

Programme: Bachelor of Science (First Year)

Subject: Microbiology

Course Code: MIG 102

Course Title: Industrial and Food Microbiology

Unit 7 - Microbiology of milk

Module Name: Grading of milk by dye reduction test: MBRT and Resazurin test

Name of the Presenter: Ms. Shilpa T. Shirodkar

The microbiological quality control testing of milk can be divided into two different groups: for example direct tests (quantitative) and indirect tests (qualitative). Dye reduction tests are indirect methods of assessing the microbiological quality of milk. These are based on the metabolic activity of the microorganisms. A correlation is made between the time required for the reduction of dye and probable number of bacteria in milk.

The principle of these tests is to add dyes, like methylene blue, resazurin or trimethyltetrazolium chloride, to milk or liquid dairy products, and to measure the color change after incubation. The color change is based on the dehydrogenase activity of the bacteria present in sample.

Most commonly used dye reduction tests are methylene blue reduction test (MBRT) and resazurin test.

1. Methylene Blue Reduction Test (MBRT)

It is commonly used as a quick method to assess the microbiological quality of raw and pasteurized milk. This test is widely used at the dairy reception dock, processing units and milk chilling centres where it is followed as acceptance/rejection criteria for the raw and processed milk.

Principle

This test is based on the fact that the blue colour of the dye solution added to the milk get decolourized when the oxygen present in the milk get exhausted due to microbial activity. Oxidation reduction potential of a substrate may be defined generally as the chemical process in which the substrate either loses or gains electrons. When an element or compound loses electrons the substrate is said to be oxidized, while a substrate that gains electrons becomes reduced. Due to the metabolic activity of bacteria present in milk, more and more oxygen is used up, which causes in depletion of oxygen in milk. Hence methylene blue starts acting as

electron acceptor instead of oxygen. As the oxidation reduction potential decreases methylene blue gets reduced and gets converted into leucomethylene blue which is a colourless compound. The greater is the number of microorganisms in milk, the greater is the metabolic activity and the faster is the reduction of methylene blue. Thus, the time of reduction is taken as a measure of the number of microorganisms in milk. The disappearance of color in a short time indicates a high microbial load.

Procedure

Take 10 ml of milk into a test tube and add 1 ml of standard methylene blue solution. Mix the milk and methylene blue solution. Place the test tube in a thermostatically maintained water bath at 37°C and note down the time of incubation. Observe the test tubes after 30 min for decolourization reduction of dye. If there is no decolourization the tubes are kept for further incubation. After 30 min, continue to observe for the reduction of dye at an interval of every one-hour. The milk shall be regarded as decolorized, when the entire column of milk is completely decolorized or is decolorized up to 5 mm of the surface.

Grading of milk based on MBRT

Quality of milk	Reduction time
Excellent	Not decolorized in 8 hours
Good	Decolorized in less than 8 hours but not less than 6 hours
Fair	decolorized in less than 6 hours but not less than 2 hours
Poor	decolorized in less than 2 hours

Factors affecting the MBRT

These are factors that affect the MBRT and therefore, the steps of operation should be uniform.

1. Since, the oxygen content must be used up before the colour disappears; any manipulation that increases the oxygen content affects the test.
 - a. Cold milk holds more oxygen than warm milk
 - b. Pouring milk back and forth from one container to another increases the oxygen, and
 - c. During milking time much oxygen may be absorbed.
2. The rate of reduction of dye depends on the type of microorganism
 - a. Coli forms appear to be the most rapidly reducing microorganisms,
 - b. Closely followed by *Lactococcus lactis* spp. *lactis*, some of the faecal Streptococci, and certain micrococci.
 - c. Psychrotrophs reduce methylene blue very slowly.
3. Presence of a large number of leucocytes as in mastitic milk will affect the reduction time materially.

4. Light hastens reduction process and therefore, the tests should be carried out in relatively low light.
5. Concentration of dye should be uniform as an increased concentration lengthens the time of reduction.
6. Increasing the incubation temperature augments the activity of the bacteria and therefore shortens the reduction time.
7. Creaming of milk causes a number of micro-organisms to be removed from of milk and brought to the surface with the rising fat. This factor causes variations in the reduction time, since the bacteria are not evenly distributed.
8. The accuracy of test is increased, reduction time shortened and decolourization more uniform, if the samples are periodically inverted during incubation.

2. Resazurin Reduction Test (RRT)

Resazurin reduction test is another method of dye reduction test and the principle of this test is nearly similar to methylene blue reduction test. In MBRT the time for reduction of the dye is measured, while in RRT, at a fixed period time, specific shade of colour and its intensity is measured. There are two variations in RRT of testing milk. One is 10 min RRT that can be used as a rapid platform test for quick assessment of milk at the raw milk reception dock. The other one is a one hour RRT performed in the lab.

Principle

Unlike methylene blue the resazurin undergoes reduction through a series of colour shades viz., blue, purple, lavender and pink before completely getting reduced to colourless. Resazurin dye which is blue in colour reduces to pink colour compound (resorufin). When the redox potential is reduced further the colour of dye changes to colourless (dihydroresorufin). Usually, the degree of reduction of the dye is measured after a fixed time of incubation of milk sample in the presence of dye. The reduction of dye to a particular shade of colour is dependent upon the extent of depletion of oxygen by metabolic activity of microbes. The colour change is measured with the help of a Lovibond colour comparator and a standard resazurin disc.

Procedure

1. Take 10 ml of milk into a test tube and add 1 ml of Resazurin solution.
2. Put air tight closure to prevent oxygen entry
3. Mix the milk and Resazurin solution.
4. Place the test tubes in a thermostatically maintained water bath at 37 °C and note down the time of incubation.
5. At the end of incubation match the colour of the milk with one of the colour standards of Resazurin disc.

Grading of milk based on RRT

Disc no.	Colour	Bacterial quality of milk
6.	Blue	Excellent

5.	Lilac	Very good
4.	Mauve	Good
3.	Pink mauve	Fair
2.	Mauve pink	Poor
1.	Pink	Bad
0.	White	Very bad

Advantages of dye reduction test

- 1) These tests are cheaper and less time is required.
- 2) In case of SPC, clumps of microbes are recorded as one colony, whereas the rate of decolorizing of dye is due to the combined metabolic effect of each bacterium in the clump.
- 3) With the help of these tests the activity is measured rather than the number of bacteria.
- 4) Unlike the artificial media used in SPC, in milk the natural environment for microbes is present.
- 5) In case of RRT, the results can be measured in a shorter time.
- 6) Some of the bacteria capable of reducing the dye may not develop colonies on the medium used in SPC.

Disadvantages

- 1) Rate of reduction of dye varies considerably and is related to species and the rate at which different micro-organisms grow at a particular temperature.
- 2) Inhibitory substances like penicillin and other antibiotics prevent the growth of bacteria and thus increase the reduction time.
- 3) These tests do not give indication for the type of micro-organisms present.
- 4) Temperature of incubation used during these tests is not the optimum for majority of the micro-organisms present in milk.
- 5) Not suitable for testing quality of pasteurized milk intended for processing because of the low number of micro-organisms.
- 6) Require continuous attention until reduction takes place.