# PRACTICAL 11 ANALYSIS OF FOOD SAMPLES

**Structure**



## 11.1 INTRODUCTION

Practical 10 dealt with Microbiological study of water. Now, in this practical, we shall deal with the endogenous and exogeneous microorganisms which are present on the food as contaminants. We know that these contaminants can lead to the spoilage of food and food borne illnesses. Hence, we need to become familiar with different sources of microbial contaminations and the ways for their detection and enumeration to access the quality of food and sanitary conditions maintained during food production. This practical focuses on these aspects. We shall learn about different tests and procedures we can use in the laboratory to detect microorganisms in food and determine their quality.

**Objectives**

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

- recognize the microorganisms responsible for outbreaks of food borne infections and food poisoning,
- determine the amount of microbes in food sample, and
- analyze the microbiological quality of the sample.

## 11.2 SOURCES OF MICROORGANISMS

Microorganisms are virtually present everywhere in nature including air, water and soil. The microbial flora is also associated with the food from the time of harvest from the plant or slaughter of an animal till it is used as a food. Foods are biological materials derived from once living organisms and are perishable commodities, i.e., subject to spoilage

and decomposition. Spoilage refers to *change in the quality of food, in terms of food appearance, texture, flavour, odour and even alteration in nutritional properties.* Spoilage leads to biological, chemical and physical changes in food. The major causes of food spoilage are *microbial growth* and *enzymatic and chemical reactions*. Besides spoilage causing microorganisms, other microbes of importance are pathogenic microorganisms on food. These organisms, e.g., *Staphylococcus, Campylobacter, Clostridium perfringens, E. coli* etc. are responsible for outbreaks of food borne infections and food poisoning.

Contamination of food can occur at different levels. In fact, microbes present on foods include:

*Microorganisms associated with the raw foods*

Foods of animal and plant origin are in direct contact with air, soil and water and get contaminated with the microorganisms present in these environments. Contact with already contaminated surfaces also adds the microorganisms. Further, plants can also become contaminated with the animal manure used as fertilizers. Similarly, animals obtain microorganisms through consumption of contaminated food and by contact with insects, pests and other animals and their faeces.

*Microorganisms acquired during transport, handling and processing of raw foods*

Microorganisms can come from truck, tanker, food handlers and equipment and utensils used in the harvesting, transport and processing of foods. Personnel who come in contact with the food directly or indirectly can transmit the microorganisms present on their skin, hands, mucous membrane, intestine and also on clothing. Food handlers with poor hygiene and unsanitary habits are most likely to contaminate the food with enteric microorganisms.

Microorganisms present on the equipments and utensils, like, knives, containers, trays, pipes, tables, conveyor belts, filters, grinders etc., processing surfaces and environment and also from packaging materials can enter into the food.

*Microorganisms that survived the preservation and shortage treatment applied to the food*

Different microorganisms have different susceptibility to the preservative methods employed. Some microbes may survive depending upon the time and severity of the method used.

These spoilage microorganisms result in the commercial loss to producers, processors and consumers. Also, illness leads to loss of productivity. Therefore, it is necessary to check the number and activity of these microorganisms by employing various preservative methods. For this, understanding of the sources of microbes, the factors influencing the growth of microorganisms and the means of controlling them is must. Also, one should have the knowledge about HACCP (Hazard Analysis and Critical Control Point – a systematic approach to ensure food safety in a food processing environment) and Hurdle Strategy (using a combination of preservation methods to inhibit microorganisms). So then next let us review the factors affecting growth of microorganisms.

## 11.3 FACTORS AFFECTING MICROBIAL SURVIVAL AND GROWTH

Microbes commonly present on the food, we have seen, include members of the genera *Bacillus, Clostridium, Pseudomonas, Serratia, Proteus, Enterobacter, Micrococcus, Alcaligenes, Citrobacter, Rhizopus, Penicillium, Botrytis, Fusarium, Trichothecium* etc. The types and number of microorganisms present in food depends on various factors. These are –

1. *Nutritional Availability* – Food borne microbes are chemotrophs. Generally the types of microorganisms present on a particular food are the ones that can utilize the food components optimally. For some microbes food merely acts as a carrier.

2. *Water activity ($a_w$)* – It is the amount of water available to the organism. In general, requirement for water activity is more for bacteria (minimum 0.90) than yeasts (minimum 0.8) and moulds (0.70) with few exceptions. No microorganisms are able to grow at $a_w$ below 0.61.

3. *pH* – Presence of microorganisms also depends on the pH of the medium. Most bacteria grow optimally near neutral pH (optimum 6.5 – 7.5) and usually do not survive below pH 4.0. Yeasts (3 – 8.5, optimum 4.0 – 6.5) and moulds (2 – 11, optimum 4.5 – 6.8) can grow at more acidic pH.

4. *Oxygen* – The amount of oxygen in the environment influences the microbial growth. Depending on the oxygen requirement, you may recall studying that bacteria may be aerobe, facultative anaerobe, anaerobe or micro-aerophilic. Moulds are aerobic whereas yeasts are facultative anaerobes.

5. *Temperature* – Survival and growth of microorganisms is also affected by the temperature. According to their temperature requirement, microbes may be categorized as:

   Psychrophiles – Organisms that prefer low temperature.

   Psychrotrophs – Organisms that prefer high temperature but can grow at low temperature also.

   Mesophiles – Organisms that grow at temperature 20-40°C. Most of the disease-causing organisms belong to this category.

   Thermotrophs – Organisms that tolerate high temperature.

   Thermophiles – Organisms that grow at high temperature.

6. *Food Effects* – Nature of the food also affects the microbial survival and growth. Each food has its own characteristics which determine what type of microbes are present in that food e.g. foods having high protein content have a buffering effect. Dry foods do not contain significant number of organisms. Sugary foods contain microorganisms able to tolerate high osmotic pressure.

Having looked at the factors influencing the growth and survival of microorganisms, next, let us get to know how to detect microbes in food.

## 11.4 ENUMERATION OF FOOD MICROBIOTA

Quality of food depends on its physical, chemical, microbiological and sensory quality. The examination of food for the type and number of microbes and their products is required to analyze the microbiological quality of the food. Number of methods can be employed for enumeration of microbes in food, but none of the method can give the exact number of microbes in a food product. Every method has its own limitations.

Following methods can be employed for determining the amount of microbes in the food samples and analyzing their microbiological quality:

1. Standard or Total Plate Counts (SPC/TPC)

2. The Most Probable Number (MPN)

3. Dye Reduction Techniques, and

4. Direct Microscopic Counts (DMC)

We have already learnt about a few of these methods earlier in this manual. First three methods give the viable count, whereas, total number (viable and dead cells) is obtained from DMC.

Let us learn about these methods.

### 11.4.1 Standard or Total Plate Counts (SPC/TPC)

It is the most widely used method to know the microbiological quality of the food sample. It is quick and efficient method, giving the viable count present in the food sample. High TPC generally indicates poor quality of the food. The method involves following steps:

(i) Homogenization or blending of food sample.

Food homogenization — Liquid food samples are mixed manually before analyzing whereas solid foods are homogenized in a diluent to release microorganisms from food matrix and also to dispense clumps into smaller units. Blenders and Stomacher are commonly used to prepare the food sample for analysis. In Blender, revolving blades cut and mix the food with diluent. On the other hand, stomacher homogenizes specimen in a special sterile plastic bag by the vigorous back and forth mechanical action of 2 paddles. This shears the food specimen and the microorganisms are released into the diluent. Stomacher is generally preferred over blending because –

(a) normal operational time is less,

(b) homogenate can be stored in the stomacher bag in a refrigerator,

(c) it is less lethal to microbes than blender, and

(d) it homogenizes meat for dye reduction test which is not possible with the blender.

(ii) Serial dilution of homogenized sample in an appropriate diluent.

*Preparation of dilutions* — Decimal dilutions are prepared in a diluent [peptone water (1%) and 0.5% NaCl] and some dilutions are plated. Selection of the dilutions to be plated is made on the basis of expected microbial load in the sample.

(iii) Plating on to suitable agar medium by pour plate or surface plating.

(iv) Incubation of plates at appropriate temperature (usually 35-37$^{o}$C) for a given time (24-48 hours).

(v) Counting of all visible colonies manually or using a Quebec or electronic counter.

(vi) Calculating CFU/ml or CFU/gm of the food sample.

This method has certain limitations –

1. Microbial count depends on the type of media, incubation temperature, incubation time, distribution of the organism in the food sample and sampling method used.

2. Fastidious microorganisms may not be able to grow on the agar medium used.

3. Strict anaerobes may be overlooked because incubation conditions favours mesophilic aerobes.

4. Fungi may be underestimated because longer incubation time and lower temperature is required for fungal growth.

5. TPC indicates the size of the population but not the exact number of microbial cell because a colony may result from a clump of cells. Both pour plate and spread plate can be used for plating. Spread plate has advantage over pour plate as heat sensitive psychrophiles can be examined. Also, colonial features can be used for presumptive identification. However, there may be a problem of spreaders and crowding of colonies especially when the agar is not completely dry which makes counting difficult. Further, microaerophiles grow slowly in spread plate.

An alternative method to the conventional SPC is a Petrifilm (dry film) method. It is a non-petri dish plating system where a layer of nutrient gel is present on a paper backing covered by a thin flexible film. Indicator dye - tetrazolium – is present which imparts red colour to the colony. All red spots are counted as coloues. Petri films can also be used for detection and enumeration of specific groups such as coliforms.

Another method uses spiral plater, which is a mechanical device that distributes the liquid inoculum on the surface of the rotating agar medium plate. Special counting grid is used for enumeration of colonies. Here less agar is used as compared to standard plating and also more samples per hour can be examined. The method is, however, more suited for liquid foods.

Membrane filters can be used for determining viable count. Let us look at the different membrane filters which can be used.

*Polycarbonate nucleopore filtre*: A given sample is filtered through a membrane filter, usually polycarbonate nucleopore filter with a pore size that will retain bacteria (generally 0.22 μm or 0.45 μm). The process is illustrated in Figure.11.1. Viable counts are made by placing the filter on agar medium plate or on an absorbent pad saturated with the culture medium. Alternatively, direct microscopic counts are made, the efficiency of which can be improved by using fluorescent dyes and epifluorescence microscope. Membrane filter technique can be used only for small samples of dilute homogenates.

**Figure 11.1: Membrane Filter**

*Microcolony DEFT:* It allows determination of viable cells. Food homogenate is filtered through a DEFT membrane and then placed on the surface of appropriate culture medium plate. Microcolonies develops and are counted with a microscope. Another variation is a microcolony epifluorescence microscopy where acridine orange is used for staining and the microcolonies are enumerated by epifluorescence microscopy. Results are obtained within 6-12 hours.

*Hydrophobic Grid Membrane Filter (HGMF):* It is also used to enumerate microorganisms from a variety of food samples. By using this method, organisms as few as 10 cells/gm can be detected and results can be obtained within 24 hours. The filter contains 1600 wax grid. Following filtration, membrane is placed on suitable media for colony development overnight. The grids that contain colonies are enumerated and the MPN is calculated.

Microscope Colony Counts – The method involves counting of micro-colonies that develop on agar layered over a microscope slide.

Agar Droplets — The method was developed by *Sharpe* & *Kilsby*. In the method, food homogenate is diluted in a set of three tubes of molten agar. From each tube, a line of 5 droplets is transferred to empty Petri-plate with the help of a sterile capillary pipette. Colonies are counted with the help of 10x viewer after incubation for 24 hours. The method is faster than conventional TPC and also no dilution blanks are required. Only one Petri-dish per sample is needed.

### 11.4.2 Most Probable Number (MPN) Techniques

Different procedures and media may be used depending on the food tested and the information sought. Media which can be used include Brilliant green lactose bile broth, Eosin methylene blue agar, lauryl sulfate tryptose broth, violet red bile agar or lactose broth etc.

Presumptive count of coliforms in food is determined using Lauryl Sulphate Tryptose (LST) broth and Violet Red Bile (VRB) agar. Former helps in detection of microbes that produces gas from lactose (gas in Durham tube) while acid production is detected by VRB agar (purple red colonies surrounded with a precipitate). Additional testing is done by inoculating from the positive presumptive tests into Brilliant Green Lactose Bile (BGLB) broth for confirmation. Gas in Durham tube confirms the test. Samples from the positive confirmed test are then streaked on to Eosin Methylene Blue (EMB) agar for completing the test. Lactose fermenters produces the black or dark center colonies (Positive result). Black colonies with green sheen are probably of *E. coli*.

The MPN technique (multiple tube technique) can be used to determine the presumptive coliform count in the food. The sample is homogenized adequately and decimally diluted in saline solution or peptone water. Measured portion (e.g. 1 ml) of the solution and dilutions is transferred into multiple tubes (3 or 5) of selective and differential medium e.g. LST and incubated at 35°C for 48 hours. Production of gas in inverted Durham tube by lactose fermentation indicates coliforms. By using standard MPN tables, as given herewith (Table 11.1), MPN/ml or MPN/g of food samples is derived from number of positive tubes at 3 successive dilutions.The method was given by *Mc Crady* in 1915 and is statistically based. Three tubes or five tubes method can be used by inoculating 3 serial dilution of the sample into 9 or 15 tubes of appropriate medium respectively. The number of organisms in the original sample is determined by using standard MPN table as we have already studied in the last practical.

**Table 11.1: Most probable Number (MPN) Estimates for Three Fermentation Tubes per Dilution and Dilutions Representing 0.1 ($10^{-1}$), 0.01 ($10^{-2}$), and 0.001($10^{-3}$) Samples**

| Number of Positive Tubes/3 Tubes | | | | Number of Positive Tubes/3 Tubes | | | |
|---|---|---|---|---|---|---|---|
| **0.1 g** | **0.01g** | **0.001g** | **MPN/g** | **0.1 g** | **0.01g** | **0.001g** | **MPN/g** |
| 0 | 0 | 0 | <3 | 3 | 0 | 0 | 23 |
| 0 | 1 | 0 | 3 | 3 | 0 | 1 | 38 |
| 1 | 0 | 0 | 3.6 | 3 | 1 | 0 | 43 |
| 1 | 0 | 1 | 7.2 | 3 | 1 | 1 | 75 |
| 1 | 1 | 0 | 7.4 | 3 | 2 | 0 | 93 |
| 1 | 2 | 0 | 11 | 3 | 2 | 1 | 150 |
| 2 | 0 | 0 | 9.2 | 3 | 2 | 2 | 210 |
| 2 | 0 | 1 | 14 | 3 | 3 | 0 | 240 |
| 2 | 1 | 0 | 15 | 3 | 3 | 1 | 460 |
| 2 | 1 | 1 | 20 | 3 | 3 | 2 | 1100 |
| 2 | 2 | 0 | 21 | 3 | 3 | 3 | >1100 |

Confidence intervals are not included to simplify the table.

*Advantages:*

1. It is simple and method of choice for determining fecal coliform densities.
2. Results of different laboratories are comparable.
3. Appropriate selective and differential media can be used for specific group of organisms.

*Limitations:*

1. Large quantity of glassware is required.
2. Colony morphology cannot be determined.
3. It lacks precision.

### 11.4.3 Dye Reduction Techniques

The method is used to estimate viable organisms possessing reducing capabilities in a sample by using either of the two dyes – *methylene blue* and *resazurin*. Reduction of methylene blue results in a colour change from blue to white, whereas, resazurin changes from slate blue to pink or white. The time for dye reduction to occur is inversely proportional to the number of organisms in the sample.

Let us understand these techniques in more details.

Dye reduction test is based on the oxidation reduction activities of the bacteria present in the milk. Raw milk contains large number of bacteria like *Escherichia coli*, *Streptococcus lactis* and other enteric organisms. These bacteria produces a reduced environment by utilizing oxygen which is present in small amount in milk. In this reduced environment, dye methylene blue - a redox indicator - loses its colour and is said to be reduced (blue colour in oxidized state and white in the reduced condition). The time taken to reduce the methylene blue depends upon the bacterial load of the milk. More is the number of bacteria in the milk, faster is the reduction of dye. Methylene blue reduction test has an advantage of being rapid (takes about 6-8 hours or less) and inexpensive. The time of methylene blue reduction is indicative of the milk quality. Table 11.2 gives the classification of milk quality according to the methylene blue reductase time.

**Table 11.2: Classification of milk quality according to the methylene blue reductase time.**

| S.No. | Methylene Blue Reduction Time | Classification of milk sample | Approximate number of bacteria/ml |
|---|---|---|---|
| 1. | 0-30 mins. (1/2 hr.) | Very poor quality | >20,000,000 |
| 2. | 31-120 mins. (1/2 – 2 hrs.) | Poor quality | >4,000,000 |
| 3. | 121-360 mins.(2-6 hrs.) | Fair quality | >500,000 |
| 4. | 360 – 480 mins. (6-8 hrs.) | Good quality | <500,000 |

The limitations and advantages of this technique are enumerated next.

*Limitations:*

1. It is not suitable for food sample having inherent reductive substances, e.g. raw meat.
2. All the organisms do not reduce the dye equally.

*Advantages:*

1. It is simple, rapid and inexpensive.
2. It gives the viable cell count which reduce the dyes.

It is a method of choice for assessing the microbial quality of the milk.

### 11.4.4 Direct Microscopic Counts (DMC)

The process includes making a smear of food sample on a microscope slide followed by staining with an appropriate dye and viewing and counting under the microscope. It is widely used for assessing the microbial quality of the milk and dairy products (Breed Count).

The method involves spreading 0.01 ml of the milk sample on 1 cm area of a slide. After drying and staining the slide, average number of bacteria per microscopic field is counted. The diameter and area of the microscopic field is calculated by micrometry. Number of bacteria per ml of the milk sample is calculated as:

*Number of bacteria/ml* = average number of bacteria per microscopic field x number of field x dilution factor.

*Number of Microscopic fields* = area over which milk is spread ÷ area of one microscopic field = 100 $mm^2$ ÷ 0.02 $mm^2$ = 5000 field

*Dilution factor* = 100 (0.01 ml of the sample is plated)

It is rapid and simple method by which cell morphology can be detected. Efficiency can be increased by using fluorescent dye. The method, however, gives the count of both viable and nonviable cells. Sometimes food particles may not be distinguishable from microorganisms. Some microorganisms may not take stain and not be counted. Inspite of these drawbacks, it is used for quick assessment of microbial quality of foods.

The discussion above focused on the methods we can use to assess the microbiological quality of food samples. Before we move on to the exercises, let us review our understanding of what we have learnt in this practical by answering the questions given in Review Questions section 11.5.

## 11.5 REVIEW QUESTIONS

1. What is the significance of examining the microbes in a food product?

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2. Name different sources of microbial contaminants in a food.

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3. What are the factors, which affect the survival of microbes on the food?

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4. Mention different categories of microbes on the basis of their temperature requirement.

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5. Name various methods used for microbial enumeration in food and what is the basis of these tests?

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6. Why food homogenization is necessary?

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7. Which diluents can be used for making dilutions? Why serial dilution is important?

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Now let us get started with the exercises.

# EXERCISE 1

## GRAM STAINING OF BACTERIAL CULTURES

Date : .....

**Aim** : To determine the total number of bacteria in a given food sample.

**Requirements**

*Materials* : Food samples, like, ice cream, ghee, dahi, processed foods, milk etc.

*Media and Reagents* : Nutrient agar plates, diluent tubes.

*Equipments & Glassware* : Bunsen burner, sterile pipettes, incubator, test tubes, test tube rack, glass marker, rubber bulb, alcohol jar.

**Theory/Principle:**

Foods are rich in various nutrients and can support the growth and survival of microorganisms. The microbial load on foods vary depending on the way they are produced, processed, transported and handled. These microorganisms can deteriorate the food and may be responsible for causing food borne diseases, which sometimes, may be serious and fatal. Therefore, it is essential to analyze the food samples for the microbial quality. Presence of coliforms by using multiple tube technique indicate the possibility of feacal contamination and presence of pathogens in a sample. By SPC, presence of large number of microbes suggests poor sanitary and hygienic conditions, temperature abuse, inadequate processing or post processing contamination and there are increased chances of food spoilage. One type of medium and incubation conditions may not necessarily reveal the total number of microbes in a food sample and may not be suitable for all samples. The various procedures and techniques can be used for microbes determination in a food sample depending on the purpose.

The Standard Plate Count (SPC) is one of the most widely used method for enumerating the microbes and to know the microbiological quality of the food samples. Both pour plate and spread plate techniques can be employed.

**Procedure:**

Now carry out the exercise following the steps enumerated herewith:

1. Label the nutrient agar plates with the name of the food sample and the dilution to be plated.
2. Weigh 20 gm of each food sample and mix it with 180 ml of sterile water in the blender jar by blending for 2 minutes. This gives 1:10 ($10^{-1}$) dilution of each food sample. Physiological saline or peptone water can be used as a diluent. Homogenization can also be done in stomacher.
3. From $10^{-1}$ dilution, prepare further dilutions in a series from $10^{-2}$ to $10^{-7}$. This is done by adding 1 ml of 1:10 dilution into a diluent tube marked $10^{-2}$ and shake vigorously 1 ml of $10^{-2}$ (1:100) is then transferred to a tube marked $10^{-3}$ (1:1000) and so on. After each transfer shake the suspension vigorously.
4. Spread 0.1 ml of each dilution on the nutrient agar plate using sterile bent glass rod (spreader). Other media like brain heart infusion broth or plate count agar can also be used.

5. Incubate the plates at appropriate temperature (about 37°C) for 24 to 48 hours.
6. Observe the plates for colony count and determine the CFU/gm of the food by using only those plates that fit the criteria of the counting rules (i.e. colonies count between 30 and 300).

**Precautions:**

1. The dilutions should be chosen on the basis of expected counts in the food sample.
2. Food should be kept in the laboratory refrigerator or in cold till assay starts.
3. Food sample should be kept in a sterile container and should be collected aseptically.
4. Weighing of the sample should be done in a sterile stomacher bag (for stomacher samples) or into a sterile petri plate (for blender sample).
5. While using pour plate technique, do not remove all the media tubes simultaneously to avoid premature solidification of the medium.

**Observations and Results:**

Record your observations in the format given herewith.

| Observations | | | | Result |
|---|---|---|---|---|
| **Food Sample** | **Dilution** | **Colony count/ Plate** | **CFU/ml** | **CFU/gm of food** |
| | | | | |

**Inference / Conclusion:**

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Also answer the questions given in the review questions.

## Review Questions

1. What are the limitations of standard plate count?

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2. Why the counts are presented in the form of CFU/ml?

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**Submit the exercise for evaluation.**

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**Counsellor Signature**

# EXERCISE 2

# DETERMINATION OF FUNGAL AND YEAST COUNT IN A GIVEN FOOD SAMPLE

Date : .....

**Aim** : To enumerate the fungal and yeast count in a given food sample.

**Requirements**

*Material* : Food sample e.g. fruit juice, canned food.

*Reagent* : Acidified potato dextrose agar (PDA) or antibiotic plate count agar (PCA).

*Equipments & Glassware* : Bunsen burner, glass spreader, petri plates, 9 ml dilution blanks, alcohol jar, sterile pipettes (1ml), pipette bulb, incubator.

**Media preparation**

*Potato Dextrose Agar (PDA), acidified.*

| | |
|---|---|
| Glucose | 20.0 gm |
| Infusion from potato (peeled) | 200.0 gm |
| Agar | 15.0 gm |
| 10% sterile tartaric acid | 1.85 ml/100 ml |
| Distilled Water | 1000.0 ml |

pH 5.6

*Antibiotic PCA*

| | |
|---|---|
| Glucose | 1.0 gm |
| Tryptone | 5.0 gm |
| Yeast Extract | 2.5 gm |
| Agar | 15.0 gm |
| Distilled Water | 1 litre |

pH 7.0

Dissolved all the ingredients in 1litre of distilled water. Following autoclaving add 2 ml of a 500 mg solution of chlortetracycline HCl and chloramphenicol per 100 ml of PCA.

**Theory/Principle:**

Fungi are widespread and present on food, equipments and processing and storage facilities. These are the major cause of food spoilage and result in great economic loss. Further, the growth of fungi on foods may result in production of toxins called *mycotoxins*, many of which are carcinogenic. The presence of large number of moulds and yeasts in food suggests poor sanitary conditions or temperature abuse. Analysis of food for moulds and yeasts is, therefore, needed to access the safety and quality of these foods.

Non-selective, non-differential medium like acidified PDA and antibiotic plate count agar can be used for fungal detection and enumeration. Here low pH and antibiotics inhibit the growth of bacteria, not of yeasts and moulds.

**Procedure:**

Now carry out the exercise following the steps enumerated herewith:

1. Shake the food sample and aseptically transfer 1 ml of it to a 9 ml dilution blank tube marked $10^{-1}$. Similarly prepare further tenfold dilutions till $10^{-6}$.
2. Dispense 0.1 ml of each dilution on to the labeled agar plates.
3. Spread the inoculum evenly on the plates using a sterile bent glass spreader. Sterilization of a spreader is done by dipping it in the alcohol jar and passing through the flame. Caution – Always keep the alcohol away from the flame.
4. Incubate plates at $28 \pm 2^{o}C$ for 4-5 days undisturbed.
5. Count the number of yeasts and moulds. Yeast colonies are similar to bacteria whereas mould colonies are larger and fuzzier. Counting can be done from underside of plates if moulds are overgrown.
6. Calculate the CFU/ml of the food sample. Try to identify the fungi.

For solid foods, homogenates are prepared first and then the same procedure is followed.

**Precautions:**

1. Maintain aseptic conditions.
2. Keep alcohol away from flame.
3. To avoid infections and allergies, care should be taken while handling the plates containing yeast and moulds.
4. Calculations should be made only from appropriate countable plates.
5. Avoid transferring food particles from the homogenized sample into dilution tubes and on the agar plates (presence of food particles on agar medium masks the colonies).

**Observations and Results:**

Record your observations in the format given herewith:

| Observations | | | | Results |
|---|---|---|---|---|
| **Dilution** | **Volume plated** | **No. of colonies** | **Counts CFU/ml** | |
| | | | | |

**Inference / Conclusion:**

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Also answer the questions given herewith under the Review Questions section.

**Review Questions**

1. Why PDA is used for the detection of fungal count?

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2. What other media can be used for detection and enumeration of fungi and yeasts?

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3. What is the importance of fungi and yeasts in food?

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**Submit your exercise for evaluation.**

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**Counsellor Signature**

# EXERCISE 3

## DETERMINATION OF COLIFORMS IN THE GIVEN FOOD SAMPLES BY PRESUMPTIVE TESTING

Date : .....

**Aim** : To determine the presence of coliform bacteria in the given food sample(s) by presumptive testing.

**Requirements**

*Material* : Food samples e.g. vegetables.

*Media* : LST broth, peptone water (diluent), VRB agar, BGLB

*Equipment & Glassware* : Bunsen burner, incubator, pipettes, weighing balance.

**Composition of Media**

A. Lauryl Sulfate tryptose (LST) broth

| | |
|---|---|
| Lactose | 5.0 gm. |
| Potassium phosphate, dibasic | 2.75 gm |
| Potassium phosphate, monobasic | 2.75 gm |
| Sodium chloride | 5.0 gm |
| Sodium lauryl sulfate | 0.1 gm |
| Tryptose | 20. gm |
| Distilled water | 1000.0 ml |
| pH | 6.8 |

B. Violet Red Bile (VRB) agar

| | |
|---|---|
| Bile Salt | 1.5 gm |
| Crystal Videt | 0.002 gm |
| Lactose | 10.0 gm |
| Neutral Red | 0.03 gm |
| Peptone | 7.0 gm |
| Sodium Chloride | 5.0 gm |
| Yeast Extract | 3.0 gm |
| Agar | 10.0 gm |
| pH | 7.4 gm |

Dissolve in IL of distilled water. Heat to boiling (22 minutes), do not autoclave and dispense agar to plates.

Brilliant green lactose bile (BGLB) broth

| | |
|---|---|
| Brilliant green | 0.0133 gm |
| Lactose | 10.0 gm |
| Oxyall | 20.0 gm |
| Peptone | 10.0 gm |
| Distilled Water | 1000 ml |
| pH | 7.2 |

**Theory/Principle:**

Coliforms are gram negative, non-spore forming, facultative anaerobic bacteria which produce acid and gas on lactose fermentation at 37°C within 48 hours. It includes *E. coli*, *Klebsiella* and *Enterobacter* and are used as indicator of fecal contamination. Though

coliforms commonly inhabit the intestinal tract, their presence on food may be because of non-feacal origin. Therefore, the results of coliform counts should be cautiously interpreted.

As discussed in practicaly chapters, coliform detection in food and water include 3 tests. Write the principle behind the 3 tests in the space provided.

1. *Presumptive Test* .....

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2. *Confirmatory Test* .....

   .....

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3. *Completed Test* .....

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Now write about the differential media which can be used.

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**Procedure:**

1. Homogenize the food sample in a blender by mixing 11 gm of food sample with 99 ml of peptone water or sterile water. This is 1:10 ($10^{-1}$) dilution.
2. Prepare further serial decimal dilutions, i.e., $10^{-2}$, $10^{-3}$, $10^{-4}$ using 9 ml peptone water tubes.
3. Label 9 tubes of LST broth and inoculate these with 1 ml each of $10^{-2}$, $10^{-3}$ and $10^{-4}$ in triplicates. LST tube inoculated with 1 ml of *E. coli* is taken as a positive control.
4. Incubate the tubes at 35°C for 48 hours and observe for the gas production. Gently shake the tube to help release the gas from the medium.
5. Note the number of positive tubes in each set and determine the MPN/gm of the food from the MPN Table 11.1 given on Page 205 earlier. Determine the dilution factor multiplier and multiply the tube values with it to convert the dilution. e.g. if the lowest dilution $10^{-4}$ then multiply the table value by $10^{-2}$ analyzed is $10^{-2}$ then multiply the table value by $10^{1}$. Similarly if the lowest dilution is $10^{-3}$, then multiply the table value by $10^{-1}$. This will give the presumptive MPN/gm.
6. Also transfer 1 ml each of $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions to sterile petri plates. Pour the molten VRB agar and mix the sample gently with the medium. *E. coli* culture is taken as a positive control.
7. After solidification, incubate the plates at 35°C for 24 hours. Count the presumptive coliform colonies on sample's plates by matching the purple red colony colour surrounded by a halo of precipitated bile salt to those of positive control.
8. Determine the coliform CFU/gm of food by considering the countable plates.
9. Confirm the positive MPN/VRB agar results in confirmed test.

**Confirmed Test**

i. Label BGLB tubes and inoculate them from three positive LST tubes and three presumptive coliform colonies from the VRB agar for confirmation.

ii. Incubate at 35°C for 24 hours.

iii. Examine the BGLB tubes for gas production. If all the tubes are positive, the previous presumptive MPN/gm or CFU/gm is confirmed. If one or two tubes are positive, calculate MPN/gm or CFU/gm of food accordingly on the basis of the proportion of confirmed gassing in BGLB broth tubes. If all BGLB tubes show negative results then coliforms are considered to be absent.

**Completed Test**

i. For completed test, inoculate the contents of positive BGLB on EMB agar plates.

ii. Incubate at 35°C for 24 hours.

iii. Observe for dark centered colonies with or without green sheen. *E. coli* produces green sheen. *Enterobacter* colonies appear pink and mucoid. Non-coliform colonies are colourless.

**Precautions:**

1. Make dilutions carefully.
2. Solidification of VRB agar should be prevented before pouring in plate.

**Observations and Results:**

Using standard MPN table given earlier in –section 11.4.2, record your observations and results for the food samples in the format provided herewith. Under the observation record the number tubes giving colour change in a set of three dilutions used. Next find in the MPN value from the Table 11.1. Further find out the dilution factor and multiply this with MPN value to get MPN/gm of food sample.

| **Observations** | | | **Results** |
|---|---|---|---|
| **Presumptive Test** | | | **Presumptive MPN/gm** |
| **Dilutions** | **No. of Positive** | **MPN value from Table 11.1** | |
| **LST** | **Tubes** | | |
| **$10^{-2}$** | | | |
| **$10^{-3}$** | | | |
| **$10^{-4}$** | | | |
| **VRB Agar** | | | **CFU/gm=** |
| **$10^{-2}$** | | | |
| **$10^{-3}$** | | | |
| **$10^{-4}$** | | | |
| **Confirmed Test- BGLB broth** | | | |
| **With LST** | | | **MPN/g =** |

| | |
|---|---|
| **With VRB** | **CFU/g =** |
| **Completed** | |
| **With LST** | **MPN/g =** |
| **With VRB** | **CFU/gm=** |

**Inference/Conclusion:**

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**Review Questions**

1. What is the importance of performing presumptive test on foods?

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2. Which media are used for confirmed and complete tests?

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   .....

**Now submit the exercise for evaluation.**

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**Counsellor Signature**

# EXERCISE 4

## DETERMINATION OF THE QUALITY OF MILK SAMPLE BY METHYLENE BLUE REDUCTASE TEST

Date : .....

**Aim** : To determine the quality of given milk samples by performing methylene blue reductase test.

**Requirements**

*Material* : 2 milk samples – raw milk and pasteurized milk.

*Reagent* : Methylene blue solution (1:250,000).

*Equipments & Glassware* : Sterile screw cap test tubes, sterile pipettes (10 ml and 1 ml), water bath (37°C), bunsen burner, sterile distilled water, test tube rack.

**Theory/Principle:**

Milk is an ideal medium for the survival and growth of microorganisms. It contain all the nutrients, i.e., carbohydrates, proteins, vitamins, minerals and fat and has a pH of 6.8. The number and kinds of microorganism in milk depends on the milking conditions and the ways it is transported, processed and handled. Heating and pasteurization reduces the microbial load. Milk, if not handled and processed properly, is a carrier of number of diseases like diphtheria, dysentery, Q fever, tuberculosis, typhoid etc. Quality check of the milk is needed to prevent these diseases. Commonly used methods include standard plate count, breed or direct microscopic count, coliform test and dye reduction test.

**Procedure:**

1. Prepare methylene blue solution by dissolving 1 mg methylene blue in 250 ml of sterile distilled water.

2. After thorough mixing, transfer 10 ml of each sample into labeled sterile screw cap test tubes and add 1 ml of methylene blue in each tube. For control, take 10 ml boiled milk of each sample and add 1ml of methylene blue (positive control). Negative control contains 10 ml of milk sample and 1 ml of the water.

3. Close the tubes with stoppers and mix the content of the tubes by inverting the tubes gently for 4-5 times.

4. Incubate the tubes in a water bath at 37°C for 6-8 hours. Observe the tubes for reduction of methylene blue (i.e. change in colour) after every half an hour for 6-8 hours. Compare the sample tube with positive and negative control for change in methylene blue colour. Record the time of dye reduction and accordingly assess the quality of the milk samples.

**Precautions:**

1. Methylene blue dye should not be very concentrated.

2. Mix the dye properly with milk sample.

**Observations and Results:**

Record your observations in the format given herewith:

| Observations | | Result |
|---|---|---|
| **Food Sample** | **Reduction Time** | |
| **Raw Milk** | | |
| **Pasteurized milk** | | |

**Inference / Conclusion:**

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**Review Questions**

1. What is the basis of dye reduction test?

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2. When the milk is said to be of good quality by dye reduction test?

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3. Name the dyes used in dye reduction test.

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**Submit the exercise for evaluation.**

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**Counsellor Signature**

# EXERCISE 5

## DETECTION OF NUMBER OF BACTERIA IN MILK BY BREED COUNT

Date : .....

**Aim** **:** To detect the number of bacteria in milk by breed count.

**Requirements**

*Material* : Milk samples

*Reagent* : Methylene blue, xylol, alcohol

*Equipments & Glassware* : Bunsen burner, lmmersion oil, glass slide, capillary pipette, graph paper, microscope.

**Theory/Principle:**

Total number of bacteria in the milk sample can be calculated by breed count technique.

**Procedure:**

1. Place a clean, non-greasy slide over the graph paper.
2. Spread 0.01 ml of the milk over an area of 1 $cm^2$ as a thin even film.
3. Dry the smear by gently passing through the flame.
4. Defat the smear by flooding with xylol for 1 minute.
5. Wash the slide with alcohol to remove xylene followed by washing with water to remove the alcohol.
6. Stain the smear with methylene blue for 15-30 second.
7. After washing with water, observe the dried preparation under oil immersion. Count the number of bacteria in about 25 microscopic field.
8. Determine the average number of bacteria per microscopic field and multiply with microscopic factor (500,000) to get bacterial counts per ml of the milk sample.

**Precautions:**

1. Make a thin film of milk on the slide.
2. Do not over stain with methylene blue. If over staining has occurred, flood it with ethanol, to get proper degree of colour.

**Observations and Results:**

Record your observations in the format given herewith:

| Observations | Results |
|---|---|
| **No. of bacteria in 25 fields** | |
| **Average number of bacteria/field** | |
| **Microscopic factor** | |
| **Number of bacteria/ml of the original milk sample** | |

**Inference/Conclusion:**

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**Review Questions**

1. Mention the principle behind Breed Count.

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2. What are the limitations of Breed Count?

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**Submit the exercise for evaluation.**

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**Counsellor Signature**