH less than 7. Acidic buffer solutions are commonly made from a weak acid and one of its salts - often a sodium salt. A common example would be a mixture of acetic acid and sodium acetate in solution. You can change the pH of the buffer solution by changing the ratio of acid to salt, or by choosing a different acid and one of its salts.

Alkaline (basic) buffer solutions

An alkaline buffer solution has a pH greater than 7. Alkaline buffer solutions are commonly made from a weak base and one of its salts. A frequently used example is a mixture of ammonia solution and ammonium chloride solution. A buffer solution has to contain things which will remove any hydrogen ions or hydroxide ions that you might add to it - otherwise the pH will change. Acidic and alkaline buffer solutions achieve this in different ways.

Acidic buffer solution:

Mixture of acetic acid and sodium acetate is typical Acidic buffer solution. Acetic acid is a weak acid, and the position of this equilibrium will be well to the left:



Adding sodium acetate to this adds lots of extra acetate ions. According to Le Chatelier's Principle, that will tip the position of the equilibrium even further to the left (common ion effect).The solution will therefore contain these important things:

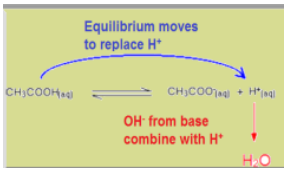
• lots of un-ionised acetic acid;
• lots of acetate ions from the sodium acetate;
• enough hydrogen ions to make the solution acidic.

Other things (like water and sodium ions) which are present aren't important to the argument.

Adding an acid to this buffer solution: The buffer solution must remove most of the new hydrogen ions otherwise the pH would drop markedly. Hydrogen ions combine with the acetate ions to make acetic acid.

That means a strong acid has been turned to a weak acid. Since most of the new hydrogen ions are removed, the pH won't change very much - but because of the equilibrium involved, it will fall a little bit.

Adding a base to this buffer solution alkaline solutions contain hydroxide ions and the buffer solution removes most of these by reacting with H^+ to form water. As soon as this happens, the equilibrium of the acid dissociation tips to replace H^+ ions .This keeps on happening until most of the hydroxide ions are removed. That means a strong base has been turned to water.



Because most of the added hydroxide ions are removed by this way, the pH doesn't increase very much.

Alkaline (basic) buffer solutions

Mixture of ammonia and ammonium chloride solutions is typical Alkaline (basic) buffer solutions. Ammonia is a weak base, and the position of this equilibrium will be well to the left:

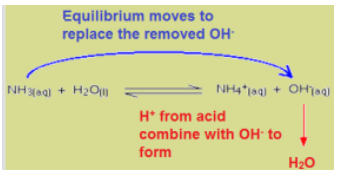
Adding ammonium chloride to this adds lots of extra ammonium ions. According to Le Chatelier's Principle, that will tip the position of the equilibrium even further to the left (common ion effect).

The solution will therefore contain these important things:

- lots of unreacted ammonia;
- lots of ammonium ions from the ammonium chloride;
- Enough hydroxide ions to make the solution alkaline.

Other things (like water and chloride ions) which are present aren't important to the argument.

Adding an acid to this buffer solution the hydrogen ions that you are adding are removed by reacting with OH- (see previous equation) to form water. That means a strong acid has been turned to water.

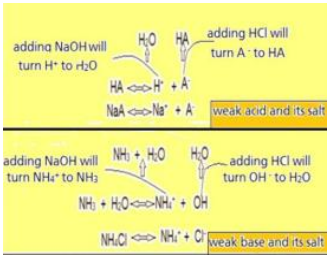


This keeps on happening until most of the hydrogen ions are removed.

Adding a base to this buffer solution. The hydroxide ions from the alkali are removed by a simple reaction with ammonium ions.

That means a strong base has been turned to a weak base. The figure on the left summarizes the mechanism by which the buffer stabilize the pH and the

following table shows some common acids and bases used for the preparation of buffer.



Examples:

In animals, a complex and a vital buffer system is found in the circulating blood. The components of these systems are carbonate, bicarbonate, oxygenated and nonoxygenated hemoglobin and the plasmaproteins. The common laboratory buffers include bicarbonate buffers, phosphate buffers, Tris buffers etc.

Media used for cultivation of Bacteria, Fungi, Actinomycetes, Yeasts, Algae and photosynthetic bacteria

Media used for cultivation of Bacteria

1. **Nutrient agar**

Distilled Water	100 ml
Peptone	2 gm
Yeast extracts	1 gm
OR	
Meat extracts	0.3 gm
NaCl	0.5 gm
Agar agar powder	2.5 gm
pH	7

2. **MacConkey's agar**

Distilled water	100 ml
Peptone	2 gm

Sodium taurocholate	0.5 gm
Lactose	1 gm
pH	7
Neutral red (2 % in 50 % Ethanol)	0.3 ml
Agar agar powder	2.5 gm

Media used for cultivation of Fungi

1. **Sabraud's agar**

Distilled water	100 ml
Glucose	4 gm
Peptone	1 gm
Agar agar powder	2 gm
pH	5.4

2. **Potato Dextrose agar**

Distilled water	100 ml
Potato (peeled)	20 gm
Dextrose	2 gm
Agar agar powder	1.5
pH	5.4

Media used for cultivation of Actinomycetes

1. **Bennet's agar medium**

Distilled water	100 ml
Glucose	1 gm
Casein	0.2 gm
Yeast extracts	0.1 gm
Beef extracts	0.1 gm
Agar agar powder	2 gm
pH	7.3

2. **Dextrose tryptone agar**

Distilled water	100 ml
Glucose	1 gm
Tryptone	0.5 gm
K_2HPO_4	0.05 gm
NaCl	0.05 gm
$FeSO_4. 7H_2O$	0.01 gm
Agar Agar powder	2 gm
pH	7.2

Media used for cultivation of yeasts

1. **Malt extract agar**

Distilled water	100 ml
Malt	1.5 gm
K_2HPO_4	0.1 gm
NH_4Cl	0.1 gm
Citric acid (0.1 N)	1.5 ml
Agar agar powder	2 gm

2. **Penicillin Streptomycin Blood Agar**

Nutrient agar	90 ml
Blood	10 ml
Penicillin	300 units
Streptomycin	300 microgram

Media used for cultivation of algae

1.Chu's medium

Distilled water	1000ml
Calcium nitrate	0.04gm
K_2HPO_4	0.01gm
Na_2CO_3	0.02gm
$MgSO_4.7H_2O$	0.025gm
Sodium Silicate	0.025gm
Ferric citrate	0.003gm
A 5 trace element stock Solution (optional)	1.0 ml

A 5 trace element stock solution

Distilled water	1000ml
Boric acid	2.86gm
$MnCl_2$	1.81gm
$ZnSO_4$	0.222gm
Molibdenum trioxide(85%)	0.177gm
Cupric sulphate	0.079gm
pH	8.5 to 9

1. Modified Bristol's medium

KH_2PO_4	0.50gm
$NaNO_3$	0.50gm
$MgSO_4.7H_2O$	0.15gm
$CaCl_2.6H_2O$	0.05gm
NaCl	0.05gm
$FeCl_3.6H_2O$	0.01gm

Media used for cultivation of photosynthetic bacteria

1.For Thiorhodaceae family

Water	100 ml
NH_4Cl	0.1 gm
KH_2PO_4	0.1 gm
$MgCl_2$	0.1 gm
$NaHCO_3$	0.1 gm
$Na_2S.\ 9H_2O$	0.1 gm
pH	8

1.For Chlorobeacae family

Water	100 ml
NH_4Cl	0.1 gm
NaCl	0.03 gm
KH_2PO_4	0.1 gm
$MgCl_2$	0.05 gm
$NaHCO_3$	0.2 gm
$Na_2S.\ 9H_2O$	0.1 gm
Fe	50 µ g
pH	7.3

Cultivation of anaerobes

The isolation of oxygen was successfully done by the scientist Priestley in 1774. Immediately in 1775 Lavoisier observed the role of oxygen in combustion and respiration. His observation led to conclusion that free air or oxygen is necessary for all life.

However in 1861, Pasteur proved that certain yeasts and bacteria could multiply in absence of air. He devised the term 'anaerobiosis', to describe the life without air. It was one of the epoch making discovery! When further study of physiology of these micro-organisms was done, it was startling to old ideas of cell physiology and biochemistry.

Many micro-organisms living without air were then discovered.

Relation of Micro-organisms to Oxygen

The atmosphere of earth contains 20 % (v/v) of oxygen. All animals and plants need molecular oxygen for respiration. Therefore it was previously thought that there will be no life without oxygen.

However, microorganisms exhibit wide variations in molecular oxygen requirement with respect to their relation to free oxygen. They are classified mainly in 4 groups.

1. Strictly aerobic organisms: These organisms cannot grow in absence of free oxygen. In these organisms oxygen acts as a final hydrogen (electron) acceptor and there is a complete oxidation of food to $C0_2$ and water. Their enzyme systems can transfer hydrogen (electron) only to molecular oxygen during respiration. They obtain maximum energy from food as fuel.

2. Facultative organisms: The facultative can grow aerobically as above or in absence of free oxygen because they can use some other reducible substances like sulphur, carbon or sodium nitrate as final hydrogen acceptor. Thus, they can grow aerobically as well as anaerobically as their enzyme systems can work in both situation. But they grow better in aerobic condition as gaining more energy by complete oxidation of food, while, in anaerobic condition obtain less energy by incomplete oxidation of food (fermentation).

3. Strictly anaerobic organisms: Strict or obligatory anaerobic organisms can grow in absence of oxygen or cannot grow in presence of free molecular oxygen. Oxygen is toxic to them because (i) certain of their enzymes are inactivated (blocked) in presence of free oxygen or (ii) their enzyme system cannot transfer hydrogen (electrons) to free oxygen. Therefore they must be using some alternative hydrogen acceptors. Toxicity of oxygen to strict anaerobes is due to production of certain molecules during oxygen involving reactions.

Ex, Sometime single electron is added to oxygen, resulting in the formation of superoxide radical as shown below.

O_2 + e- ⟶ 0_2^-

oxygen electron superoxide radical

Superoxide itself may damage the cells or may give rise to formation of other toxic molecules like hydrogen peroxide (H_20_2) and hydroxyl radical (OH').
Hydrogen peroxide is highly toxic, therefore strict anaerobes immediately commit suicide by producing H_20_2.

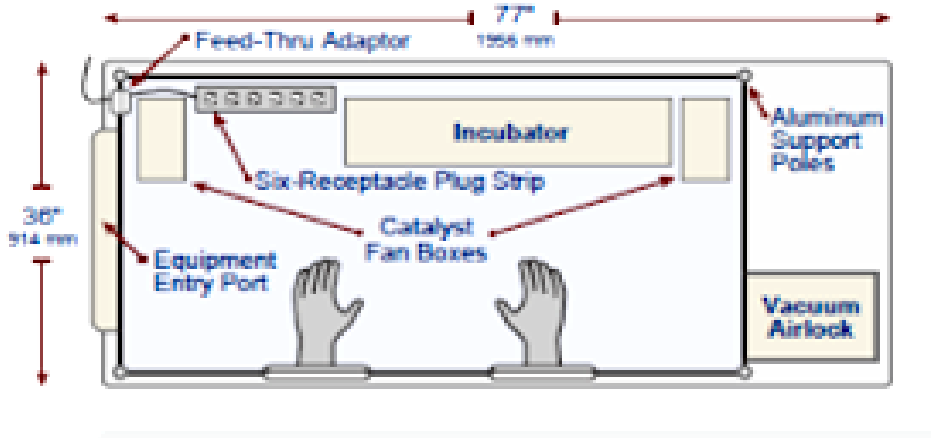
4. Micro-aerophilic organisms: These organisms are not strict anaerobes, but require lower oxygen tension. They require oxygen at very low level (2-10 %) of concentration. They possess enzymes that are inactivated under strong oxidizing conditions and can be maintained in functional state at low pressure of oxygen.

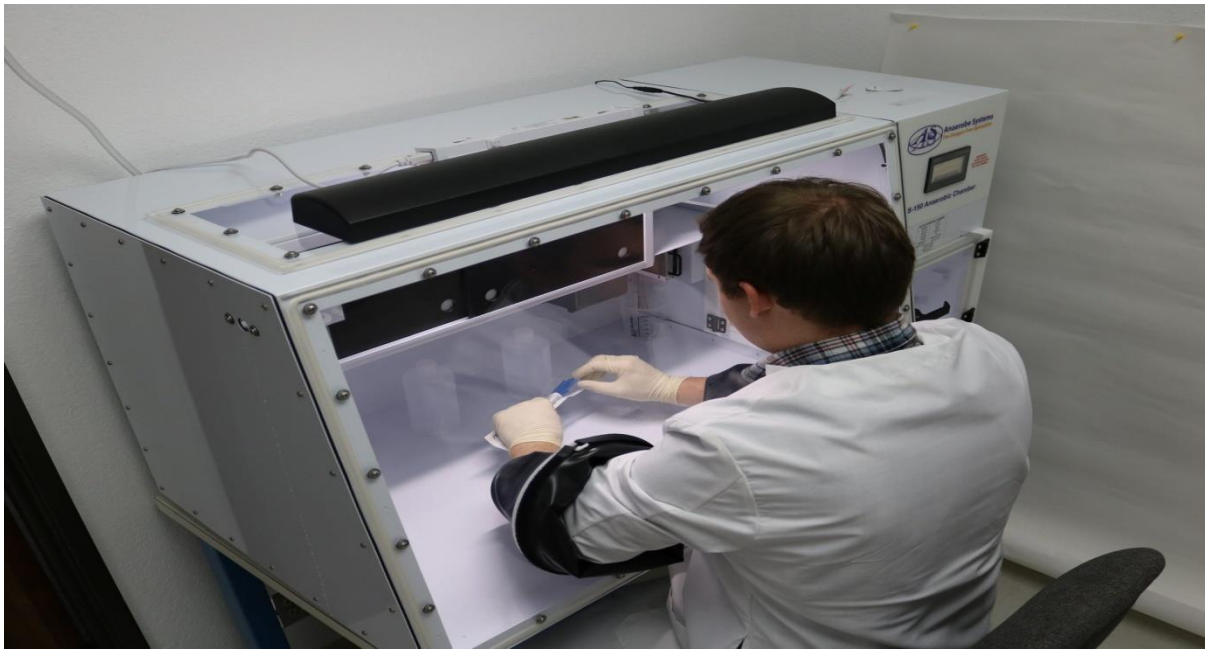
Table Types of micro-organisms with respect to their relationship to oxygen along with examples.

Types of organisms	**Examples**
(a) Strict aerobes	**(i) Most species of genus Bacillus (ii) Genus Brucella (iii) Genus Micrococcus (iv) Genus Pseudomonas**
(b) Facultative	**(i) Genus Staphylococcus (ii) Genus Streptococcus (iii)All Coliforms (iv) Genus Lactobacillus**
(c) Strict anaerobes	**(i) Genus Clostridium (ii) Genus Actinomyces (iii) Genus Desulfovibrio (iv) Genus Neisseria**
(d) Microaerophilic	**(i) Genus Leptospira (ii) Genus Compylobacter.**

Anaerobic Chamber

1. Anaerobic chamber is an ideal anaerobic incubation system, which provides oxygen- free environment for inoculating media and incubating cultures.
2. It refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Glove ports and rubber gloves are used by the operator to perform manipulations within the chamber.
3. There is an air-lock with inner and outer doors.
4. Culture media are placed within the air-lock with the inner door. Air of the chamber is removed by a vacuum pump connection and replaced with N_2 through outer doors.
5. The culture media are now transferred from air-lock to the main chamber, which contains an atmosphere of H_2, CO_2, and N_2. A circulator fitted in the main chamber circulates the gas atmosphere through pellets of palladium catalyst causing any residual O_2 present in the culture media to be used up by reaction with H_2.
6. When the culture media become completely anaerobic they are inoculated with bacterial culture and placed in an incubator fitted within the chamber.
7. The function of CO_2 present in the chamber is that it is required by many anaerobic bacteria for their best growth. A schematic representation of an anaerobic chamber showing its various parts is given in Fig





3) Anaerobic Bags or Pouches:

Anaerobic bags or pouches make convenient containers when only a few samples are to be incubated anaerobically. They are available commercially. Bags or pouches have an oxygen removal system consisting of a catalyst and calcium carbonate to produce an anaerobic, CO_2-rich atmosphere.

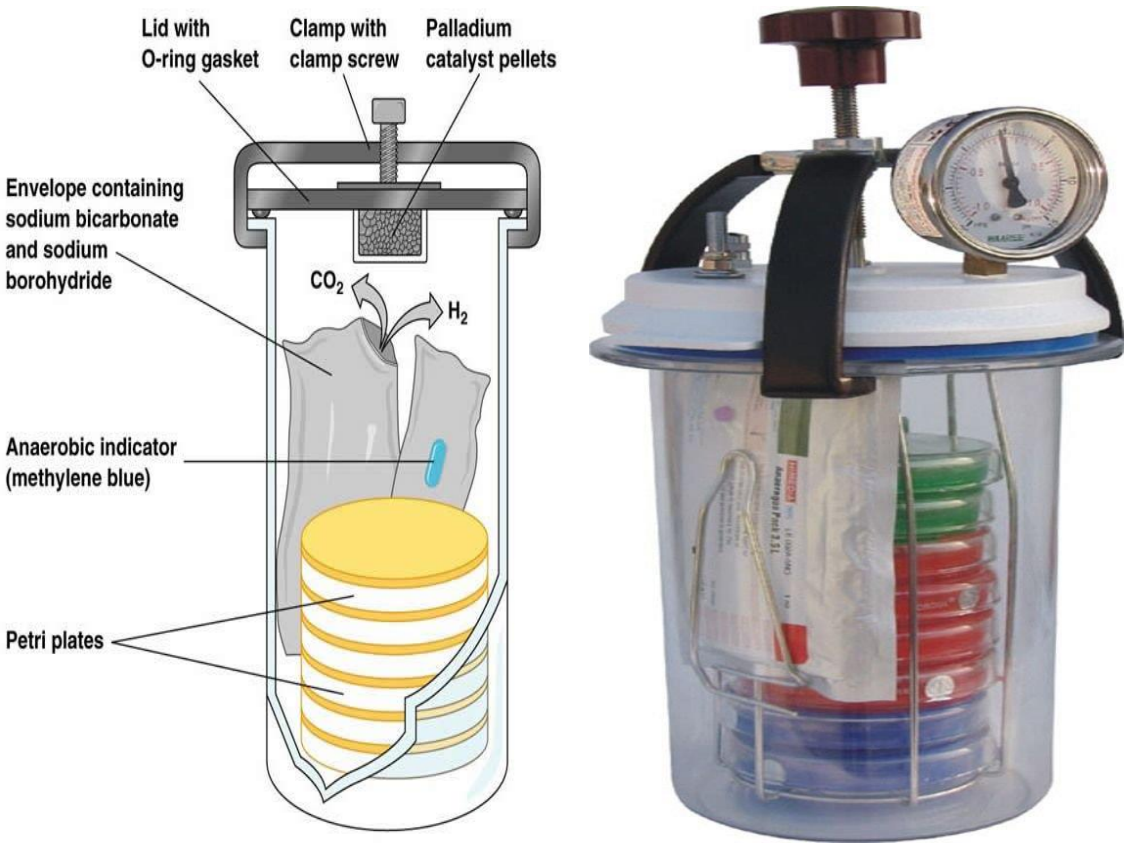
One or two inoculated plates are placed into the bag and the oxygen removal system is activated and the bag is sealed and incubated. Plates can be examined for growth without removing the plates from bag, thus without exposing the colonies to oxygen.

But as with anaerobic jar, plates must be removed from the bags in order to work with the colonies at the bench. These bags are also useful in transport of biopsy specimen for anaerobic cultures.

4. Anaerobic Jars (or GasPak Anaerobic System)

i. When an oxygen-free or anaerobic atmosphere is required for obtaining surface growth of anaerobic bacteria, anaerobic jars are the best suited. The most reliable and widely used anaerobic jar is the Melntosh-Fildes' anaerobic jar.

ii. It is a cylindrical vessel made of glass or metal with a metal lid, which is held firmly in place by a clamp.



iii) The lid possesses two tubes with taps, one acting as gas inlet and the other as the outlet.

iv) On it's under surface it carries a gauze sachet carrying palladium pellets, which act as a room temperature catalyst for the conversion of hydrogen and oxygen into water. Palladium pellets act as catalyst, as long as the sachet is kept dry.

v) Inoculated culture plates are placed inside the jar and the lid clamped tight.

vi) The outlet tube is connected to a vacuum pump and the air inside is evacuated.

vii) The outlet tap is then closed and the gas inlet tube connected to a hydrogen supply. Hydrogen is drawn in rapidly. As soon as this inrush of hydrogen gas has ceased the inlet tube is also closed.

viii) After about 5 minutes inlet tube is further opened. There occurs again an immediate inrush of hydrogen since the catalyst creates a reduced pressure within the jar due to the conversion of hydrogen and leftover oxygen into water.

ix) If there is no inrush of hydrogen, it means the catalyst is inactive and must be replaced.

x) The jar is left connected to the hydrogen supply for about 5 minutes, then the inlet tube is closed and the jar is placed in the incubator. Catalysis will continue until all the oxygen in the jar has been used up.

xi) The gasPak is now the method of choice for preparing anaerobic jar. The gasPak is commercially available as a disposable envelope containing chemicals, which generate hydrogen and carbon dioxide when water is added. After the inoculated plates are kept in the jar, the gasPak envelope with water added, is placed inside and the lid screwed tight.

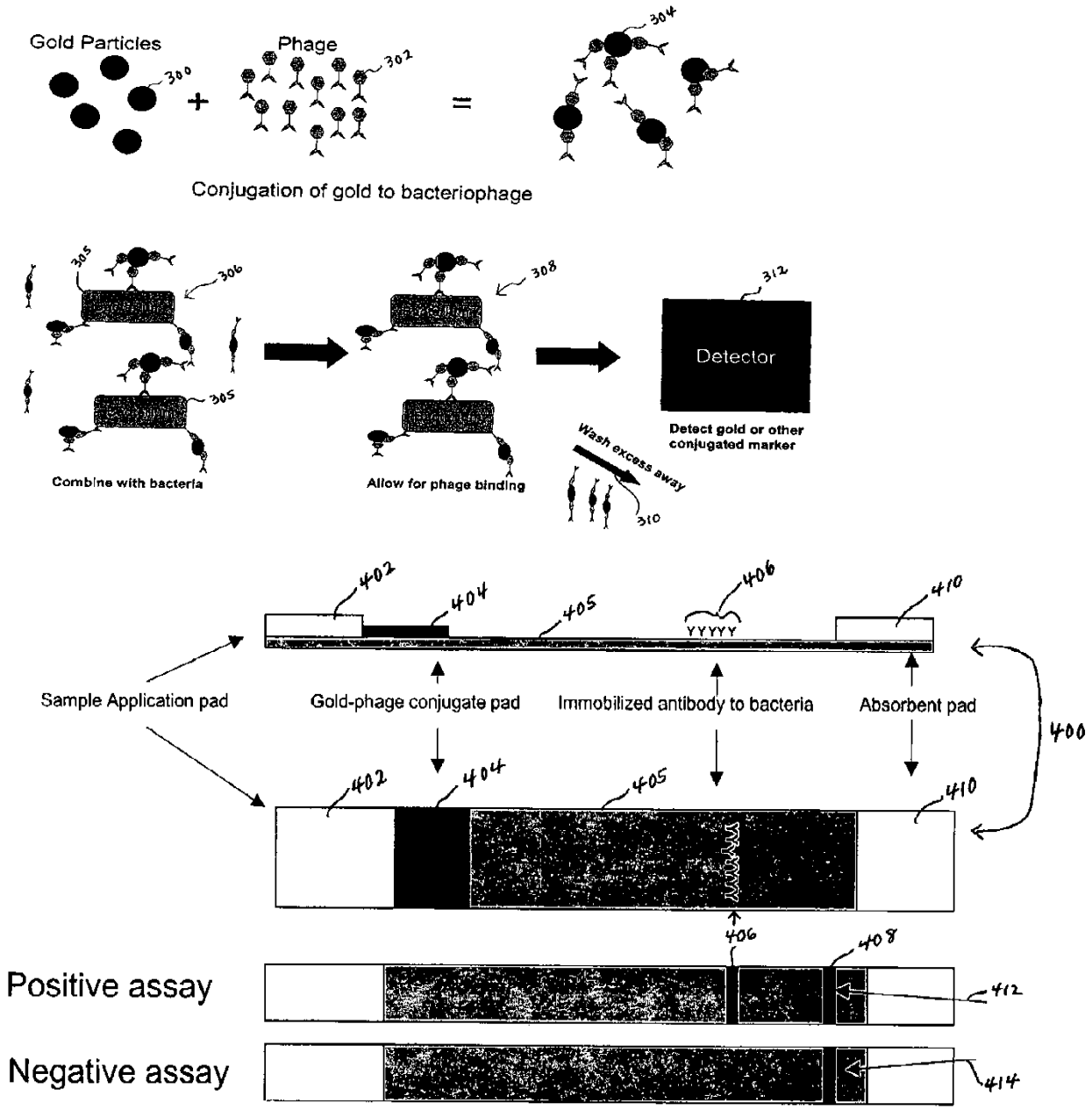
xii) Hydrogen and carbon dioxide are liberated and the presence of a cold catalyst in the envelope permits the combination of hydrogen and oxygen to produce an anaerobic environment.

❖ **Method for detecting microscopic organisms by using bacteriophages Kent J. Voorhees apparatus:**

Methods and apparatus are provided for detection of microorganisms in a sample. Methods and apparatus of the invention are based on the specificity that phage for example bacteriophage, have for target microorganisms, for example bacterium.Phage adsorption to target microorganisms act as signal, for the presence of the target microorganism. Typically, the phages are labeled with a detectable signal. Apparatus of the invention are directed toward concentrating the phage adsorbed microorganisms at a predetermined site for flag dependent observation.

A method of determining the presence of a target microorganism in a sample, the method comprising: combining with the sample an amount of flagged phage capable of binding to an outside surface of the target microorganism to create a flag labeled target microorganism; providing conditions and an amount of time sufficient to allow the flagged phage to bind to the outside surface of the target microorganism; and assaying the phage exposed sample to detect the presence of the flag labeled microorganism, without detecting phage amplified within the target microorganism and, wherein presence of the flag bound to the outside surface of the target microorganism indicates presence of the microorganism, wherein the detecting the presence of

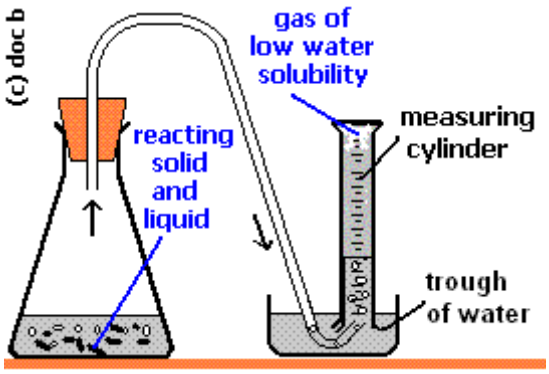
the flag labeled microorganism is by concentrating the flag labeled microorganism to enhance the capacity to detect the flag labeled microorganisms, and wherein the concentrating of flag labeled microorganisms is on a flow strip device having one or more immobilization zones, wherein the flag labeled microorganisms are captured and concentrated within the one or more immobilization zones. The method of claim 1 wherein the flagged phage is a phage labeled with colorimetric particles. The method of claim 2 wherein the colorimetric particles are colloidal gold particles. The method of claim 1 wherein the flagged phage is a phage labeled with a fluorescent tag.



Measurement of gas production by Wilkins method

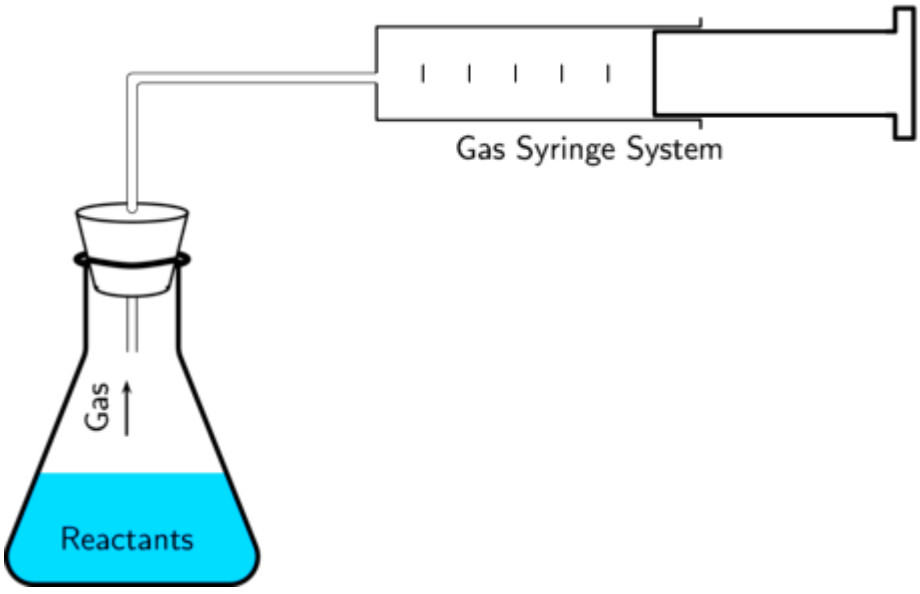
Wilkins (1974) described a different approach to measure fermentation kinetics in vitro, whereby fermentation took place in a sealed vessel and gas produced was determined using a pressure transducer to measure accumulation of pressure in the vessel headspace. This principle of measuring pressure with a sensor or transducer has been widely adopted as a simple, yet sensitive, method of determining fermentation kinetics.

Water Displacement



The simplest method is displacement of water inside an Inverted graduated measuring cylinder or graduated test tube placed inside a water bath. This method is suitable for gases such as Hydrogen or Nitrogen but does not work for water soluble gases such as ammonia or chlorine.

Syringe Barrel Displacement



The liberated gas in a lab-scale reaction is led to a syringe connected to the reaction vessel. Due to an increase in pressure the gas begins to displace the plunger of the gas tight syringe. On completion of the reaction the syringe barrel gets displaced to its final position and the volume of liberated gas can be noted. Correction is applied for temperature and pressure which are conventionally reported under NTP (Normal temperature and pressure conditions, i.e., 20 degrees centigrade and 1 atmosphere pressure).