
UNIT 8 MICROBIOLOGICAL ANALYSIS OF MILK AND MILK PRODUCTS

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8.0 OBJECTIVES

After reading this unit you will be able to:

- 1 perform the microbiological analysis of milk and milk products;
- 1 appreciate the utility of such analysis;
- 1 acquire the knowledge of various microbiological tests;
- 1 decide the test to be conducted in a given situation; and
- 1 screen and grade the dairy products on the basis of microbiological criteria.

8.1 INTRODUCTION

Microbiological analysis of dairy products is carried out to determine the degree and types of bacterial contamination in milk and milk products and to estimate the physical and chemical changes brought in milk products as a result of microbial growth. The information obtained by microbiological analysis can be employed for a number of purposes such as

- 1 Market milk control and grading
- 1 Improvement of milk production environment and practices
- 1 Screening of milk supplies to assess their suitability for processing or manufacture of milk products

- 1 Detection of pathogenic and spoilage microorganisms
- 1 To ensure compliance of finished products with microbiological standards

8.2 MICROBIOLOGICAL TESTS

Various microbiological tests performed in dairy industry can be broadly categorized in to following groups:

- 1 Direct enumeration of Total Bacterial Count e.g. Direct Microscopic Count
Estimation of number of viable bacterial cells e.g. Standard Plate Count
- 1 Assessing the microbial metabolic activities e.g. Dye Reduction Test
- 1 Detection of specific Contaminants e.g. Coliforms, Pathogens
- 1 Estimation of biochemical changes or metabolites formed in dairy products as a result of microbial growth e.g. acidity, gas production, toxin production etc.

i) Direct Microscopic Count (DMC) Method

The DMC method enables rapid enumeration of bacterial cells along with their study of morphology of the total bacterial count in milk and cream with minimum equipment. It consists of examination of stained films of a measured volume of milk or milk product (0.01 ml) spread over 1 cm² area and dried on a glass slide under microscope. Somatic cells, shapes and arrangement of bacterial cells present in films can be easily and rapidly visualized and recorded. The microbial morphology and arrangement give the clue to possible cause of high count while high somatic cell indicates udder infection e.g. mastitis. For determination of average number of bacterial cells or clumps of cells about 5 to 50 microscopic fields are scanned (fewer the number of cells, more fields to be scanned). The diameter of a field is measured with the help of a stage micrometer to calculate microscopic factor (MF). The DMC/ml is then calculated as follows:

$$\text{DMC/ml} = N \times \text{MF}$$

Where N= Average number of cells per field

MF= Microscopic Factor

MF= Area of Smear/Area of Microscopic x 1/Volume of milk (0.01 ml)

$$= 10,000 / 3.1416 \times r^2$$

This technique is very useful for screening of milk supplies on the receiving platform of a dairy plant as well as for grading of milk. However, the limitation of this method is that both dead as well as viable cells are counted.

ii) Standard Plate Count (SPC) Method

In this method a known quantity of milk sample is diluted to known degree and equal portions of each dilution is poured in to a petriplate followed by addition of nutrient agar medium, a technique known as pour plate method. The medium is allowed to solidify after mixing the contents by gentle rotation of the plate. The

organisms present in the sample are expected to be trapped in the agar gel. The plates are subsequently incubated at 37 C for 48 to 72 hours. In principle each organism is expected to take up a separate position in the medium and grow in to a mass of cells of a size sufficient enough to be counted by naked eyes, recognized as a colony forming unit (cfu). Hence, a colony count performed at this stage represents number of viable bacteria present in the given volume of milk sample. The major limitations of this method is that it is time consuming and only those bacteria which are capable of growing under given set of growth conditions (medium, incubation temperature and period) and forming colonies can be counted.

Determination of microbiological quality of milk and milk products invariably involves performing different plate counts. These include SPC, the Coliform count, and the yeast and mold count. Techniques employed for plating are identical for these tests though method of sampling and media may vary.

iii) Dye Reduction Test

There are certain dyes, which act as oxidation-reduction indicator. Bacteria consume dissolved oxygen during their growth in milk and consequently reduce the OH to a level at which these dyes are reduced and get decolorized. Such dyes can be employed to assess the biochemical activity of bacteria and thus estimate number of bacteria indirectly.

i) Methylene Blue Reduction (MBR) Test

Methylene blue is a dye, which remains blue in its oxidized state and turns colorless on its reduction. This characteristic is put to use for estimation of bacterial load of milk and milk products. When bacteria grow in milk they release hydrogen during respiration, which is simultaneously accepted by methylene blue. As a result, it is reduced to colorless or leuco compound.

The majority of bacteria, both aerobic and facultative present in milk indulge in lowering of oxidation-reduction potential of milk to such an extent that dye gets decolorized. Hence greater the number of viable cells, shorter is the time taken to reduce the dye. The result of this test is expressed in terms of time required for the color of methylene blue to disappear at incubation temperature of 37° C.

This test renders very useful information on general bacteriological quality of milk in a short period and requires fewer apparatus. Limitations of this technique include suitability only for unheated milk, no indication of type of organisms and incubation temperature favorable only for mesophilic bacteria.

ii) Resazurin Reduction (RR) Test

Resazurin is also an OH indicator and hence is liable to be reduced by bacteria. Reduction of blue dye takes place in two stages. First, the dye is irreversibly reduced to resorufin undergoing through a series of colors ranging from blue to lilac, mauve, purple and pink. During second stage, resorufin is reversibly reduced to a colorless compound, dihydroresorufin.

Various colors developed sequentially during reduction of dye can be well compared with a standard resazurin disc with the help of a small apparatus known as resazurin comparator. Results are expressed in terms of standard resazurin disc number ranging from 6 to 0. The time taken for the reduction of dye to a specific

stage (disc number) or the color change recorded on completion of incubation after a certain period can be used as a scale for measurement of bacterial activity. The test is carried out at incubation temperature of 37° C for 10 minutes, 1 hour or till complete reduction.

This test finds its application in quick grading of milk (even faster than MBR Test). However, reduction of dye is susceptible to light and confusion may arise in interpretation of results due to the fact that besides bacteria, this dye is liable to be reduced by leucocytes.

Check Your Progress – 1

Fill in the blanks:

1. Direct Microscopic Count per ml is determined by multiplying _____ with _____.
2. The formula of calculating Microscopic Factor is = _____
3. The colony formed by bacterial cells on the agar surface in SPC method is recognized as _____
4. The dye used for dye reduction tests include _____ and _____.
5. Resazurin is reduced to two compounds viz. _____ and _____.

iv. Coliform Test

The Coliform group of bacteria (Escherichia, Enterobacter, Klebsella) includes gram-negative, non-spore forming, aerobic and facultative rods capable of fermenting lactose in to lactic acid and gas.

As per American Public Health Association (APHA) method, Coliforms in milk are detected by following scheme:

Presumptive Coliform Test: One ml of milk sample or decimal dilution is poured to sterile plates followed by addition of 10-15 ml of Violet Red Bile Agar (VRBA). The content of plates is by gently rotating and tilting each dish and finally agar is allowed to solidify and incubated at 32° C for 24 h. Appearance of typically red colonies measuring about 0.5 mm is taken as positive test.

Alternatively fermentation tubes of 2% brilliant green lactose bile (BGLB) broth are inoculated with sample (1.0 ml) and production of gas after incubation of 48 h at 32° C is considered as positive indication for presence of Coliforms in milk.

Confirmed Test: A confirmed test of doubtful colonies from VRBA is carried out by transferring each of five colonies to tubes of 2 % BGLB broth and observing for gas production.

Completed Test: Finally, material from typical colonies on solid media or from BGLB broth tubes showing gas production is streaked on Eosine Methylene Blue Agar. Coliforms form dark colonies or dark centered colonies with colorless peripheries and red colonies on VRBA. Pure cultures so isolated should be able

to produce gas in fermentation tubes of lactose broth at 32°C within 48 h and microscopic examination should reveal only gram-negative, non-spore forming rods.

v. **Detection of Pathogens**

Milk is a favourable niche for pathogens and various pathogens found in milk have been discussed in detail in unit 2. In this section, detection of two frequently encountered pathogens viz. Salmonellae and Staphylococci have been discussed. Both of these organisms are enterotoxigenic (produce enterotoxin). Being heat labile, though these organism may be destroyed during heat treatment, yet their toxins survive Majority of heart treatment. National and international microbiological standards have specified limits for them in various dairy products.

Staphylococci are gram positive, catalase positive, coagulase positive (coagulate blood plasma) cocci occurring singly or in clusters. The undiluted sample or its decimal dilution of milk/milk product is analyzed by plate count method using selective media (Trypticase soy broth, Baird Parker Agar, Vogel and Johnson agar, Staphylococci medium No. 110). Appearance of typical black colonies on agar surface after incubation for 48 h at 37° C is taken as a positive test. Subsequently, Coagulase test is performed to confirm their presence. Colonies are picked from the agar plates, inoculated into brain heart infusion broth tube and 0.5 ml of coagulase plasma is added before incubation at 37° C for 6 h. The tubes are periodically observed for clot formation as a positive reaction. Doubtful colonies may be further subjected to additional tests such as catalase reaction, anaerobic utilization of glucose and mannitol, susceptibility to lyostaphin etc.

The detection scheme for Salmonellae is elaborate. This organism is gram negative, may or may not produce H₂S. Presence of other related organisms e.g. Escherichia, Enterobacter, Shigella and Proteus might prove interfering in interpretation of results. These organisms are differentiated on the basis of reaction they exhibit on slants of Triple Sugar Iron agar and appearance of characteristic colonies on the surface of differential agar media as shown in Tables 8.1 and 8.2.

Table 8.1: Appearance of typical colonies of salmonella on agar

Sl. No.	Medium	Type of organism	Appearance of colonies
1.	Desoxycholate agar	<i>Salmonella typhosa</i> Other Salmonellae	Pale or colourless opaque, dome shaped, with a central black dot surrounded by orange zone.
2.	Bismuth sulphite agar	<i>Salmonella typhosa</i> H ₂ S producer Non-H ₂ S producer	Black colonies with metallic sheen. Black colonies Green colonies
3.	Brilliant Green agar	Salmonellae	Pink colonies
4.	Salmonella-Shigella agar	Salmonellae	Colourless

Table 8.2: Reaction of salmonella and other related bacteria on triple sugar iron or lysin iron agar

Butt	Slant	H ₂ S Production	Genus
AG	N/AK	+/-	Salmonella
AG	A	-	Escherichia
AG	A	-	Enterobacter
A	N	-	Shigella
AG/A	N	+	Proteus

A-Acid; G-Gas; AG-Acid and Gas; N- Neutral, ALK-Alkaline

vi. Yeast and Mould Count

Potato dextrose agar (PDA) is the medium of choice for yeast and mould count as potato extract promotes the growth of these organisms. The suppression of bacterial growth which may otherwise interfere, is ensured by adjusting the pH of medium by adding 10% tartaric acid. The plate count technique is used and plates are incubated at 21^o C for 2 to 5 days.

Check Your Exercise – 2

Fill in the blanks.

- Two common genera representing coliforms are _____ and _____.
- Solid and liquid media used for presumptive coliform test are _____ and _____ respectively.
- _____ is an example of selective medium used for detection of Staphylococci.
- Staphylococci form _____ color colonies on selective agar surface.
- _____ color colonies are produced by Salmonellae on Brilliant Green Agar.

8.3 LET'S SUM UP

Microbiological analysis of milk and milk products renders valuable information on degree and type of contaminants. There are a number of tests available to estimate the bacterial load of dairy products viz. DMC, SPC, MBR, and RR tests. The presence of specific contaminants e.g. Coliform, pathogens and Yeasts and Mould is detected by plate count method employing selective solid and liquid media. The majority of these tests however, are time consuming and hence can not be relied up on to judge the milk at reception and prior to consumption. Efforts are on to overcome this limitation by developing suitable rapid enumeration and detection tests. Such approaches include use of techniques based on automated

instruments e.g. Bactoscan Method, Direct Epifluorescent Filter Technique, enzymatic method e.g. Enzyme Linked Immunosorbent Assay method, ATPase Method and genetic method e.g. Polymerase Chain Reaction Method.

8.4 KEY WORDS

DMC	: Direct Microscopic Count
MF	: Microscopic Factor
SPC	: Standard Plate Count
CFU	: Colony Forming Unit
MBR	: Methylene Blue Reduction
RR	: Resazurin Reduction
VRBA	: Violet Red Bile Agar
BGLB	: Brilliant Green Lactose Bile
PDA	: Potato Dextrose Agar
DEFT	: Direct Epifluorescent Filter Technique
ELISA	: Enzyme-Linked Immunosorbent Assay
PCR	: Polymerase Chain Reaction

8.5 SOME USEFUL BOOKS

Marth, E. H and Steele, J. L. S. (2001). *Applied Dairy Microbiology*. 2nd Edition. Marcel Dekker Inc.

Mudgal, V.D, Tomar, S.K., & Kulkarni, K (1998). *Dairy Production and Quality of Milk*. Text Book for Class XI, NCERT Publication

Marshall, R.T (1992) Standard Methods for the examination of Dairy Products, 16th Ed. APHA, USA.

Vanderzant, C & Spittstoesser, D.F (1992) Compendium of Methods For the Microbiological Examination of Foods. 3rd Ed. APHA, USA

8.6 ANSWERS TO CHECK YOUR PROGRESS

Your answers should include the following points.

Check Your Progress – 1

1. Average number of cells, Microscopic Field
2. $MF = \frac{\text{Area of Smear}}{\text{Area of Microscopic} \times \frac{1}{\text{Volume of milk (0.01 ml)}}} = \frac{10,000}{3.1416 \times r^2}$

3. Colony Forming Unit(cfu)
4. Methylene Blue, Resazurin
5. Resorufin, Dehydroresorufin

Check Your Progress – 2

1. Escherichia, Enterobacter
2. Violet red bile agar, Brilliant green lactose bile broth
3. Trypticase soy broth/ Baird Parker Agar/ Vogel and Johnson agar/ Staphylococci medium No. 110
4. Black
5. Pink