# PRACTICAL 5 PURE CULTURE TECHNIQUES

**Structure**



## 5.1 INTRODUCTION

Practical 4 focused on sub-culturing techniques. We learnt the techniques involved in sub-culturing, i.e., the process involved in transfer of culture from one medium to another or transfer of culture from the parent growth source to another. What is the role of sub-culturing? Basically, for maintaining and transferring the culture for various microbiological test procedures, sub-culturing as a technique is important. You would realize that microbiological studies require microorganisms in pure form. How to obtain these pure forms i.e., a single type of microorganism in a culture? This is the focus of Practical 5.

This Practical introduces the concept of pure culture and the ways of obtaining it. After going through the practical, you will be able to understand the importance of obtaining pure culture in careful study of an individual microbial species.

**Objectives**

After studying this practical and conducting the exercises given herewith, you will be able to:

- explain the concept of pure culture,
- describe the ways i.e., the methods employed in obtaining pure cultures, and
- prepare and maintain pure cultures.

## 5.2 PURE CULTURE TECHNIQUES: AN INTRODUCTION

Microorganisms can be isolated from various natural environments like soil, food, water, sewage, decomposing matter and even from infectious materials such as pus, sputum, urine etc. You may recall studying about these sources in Unit 3 in the theory booklet. These sources usually contain a mixed population of microorganisms containing several species in close association with one another. This mixed population, however, pose a problem for microbiologists as a pure culture (a culture containing only a single kind of microorganisms) is required for studying a particular species. Different characteristics of a microbe like colony appearance, morphological, physiological, biochemical and immunological characteristics or antimicrobial susceptibility can be studied adequately only in a pure culture. *Pure culture can be defined as a population of cells arising from a single cell*. Both conventional and more advanced approaches can be used to

prepare pure cultures. These are – (1) Plating Method, (2) Agar Shake Tube Method, (3) Most Probable Number (MPN) Method, and (4) Laser Tweezer Technology. All these methods involve two operations:

(i) isolation of a particular microorganism from a mixed population, and

(ii) cultivation of the isolated microorganism on culture media.

Let us get to know about these methods. We begin with the most common one – the plating method.

## 5.3 PLATING METHOD

Plating method is one of the commonly employed methods for getting a pure culture. The procedure involves the separation and immobilization of individual organisms on or within the nutrient agar medium. Each viable cell then multiplies and produces an isolated colony – a visible mass of microbial cells on solid surface, which is obtained by multiplication of a single organism. These colonies can then be transferred readily to nutrient broth or nutrient agar slant to get a pure culture.

There are different methods of plating, which are used for getting a pure culture. These include –

1. Streak Plate Method,
2. Spread Plate Method, and
3. Pour Plate Method.

Let us learn about these methods.

1. *Streak Plate Method* – The method was developed by *Loeffler* and *Gaffkey* in the laboratory of *Robert Koch*. The streak plate method depends on spatial separation of single cells. The mixed microbial culture is transferred to the edge of an agar plate and 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 inoculating loop as illustrated in Figure 5.1. As the microbes are rubbed off the loop on to the medium, there is a continuous reduction in the number of microbes till the last cells to be rubbed off the loop are far enough apart to form isolated/ discrete colonies. The isolated colony can be picked and restreaked on nutrient agar plate to get a pure culture.

   Streak plate method is the most commonly used method to isolate pure cultures. As discussed above, principle of streak plate method is *continuous dilution of the microbes, resulting in separation of individual cells*. These cells then form isolated colonies. Repeated picking and restreaking of isolated colony ultimately results in a pure culture. Different patterns can be used for streaking. Common ones are quadrant (four way streaks) and full plate streak methods, which are shown in Figure 5.2. There are other variants of streaking pattern also.

**Figure 5.1: Streak Plate Method**

**Figure 5.2: Different streak patterns**

The method works well when the organism to be isolated is present in large amounts in a mixture. However, when the amount of desired organism is less, its level has to be increased by using specific enrichment culture before performing streaking.

2. *Spread Plate Method* – In this method, diluted microbial suspension containing about 30 to 300 colonies is spread uniformly on the agar surface with a sterile bent rod (spreader). Look at the Figure 5.3(b), which graphically illustrates the spread plate method. By repeated picking and restreaking of a well-isolated colony, a pure culture can be obtained. The dispersed cells develop into isolated colonies. The method can also be used for quantitation of microbial number in the sample, as you will learn in the next practical.

**Figure 5.3: Pour plate and spread plate method**

Briefly, in spread plate method, a small volume of the diluted sample (about 0.1 ml) is transferred to the centre 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. 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. It is important that the surface of the plate be dry so that the culture that is spread soaks in.

The advantages and disadvantages of spread plate method are highlighted next.

| Advantages | Disadvantages |
|---|---|
| It is useful for the samples having heat sensitive microbes. | Volume no greater than 0.1 ml can be spread on the nutrient agar plate because it would not soaken well and may result colonies to coalesce as they form. |
| No subsurface colonies appear in spread plate so isolation of the organism is easy. | |

3. *Pour Plate Method* – Isolated colonies can also be obtained by pour plate method. 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 5.3(a). The microbial suspension should be diluted sufficiently to obtain separate colonies on plating.

   The pour plate method involves adding specified amount (0.1 ml or 1.0 ml) of the dilution to the sterile petri plate. 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. 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. Colonies appeared both within the nutrient agar, as well as, on the surface of agar plate.

   Microbial cells get fixed on solidification of agar and forms individual colonies on incubation. Colonies are present both on the agar surface and embedded in the nutrient medium. Look at Figure 5.4 to see how the colonies look. Colonies obtained at different dilutions are also highlighted in the Figure 5.4. Colonies growing on the surface can be used to inoculate fresh medium for pure cultures. The method can also be used for microbial cells enumeration in the original sample. We will practically try out this technique in the next practical. But here let us now highlight the advantages and disadvantages of this technique.

**Figure 5.4: Colonies obtained at different dilutions**

| Advantages | Disadvantages |
|---|---|
| As colonies grow both on the surface and beneath the agar surface, so aerobes, facultative anaerobes and non-stringent anaerobes can be studied. | Heat sensitive microorganisms may be damaged by melted agar, giving low viable count as compared to spread plate. |
| Volume greater than 0.1 ml can be used as the sample is mixed with the molten agar medium. | Appearance of colonies on differential media cannot be used satisfactorily for diagnostic purpose, if it is growing within the agar. To accomplish this, spread plate method can be used. |

Plate count may be unreliable when used to assess total cell numbers of natural samples. This is so because both viable and dead cells are present and also different cells have different nutritional and cultural requirements for growth.

We hope the discussion above provided you good insight into the plating method. Next let us get to learn about the agar shake tube method of obtaining pure cultures.

## 5.4 AGAR SHAKE TUBE METHOD

The agar shake tube method is useful for isolation of anaerobic microorganisms. What are anaerobic microorganisms? You may recall studying in Unit 3 in the theory course that anaerobic microorganisms are those which grow best in the absence of free oxygen. Few examples of anaerobic microorganisms include: *Clostridium perfringens, C. botulinum, Bacillus cereus, Campylobacter, E. coli etc.* In preparing pure culture using agar shake tube method, the dilutions of the mixed microbial suspension are made in tubes of molten agar. The colonies formed are embedded in the agar medium. The isolated colony from the highest dilution is used to make further dilution in a new set of molten agar, resulting in a pure culture.

Next, let us learn about the most probable number method of preparing pure cultures.

## 5.5 MOST PROBABLE NUMBER (MPN) METHOD

The MPN method is like agar shake tube method where no agar is used. The dilutions of the microbial suspension are made in liquid medium till the final tube in a series shows no growth. By repeating this process several times pure culture can be obtained. The method is used for estimation of number of microorganisms in foods, waste water and other samples. We will learn more about this method later in Practical 10.

Finally let us get to know about the laser tweezers technology used for preparing the pure cultures.

## 5.6 LASER TWEEZERS TECHNOLOGY

In addition to above said classical methods, advance technologies can be used for obtaining pure cultures. Laser tweezers technology is useful for isolation of slow growing bacteria and for microorganisms that are present in such a low number that these may be missed using dilution based enrichment method. Laser tweezers allow one to trap a single cell from a viewing microscopic field by the laser beam and moves it away from other contaminants. The cell is then put into a sterile medium to initiate a pure culture.

Regardless of the method used to purify a culture, once a pure culture is obtained, it is essential to check its purity, which can be done as under:

(1) Microscopically – Presence of a single type of cells in a culture.

(2) Colony characteristics on plates – Different microbes have different colony characteristics.

(3) Culturing on various media.

With this discussion, we end our study about the methods and techniques we can use to prepare pure cultures. To recapitulate what you have learnt or understood so far please answer the review questions given herewith. Write the answer to the questions in the space provided.

## 5.7 REVIEW QUESTIONS

1. Define pure culture. Why are these important?

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2. What is the principle of streak plate method?

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3. Name three plating techniques for getting pure culture.

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4. How does the agar shake method differ from streaking to obtain isolated colonies?

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5. Describe the principle of MPN.

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6. Why would the laser tweezers be a superior method to dilution and liquid enrichment for isolation of a microbe present in low numbers in a sample?

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7. How can we check the purity of a pure culture?

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Now that you have answered these questions, you are ready to take on the exercise related to preparing pure culture given next in this practical. So go ahead and get started with the exercise.

# EXERCISE 1

## ISOLATION OF PURE CULTURE OF BACTERIA BY PERFORMING STREAK PLATE METHOD

Date : .....

**Aim** : To isolate a pure culture of a given bacterium by performing streak plate method.

**Requirements**

*Culture* : 24 hours culture of *E. coli.*

*Medium* : Nutrient agar plates

*Equipments and Glassware* : Bunsen burner, inoculating loop, incubator, permanent glass marker

**Theory/Principle:**

Look up the Streak Plate Method discussed in section 5.3 in this practical. Based on your understanding of this method, briefly write the theory/principle behind this method in the space provided. Also graphically show the streaking method.

**Procedure:**

Now carry out the exercise following the steps enumerated herewith and also illustrated in the Figure.

1. Label the name of the organism on the bottom of the petri plate.
2. Sterilize the inoculating loop by holding it with right hand in flame till it red hot.
3. Hold the culture tube in left hand and remove the cotton plug with the help of little finger of right hand.
4. Sterilize the mouth of culture tube immediately in flame and take culture (loopful if the culture is liquid broth or small amount if the culture is solid medium) with the sterilized inoculating loop. Replace the cotton plug and keep the tube in test tube rack.
5. Transfer the culture to the labeled petri plate by streaking. For the same, hold the plate in the left hand at an angle of 60°C and open the lid (Petri plate cover) with the help of the thumb.
6. Place the culture at one end of the plate and spread it slightly in a rounded manner. It is the primary inoculum. (Look at area 1 in the margin Figure).
7. Flame the loop and cool it. Then streak the inoculums at an angle to the primary inoculum by making 5-6 parallel lines. 1-2 lines should pass through the primary inoculum (as shown in area 2 in margin Figure). The flaming of the loop results in desired dilution and fewer and fewer organisms are streaked in each successive area leading to final separation of the microbial cells.
8. Reflame and cool the loop. Turn the plate at right angle. Drag the inoculum in several parallel lines across the agar surface (as shown in area 3 of margin Figure) by touching the inoculum in area 2 only once or twice.
9. Again rotate the Petri plate at 90°C angle. After flaming and cooling the inoculating loop, touch the inoculum in area 3 once and streak the inoculum several times in parallel lines across the agar. Look at the margin Figure the area marked 4.
10. Without reflaming the loop again turn the Petri plate at an angle and drag the culture from previous streak series (area 4) across area 5 in a similar manner by making parallel lines.
11. Replace the lid on Petri plate and incubate the culture for 24 hours (overnight) at 37°C. Observe the plates for isolated colonies. Margin Figure shows the plate after incubation.
12. Pick and restreak the isolated colony on another nutrient agar plate in a similar manner to get the pure culture.

**Precautions:**

1. Plating of the medium should be done 24 hours before performing the streaking. There should not be any moisture on the plate.
2. Petri plate lid should not be lifted completely.
3. Labeling of the plate should be done at the bottom of the plate.
4. Flaming and reflaming of the loop should be done to get the desired separation of the organism.
5. Before using the inoculating loop, cooling should be done by touching the agar surface away from the streaks, otherwise the organisms would be killed.
6. Maintain aseptic conditions.
7. Avoid pressing the loop too firmly against the agar surface as this will damage it.
8. Never let the loop to touch any of the previously streaked ones.

**Observations and Results:**

Having carried out the exercise, now record your observations in the format given herewith. Under observations comment on the colonies formed.

| **Observations** |
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**Inference / Conclusion (Comment on the characteristics of the colony)**

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**Now submit the exercise for evaluation.**

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**Counsellor Signature**