Bacterial cultures are the ideal method for multiplying and reproducing microorganisms in laboratory conditions. Culturing bacteria is mainly used for identifying, storing, isolating single colonies, and counting the amount of organisms present. Culturing bacteria is an essential tool for determining infectious diseases of sick patients and is also an extremely important tool in microbiology labs for research and for teaching students of microbiology. Culturing bacteria is probably the most important yet basic technique used in microbiology today.

Culturing bacteria in laboratory poses two problems:
1. First, a pure culture of a single species is needed to study an organism’s characteristics.
2. Second, a medium must be found that will support the growth of the desired organism.

By a pure culture we understand, as is well-known, a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and from one only. As all bacteriologic work, of whatever kind it may be, depends on our working with such reliable pure cultures, many efforts have, of course, been made in the course of time in order to devise reliable methods of isolating a single bacterium.

History of development of pure culture techniques:

**Robert Koch:** In 1881 Koch published an article describing the use of boiled potatoes, sliced with a flame-sterilized knife, in culturing bacteria.

The surface of a sterile slice of potato was inoculated with bacteria from a needle tip, and then the bacteria were streaked out over the surface so that a few individual cells would be separated from the remainder. The slices were incubated beneath bell jars to prevent airborne contamination, and the isolated cells developed into pure colonies.

**Drawback:** Unfortunately many bacteria would not grow well on potato slices.

**Frederick Loeffler,** an associate of Koch, developed a meat extract peptone medium for cultivating pathogenic bacteria. Koch decided to try solidifying this medium.
He spread a mixture of Loeffler’s medium and gelatin over a glass plate, allowed it to harden, and inoculated the surface in the same way he had inoculated his sliced potatoes.

**Drawbacks:**
1. The new solid medium could not be incubated at 37°C (the best temperature for most human bacterial pathogens) because the gelatin would melt.
2. Some bacteria digested the gelatin.

**Minora Tarazaemon:** In 1882 Japanese innkeeper, Minora Tarazaemon first used agar as a solidifying agent. He threw out extra seaweed soup and discovered the next day that it had jelled during the cold winter night. Agar had been used by the East Indies Dutch to make jellies and jams. Fannie Eilshemius Hesse, the New Jersey born wife of Walther Hesse, one of Koch’s assistants, had learned of agar from a Dutch acquaintance and suggested its use when she heard of the difficulties with gelatin. Agar-solidified medium was an instant success and continues to be essential in all areas of microbiology.

![Image of Fannie Eilshemius and Walther Hesse](image-url)

**Fannie Eilshemius (1850–1934) and Walther Hesse (1846–1911).** Fannie Hesse suggested to her husband Walther (a physician and bacteriologist) that he should try using agar in his culture medium when more typical media failed to meet his needs.

**Julius Richard Petri:** A member of Robert Koch’s laboratory invented these dishes named petri dishes around 1887 and they immediately replaced agar-coated glass plates. They consist of two round halves, the top half overlapping the bottom. Petri dishes are very easy to use, may be stacked on each other to save space, and are one of the most common items in microbiology laboratories.

**Emil Chr. Hansen:** First investigator who devised reliable methods of isolation of pure cultures. The principle of his method was, to observe directly under the microscope the growth of the individual yeast-cell until it has formed a small colony in a gelatin droplet on the lower surface of a cover glass in a moist chamber.

**Drawback:** Yeast-cells are however far bigger than most bacteria and hence this method was suitable only for isolation of pure yeast cell cultures and not for bacteria.

**Schouten, Barber and Malone:** In this method Barber picked up successively each single one of four bacteria, which he views in a small hanging-drop of broth and inoculates them into four broth test tubes separately.

**Drawback:** This method couldn’t attain extensive application
a) It required intricate apparatus
b) It was immensely difficult to pick up such minute objects as bacteria with such relatively coarse implements as pipettes and loops.

**Burri’s India ink method:** In this method the bacteria was emulsified in diluted India ink. Then minute emulsion droplets were deposited on a gelatine plate in a Petri dish by means of
a mapping-pen. By microscopical investigation with a high power dry lens those droplets which contained only one single cell, were noted and allowed to stand until a small colony has develops.

From these colonies, subcultures were prepared or, the India ink droplet is removed, together with the bacterium, by means of a sterile cover-slip that is superimposed on the black spot of the gelatine plate, removed again together with the India ink and the bacterium, and dropped into an appropriate fluid nutrient medium.

Benefit: This is, as has been said, an excellent method, by means of which, with some practice and patience, reliable single-cell cultures of most species of bacteria can fairly easily be produced.

Drawback:
1. All bacterial species cannot tolerate the India ink.
2. The necessity of having the unhandy Petri dish standing on the microscope stage during the examination.

J. ORSKOV modified Burri’s method by means of pouring liquid gelatine upon previously sterilized slides with the help of sterile Pasteur pipettes. Then on the gelatine surface three rows of India ink droplets were deposited, which could now be much more easily and rapidly examined by shifting the mechanical stage, the selected India ink droplets being subsequently removed as usual by means of sterile coverslips.

Hort: In bacteriologic literature, Hort was the first to point out that it is possible to see bacteria distinctly on the surface of an agar plate and to watch their growth by means of a high-power system of dry lenses without any staining or contrast.

The hot agar was poured over sterile slides in as level a layer as possible, taking care to keep the agar well within the edges of the slide. The mode of procedure now varies as to whether he employs the oil-immersion lens or the dry lens system for further examination.

In the first case a series of sterile cover-slips have been previously prepared, each with a small circle etched on its surface by means of a diamond. In the centre of this circle, a minute droplet of broth is now deposited, taken from a broth culture containing the bacteria under investigation in a suitable emulsion. The inoculated cover-slip is now placed face downwards on the agar surface; the area within the small circle is thoroughly examined with an oil-immersion lens and, in case only one single organism is found within the circle, the slide is placed in a Petri dish which is then placed in the incubator. The circle is examined at short intervals, until a small colony has formed from which subcultures can be prepared.

Drawback:
1. Great care must be taken to ensure that the droplet does not run outside the etched circle when the cover slip is applied to the agar.

J. ORSKOV: A twelve-hour old broth culture of colon bacilli, was inoculated on the agar plate in a Petri dish. A big droplet of the broth culture is deposited in the centre of the circle, and, by means of a glass rod, bent at a right angle; the drop is pressed down between the parallel dotted lines. Now the glass rod is moved from side to side across the first inoculated area, and, finally, the remaining half of the dish is inoculated and it is placed in the incubator.
for about one hour at 37°C. (Inoculation can of course also be performed in a streak, which some will perhaps find more to the purpose, and in this way an appropriate difference in the density of the bacteria can likewise be obtained.) This measure is taken because the development in the case of the colon bacillus begins just after the expiration of one hour, and because bacteria are more readily discernible when in development, owing to their increased refractive power. As previously described, a suitable square of the agar is now excised and placed upon the previously sterilized microscope slide, which is most conveniently sterilized by flaming.

The slide is placed on the stage of the microscope, and an area is chosen where the organisms are placed at a convenient mutual distance, commencing the examination where they are lying close, and thence, by means of the mechanical stage, proceeding to where they are lying more scattered. Having now come upon an area where there is one organism, only, within the field of vision, and this single bacterium having been centred, the area is noted by means of the mutual relation of the regular scale and the vernier attached to the mechanical stage, and one should now be able to focus exactly in the same place again.

**Modification in focussing methodology:** Before placing the agar plate on the slide, a complex of fine lines are made by means of a diamond scratched criss-cross, preferably on the lower surface of the slide. If we now focus sharply on the scratches on the slide with the low power of the microscope, the lines in the eyepiece micrometer will be intersected by these scratches in a quite specific manner and thus we obtain two distinguishing marks instead of one. The course of procedure will now be as follows:

The agar is placed on the scratched area of the slide, and we search for a place where there is only one organism within the field of vision. The spot is marked by means of the scales of the **mechanical stage**, the **objective with the attached micrometer** is placed in the tube, the microscope is adjusted to low magnification and focussed sharply on the scratches. By means of thus marking the position of the organism we have always succeeded in hitting upon exactly the same spot for repeated examination.

METHODS OF OBTAINING PURE CULTURES:
To study bacteria in the laboratory, it is important to obtain a pure culture, a culture that contains only a single species of organism. Prior to the development of pure culture techniques, scientists studied mixed cultures, or cultures containing several different kinds of organisms. Researchers could make observations of different shapes and sizes of organisms, but they could find out little about the nutritional needs or growth characteristics of individual species. Today, pure cultures are obtained by isolating the progeny of a single cell. Simple as it seems now, the technique of isolating pure cultures was difficult to develop.

Attempts to isolate single cells by serial dilution were often unsuccessful because two or more organisms of different species were often present in the highest dilutions. Koch’s technique of spreading bacteria thinly over a solid surface was more effective because it deposited a single bacterium at some sites. However, he tried several different solid substances. Using the discovery of Angelina Hesse, the wife of an associate, he settled on agar as the ideal solidifying agent. Only a very few organisms digest it, and in 1.5% solution it does not melt below 95° C. Furthermore, after being melted, agar remains in the liquid state until it has cooled to about 40° C, a temperature cool enough to allow the addition of nutrients and living organisms that might be destroyed by heat.

There are several ways to prepare pure cultures:

A. The Spread Plate method:
1. Mixture of cells is spread out on an agar surface at a relatively low density so that every cell grows into a completely separate colony, a macroscopically visible growth or cluster of microorganisms on a solid medium.
2. Each colony arises from a single cell and represents a pure culture.
3. The spread plate is an easy, direct way of achieving pure culture of bacteria.

Steps involved in spread plate method:

a) A small volume of dilute microbial mixture containing around 30 to 300 cells is transferred to the centre of an agar plate.

b) The dilute microbial mixture is spread evenly over the surface with a sterile bent-glass rod.

c) The dispersed cells develop into isolated colonies.

d) As the number of colonies should be equal to the number of viable organisms in the sample, spread plates can be used to count the microbial population.
B. The Streak Plate method: Pure colonies also can be obtained from streak plate method. Streak Plate Method is one of the most exercised and convenient methods for the isolation of bacteria from various samples. This method depends on first sterilizing and charging the nichrome wire loop, which is then streaked/ rubbed on the solid surfaces of the agar medium in a manner that thins out and progressively dilutes the population of microorganisms in a way that helps get very well isolated colonies at the end.

There are 3 (Three) Different Methods of Streak Plate method:

(1) Direct Streaking method:
In this method, the loop full of culture suspension of microorganisms is streaked progressively to thin out the population, right from one end of the agar medium to the other end. At least 50-100 strokes are made to sufficiently sparse out the population. Upon incubation, the plate shows well isolated colonies on one ends of the medium, whereas the other part has densely populated microbial colonies.

The colonies which are very well isolated are larger, where as the ones which are crowded are smaller and merged with each other. This depends on the competition for the available space as well as the nutrients in the medium.

The Direct streaking method is used when culture suspension of bacteria is of low to moderate population density.

(2) Sector Streaking Method:
The Sector Method is performed by drawing 4 different sectors on the back-side of the petri plate containing the medium. The four sectors are drawn in such a way that at the end of every sector there is small portion termed as a tail that is the actual starting point of the next sector.

Streaking is performed from the first sector, and after that the tail of the first sector is used to initiate streaking for the next sector and so on.

This helps in reducing the microbial population entering the next sector, and thus the population of microbes get decreased by several folds. Upon incubation, we observe that with each and every successive sector, the isolation and separation of microorganisms gets better and better.

The sector method is suitable when there is a high population density in any sample or given culture suspension of micro-organisms.

(3) Four Flame Method:
The Four Flame Method is similar to the sector method, the only difference between both of them is, in Four Flame Method, the nichrome wire loop is flamed in an oxidizing flame and cooled and used again for streaking each and every sector.

This method exercises proper decrease in the number of micro-organisms, if the culture suspension is very densely populated.

Steps involved in streak plate method:

a) The microbial mixture is transferred to the edge of an agar plate with an inoculating loop or swab.
b) The microbial mixture is then streaked out over the surface in one of several patterns.
c) After the first sector is streaked, the inoculating loop is sterilized.
d) After that inoculums for the second sector is obtained from the first sector.
e) A similar process is followed for streaking the third sector, except that the inoculums is from the second sector.
f) Thus this is essentially a dilution process.
g) Eventually, very few cells will be on the loop, and single cells will drop from it as it is rubbed along the agar surface.
h) These develop into separate colonies.

In both spread-plate and streak-plate techniques, successful isolation depends on spatial separation of single cells.

Automated equipments are used at industrial level for streak plating the solid media in order to achieve better sterilization and consistency of streaking and for reliably faster work. While streaking manually it is important to avoid scratching the solid medium as subsequent streak lines will be damaged and non-uniform deposition of inoculum at damaged sites on the medium yield clustered growth of microbes which may extend into nearby streak lines.

C. The pour plate method:

This method is extensively used for obtaining pure cultures with procaryotes and fungi, a pour plate also can yield isolated colonies.

Steps involved in pour plate method:

1. The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies when plating.
2. Then small volumes of several diluted samples are mixed with liquid agar that has been cooled to about 45°C, and the mixtures are poured immediately into sterile culture dishes.

3. Most bacteria and fungi are not killed by a brief exposure to the warm agar.

4. After the agar has hardened, each cell is fixed in place and forms an individual colony.

Like the spread plate, the pour plate can be used to determine the number of cells in a population. Plates containing between 30 and 300 colonies are counted. The total number of colonies equals the number of viable microorganisms in the sample that are capable of growing in the medium used. Colonies growing on the surface also can be used to inoculate fresh medium and prepare pure cultures.

**Pour Plate Method is also known as Total Viable Count (TVC) or The Standard Plate Count Method (SPC).**

The initial part of Pour Plate Technique is the Serial Dilution of the given sample 10, 100 and 1000 times using sterile distilled water tubes. Then, the adequate amount of inoculum is taken from each dilution and is added in a sterile melted N. agar medium before it gets solidified. It is also mixed thoroughly by shaking the contents of the liquid cooled agar medium to allow uniform distribution of microorganisms. And it is poured in a sterile empty petri plate, and allowed to solidify.

Upon incubation, the colonies that are developed on the plates are of 3 (three) different types.....

(1) Surface Colonies,

(2) Sub-surface Colonies, and

(3) Bottom Colonies

However, Pour Plate Method has several important features to be noted:

(1) This method takes the advantage of the liquefying (at 92-95 C temperature) and solidifying (at 45-48 C temperature) characteristics of Agar Agar Powder present in the nutrient agar medium.

(2) Psychrophilic organisms escapes detection in this method, as they are easily killed even at the gelling temperature of agar.

(3) Numbers of colonies does not represent the numbers of microorganisms, instead they are referred to as the CFUs (Colony Forming Units), as a colony may be a result of a single cell,
a pair of cells, a chain of cells, a cluster of cells or even a bunch of cells which are adjacently placed on the medium.

(4) In case, if you are performing SPC for water, milk, food, soil or any other samples, it needs to count the exact number of colonies after the incubation of 24 hrs at 37°C. The colony count between 30-300 is used for further calculation purpose. If the number of colonies are 30, it is referred to as TLTC (Too Less To Count). And if the count of colonies exceeds 300, it is referred to as TNTC (Too Numerous to Count). And these types of results are simply omitted from the further calculations.

Number of CFUs/ml = Number of Colonies Counted \times \text{Dilution factor}/ \text{Amount of Sample Plated}

The Pour Plate Method is one of the most suitable methods for the quantification of total number of microorganisms in the given sample.