

CC9: Food Microbiology.

Unit 7: CULTIVATION OF MICROORGANISMS – PART 1

By

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Dear viewers in this chapter let us try to understand and the various methods for the isolation of microbes.

This chapter includes:

- **Sampling**
- **Methods of isolation and cultivation**
- **pure culture technique**
- **Isolation technique**
- **Enumeration of Microorganisms---- qualitative and quantitative**
- **Isolation of pure culture in media**

Introduction: Our everyday activities are influenced in some or the other way by microorganisms. Microorganisms may cause and cure infectious diseases. As is infections are produces in open wounds by microbes, so also a large number of antibiotics are produced by microbes. In addition, they are used in production of food, production of industrially useful chemicals, which are beneficial to humans. The production of foods as a result of microbial fermentation, predates recorded history. Fermentation was mainly used as a method of preservation (eg. Pickle) and to increase its taste and flavor. Beneficial bacteria are used as an aid in producing desirable characteristics in food. Some applications of microorganisms are:

- ✚ **Food products:** alcoholic drinks, dairy products, bread, vinegar, pickled foods, mushrooms, single-cell protein
- ✚ **Products from microorganisms:** enzymes, amino acids, antibiotics, citric acid
- ✚ **Mining industries:** metal extraction, Desulphurisation of coal
- ✚ **Agriculture:** Microbial pesticides, herbicides, nutrient solubilizing organisms like nitrogen fixation, phosphorus and potash solubilisation, etc.
- ✚ **Environment:** bioremediation, wastewater treatment, nitrogen fixation,

composting

7.1 Sampling

Sampling requires detail steps ensuring that the sample procured is a “fair sample” that accurately reflect the constituents of the bulk material being sampled.

The basic principles that must be addressed in sampling are as follows

- 1) The sample should represent the food as sold to the consumer.
- 2) Each part of a divided sample should be the truly representative of the original
- 3) The sampling process must not alter the sample in any way that might affect the analysis
- 4) Storage and transportation of the sample must not alter the sample in any significant way – whether through contamination, loss, deterioration or any other means.

The nature of the samples, that are appropriate, will depend on the purpose for which the analysis is undertaken. The quantity will vary according to the product and the type of analysis to be carried out. In addition, samples must be drawn under the provision of regulation that have their own provisions some of the examples are:

- The Poultry Meat (Water Content) Regulations 1983, S.I. 1983/1372
- The Milk-based Drinks Regulations 1983, S.I. 1983/1514
- The Animals and Animal Products (Examination for Residues and Maximum Residue Limits) Regulations 1997, S.I. 1997/1729
- Natural Mineral Water, Spring Water and Bottled Drinking Water Regulations 1999, S.I. 1999/1540
- Contaminants in Food Regulations 2003, S.I. 2003/1478
- Sampling regulations as per FSSAI 2006.

Based on regulations and given guidance, minimum weight of samples should be taken and packed in relatively small containers. They should be in sealed containers, at least 6 to 9 items should be taken as sample. Generally, one sample of a specific food is taken and divided into three final parts of the sample. On some occasions multiple samples are collected and preserved separately.

a) Administration of sample:

This will normally include the use of appropriately designed record forms, sample labels and laboratory submission sheets, keeping an exact record of all data. On taking a formal sample for analysis, the sample must be labeled. The label should contain, the source, the name of the sampling officer, the name of the authority, the place, date and time of sampling and an identification number. All the samples should be placed in suitable containers and secured with a tamper evident seal. Sufficient labels, forms, containers and seals need to be taken to the point at which the sampling is to be carried out. It is often useful to place the parts of the sample in a second container, such as a plastic bag, which should be sealed in such a way as to ensure that the sample cannot be tampered with, without being obvious.

In some cases specialised equipment might be needed to carry out the sampling and could include items such as a sampling spear, thief (specific piece of sampling equipment), temperature-monitoring device, scales, and weights. A camera is a useful piece of equipment for recording both the premises and details of the samples taken when appropriate.

The sample should be taken during normal working hours. Sampling visits should therefore be planned as part of the inspection program and timed having regard to all relevant and available information, which including hours of operation, hours at which certain foods may be handled and any seasonal factors. It is desirable to sample at different times of the day at manufacturing and packing premises to ensure that samples are obtained from beginning to the end of a batch.

b) Transportation of samples:

The collected sample must be send to Public Analyst or analyst department particularly where tests are to be made for. It is common practice to transport chilled or frozen samples in cool boxes or refrigerated vans. It is essential to ensure that frozen food stays below 0°C and chilled foods below 5 °C.

c) Storage of samples:

After analysis the retained final parts may need to be stored for several months prior to submission to the concerned authorities, it is important that they are appropriately stored. Final parts of food which are perishable should be kept refrigerated or in a frozen state as necessary.

d) Homogenization of Food Sample: Food sample must be homogenized for enumeration of microbial contents. The Stomacher, a relatively simple device, homogenizes specimens in a special plastic bag by the vigorous pounding do two

paddles (Fig. 1). The pounding effects the shearing of food specimens, and microorganisms are released into the diluents. The other method is using a high speed blending.

Fig.1 Stomacher

7.2.1 Methods of isolation and cultivation of pure culture

Microorganisms can also be used as indicators. Microorganisms like *E. coli* that are usually associated with the presence of pathogens are called “indicators”. Most *E. coli* do not cause illness but they are indicators for fecal contamination. Therefore, there is a need for food safety control hazards to the consumer.

Microorganism can also cause foodborne hazard that may cause a food to be unsafe for human consumption. Therefore, food quality controls deterioration of food. The development of microbial isolation techniques makes it possible to use pure culture in food production, isolation of these bacteria and identify them. It is essential to maintain the originality of known microorganisms. The study of pure cultures opened up the possibility of classifying microorganisms, on the basis of the characteristics they display in pure culture.

The first step in the cultivation of microorganisms is therefore the creation of a pure culture. A key development for the production of pure culture was the ability to grow microorganisms on a solid medium. Koch had noticed that when a nutrient surface such as cut potato was exposed to air, individual microbial colonies grew up, and he inferred from this that these had each arisen from the numerous divisions of single cells.

The use of agar was first suggested by Koch's colleague's wife, who had used it as a setting agent in jam making. Agar does not melt until near boiling point hence an ideal setting agent, resisting both thermal and microbial breakdown, shortly afterwards, Richard Petri developed the two-part culture dish that was named after him as Petri plate. Petri plates can be sterilized separately from the medium and provide protection from contamination by means of its lid. This is still a standard equipment today, although the original glass has been largely replaced by pre-sterilized, disposable plastic.

7.2.1. Isolation technique

One of the first requirements to study specific microorganisms is to separate them from the mixed microbial populations in which they are found in the environment. To achieve this goal microbiologists use culture media and aseptic transfer techniques.

a) Inoculation: It is an introduction of a very small sample of cells (the inoculum) into a receptacle containing nutrient or culture medium

The aseptic (sterile) technique is a technique designed to keep the working environment as free of contaminants as possible. This is achieved first, by sterilizing all equipment and media that will be in contact with the microorganisms. This includes minimizing the air movement on the working area. Usually the work is done within the vicinity of a flame. Aseptic technique is required for the maintenance of pure cultures and the successful isolation of specific types of microorganisms. In the present day Laminar Air flow (LAF) devices are used for all microbiological works which are stationed in clean rooms.

Pure culture: A pure culture is a culture that contains only one species of bacteria (Fig.2).

Fig.2 Pure culture

Mixed culture: A mixed culture encompasses more than one species (Fig.3.)

When isolating bacteria from the environment the microbiologist always starts with a mixed culture. The samples are diluted and plated in any one of the techniques listed below to obtain a mixed culture and also to count the total population in the given sample. A pure culture can be obtained from the mixed culture by sub-culturing and streaking for isolation.

Fig.3 Mixed culture plate

c) Use of the Loop/Stab Inoculator: Two different types of inoculators can be used depending on the purpose of the work. i.e a loop and a needle. The loop is used to a) transfer cultures from one medium to another, b) to prepare bacterial smears, and c) to streak plates. The loop is the tool of choice for working with a liquid inoculum culture. The stab is used to prepare stab cultures and to pick single colonies from a plate. Stab is usually carried out with inoculation needle (Fig. 4.).

Fig.4 Inoculation loop and needle

b) Inoculation of an agar slant: Rest the inoculum gently at the lower end of the slant and withdraw it slowly upwards moving it from side to side (the surface of the agar should not be broken). This should leave a streak on the surface of the slant as show in Fig .5.

Fig. 5 Procedure for inoculating a nutrient agar slant from a slant culture

e) Inoculation of an agar stab: Using aseptic technique pick a single well isolated colony with a sterile inoculating stab needle and stab the needle several times through the agar to the bottom of the vial or tube (Fig.6). Replace and tighten the cap. Make sure the tube and cap are well labeled

Fig. 6 Procedure for stab inoculation

f) Inoculation of an agar plate: In case of agar plates there is a greater surface area of sterile media that can be exposed to contaminations in the atmosphere as shown in Fig. 7. The key is to keep as much of the lid over (covering) the open agar plate as possible. Incubate the plates at appropriate temperature and in a secluded container. This is normally carried out in a incubator. Never open the lid on the lab bench when in an open contaminating environment.

Fig. 7 Inoculation in agar plate

7.3 Enumeration of Microorganisms qualitative and quantitative

7.3.1 Isolation technique: To study microorganisms in food samples, it is necessary to isolate them as mixed population and further from mixed into individual species. For isolation and cultivation of these microorganisms basic laboratory apparatus are required. First of all, samples are taken from the food source which needs to be tested. These samples if are not going to be analysed immediately are then stored at preferably at 0-4 °C insterile containers.

a) Serial dilution: For analysis, samples are then diluted by serial dilution method to get the microbial count. Serial dilution involves taking a sample and diluting it through a series of standard volumes of sterile diluent, e.g. distilled water or 0.9 % saline (Fig. 8.). Then a small measured volume of each dilution is used to make a series of pour or spread. By diluting the sample in this controlled way it is possible to obtain an incubated plate with an easily countable number of colonies (30–100) and then calculate the number of microbes present in the sample.

Procedure:

1. Take a sterile pipette.
2. Draw up 1 ml of a well-mixed sample/culture into the pipette.
3. Add this sample to the first tube. The total volume of this tube should now be 10 ml. This provides an initial dilution of 10^{-1} .
4. Mix the dilution thoroughly, by emptying and filling the pipette several times.
5. Take a new pipette and draw 1 ml sample of the 10^{-1} dilution and place it in the second tube which all ready contains 9 ml of diluent.
6. Mix well as before. This gives a 10^{-2} dilution.
7. Repeat this for the remaining tubes, removing 1mL from the previous dilution and adding it to the next 9 ml of diluent.

b) Pour plate method: A pour plate is one in which a small amount of inoculum from broth culture is added by pipette to the center of a Petri dish (Fig. 9). Molten, cooled agar medium in a test tube or bottle, is then poured into the Petri dish containing the inoculum. The dish is gently rotated clock wise for three times and anticlock wise once. This ensure that the culture and medium are thoroughly mixed and the medium covers the plate evenly. Pour plates allow micro-organisms to grow both on the surface and within the medium.

Fig.8 Methods for dilution

Fig.9 Pour plate method

c) Spread plate method: Spread plates, also known as lawn plates, should result in a culture spread evenly over the surface of the growth medium (Fig.10). This means that they can be used to test the sensitivity of bacteria to many antimicrobial substances, for example mouthwashes, garlic, disinfectants and antibiotics. The spread plate can be used for quantitative work (colony counts) if the inoculum is a measured volume, usually 0.1 mL, of each of a dilution is delivered by pipette. The sample is then spread using a spreader.

The advantage and disadvantage pour and spread plate method is given in (Table 1).

Fig. 10 Spread pate method

Table 1. Advantages and disadvantages of spread plate

Technique	Advantages	Disadvantages
Pour plate	Enumeration of all the microbes is possible	Organism which are embedded with in the media may not grow due to lack of oxygen
Spread plate	-Strictly aerobic organisms are favored because colonies grow on the agar surface	-The sample volume analyzed routinely is a maximum of 0.1 mL -Scoring of typical colonies not always easy

d) Plating and counting procedure: Use a known volume of each dilution in plating. By starting with the highest dilution. For statistical purposes, replicate plates should be prepared. After incubation the plates will show a range of numbers of colonies. Choose the plate that has an easily countable number (about 30–300) and carefully count every colony. Using a marker pen helps to avoid counting the same colony twice. Then calculate the number of micro-organisms in the sample by using the formula:

$$\text{Number of microbes/ml} = \text{number of colonies} \times \text{dilution of sample}$$

7.4. Isolation of pure culture in media

a) Streak plate: A sterile inoculation loop is used for preparing a streak plate as shown in fig.5. This involves the progressive dilution of an inoculum of bacteria or yeast over the surface of solidified agar medium in a Petri dish. The progress of streaking should be in such a way that colonies grow well separated from each other. The aim of the procedure is to obtain single isolated pure colonies. A culture developed from single unaltered colony is called as pure culture. A loop is flamed and then touched to the first area to retrieve a sample of bacteria. This sample is then streaked several times in the second area of the medium. The loop is then re-flamed, touched to the second area, and streaked once again in the third area. The process can be repeated in a fourth and fifth area if desired. During incubation, the bacteria will multiply rapidly and form colonies.

b) Single cell isolation: In this procedure cells from a single isolated colony is again subjected to serial dilution and plated. Colonies arising from such plate are considered as a colony from a single cell.

c) Microscopic isolation of single cell: the serially diluted sample from the above procedure is taken in a hemocytometer and a single cell is aspirated using a pipette.

Fig. 5 Streak plate method

Conclusion

This chapter dealt with the sampling of food samples for microbial analysis, its enumeration and methods for isolation of pure culture for further studies. These methods are common for the isolation of beneficial organisms and spoilage organisms. The methods have been standardized over a period of time and are consistent. It depends on the skill of the microbiologist to isolate the right organism. The sampling and its preservation is one of the most important step to isolate the organism of choice, as it can lead to wrong results. However, if all the procedures are followed meticulously it is easy to isolate and preserve an organism.



Fig.1 Stomacher



Fig.2 Pure culture

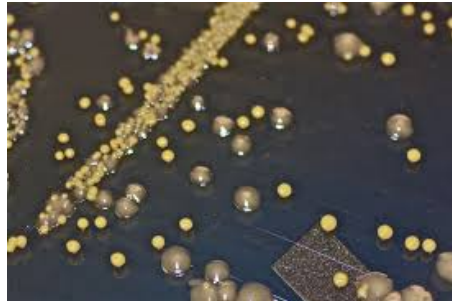


Fig.3 Mixed culture plate

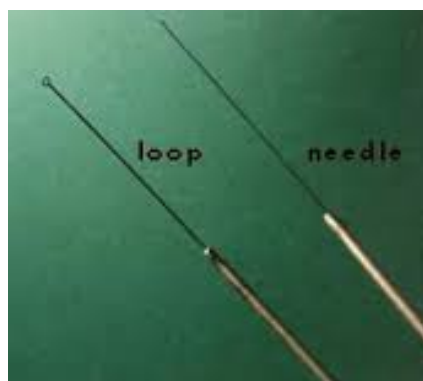


Fig.4 Inoculation loop and needle

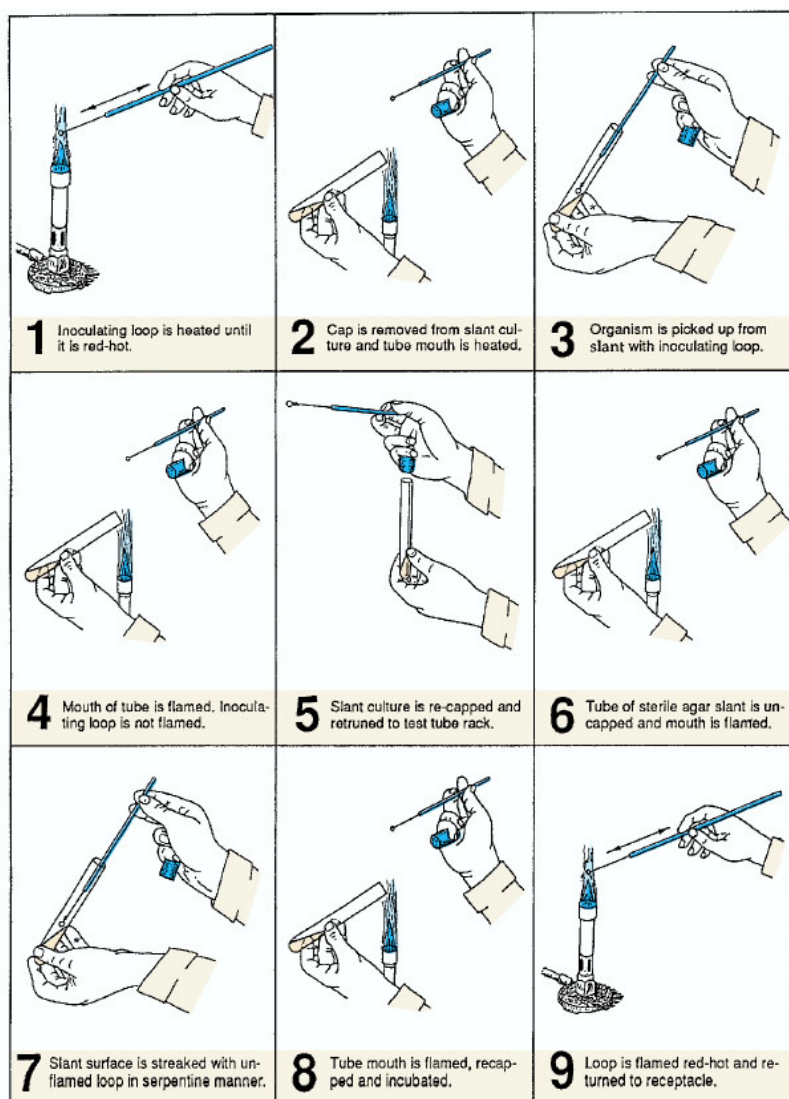


Fig. 5 Procedure for inoculating a nutrient agar slant from aslant culture

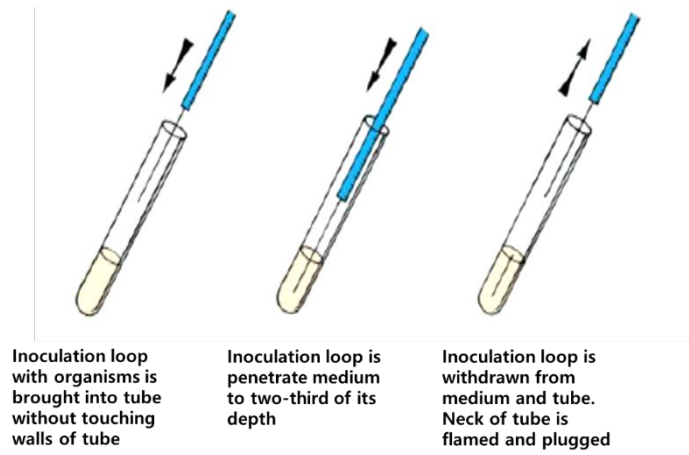


Fig. 6 Procedure for stab inoculation

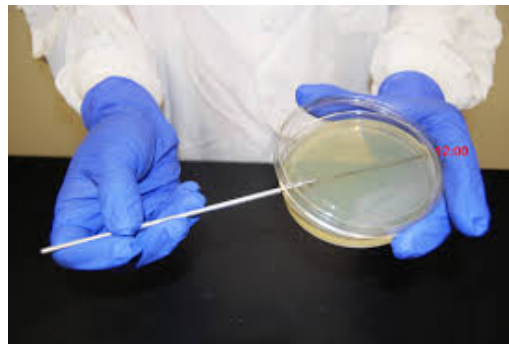


Fig. 7 Inoculation in agar plate

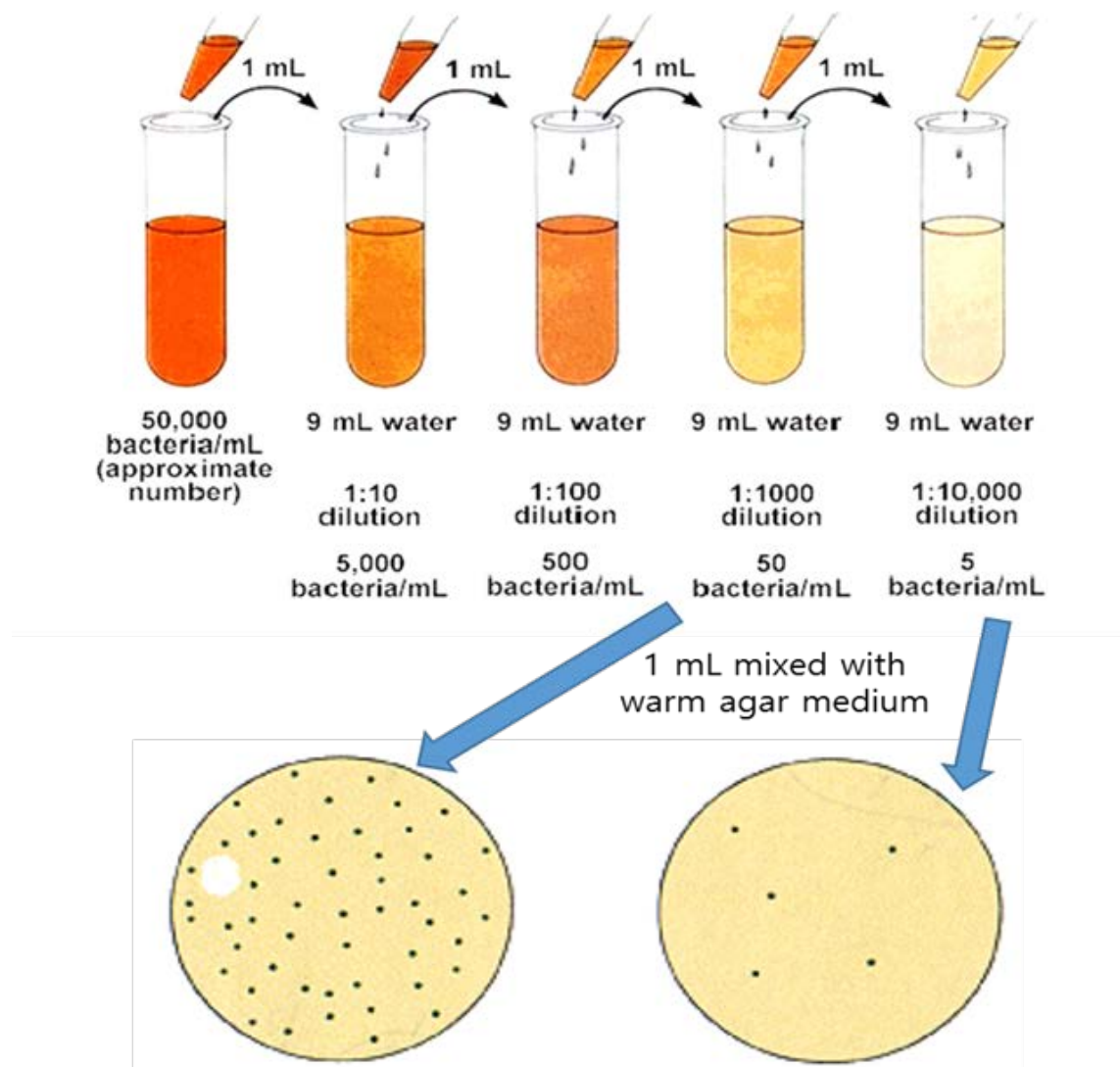


Fig.8 Methods for dilution



Fig. 9 Pour plate method



Fig. 10 Spread plate method

