

Determination of microbiological quality of food by dye reduction test, IMVIC Test

Dye reduction test is a simple and easy to perform enzymatic test useful for determining the microbiological quality of food samples such as milk. A milk sample containing large population of actively metabolizing microorganisms lower dissolved oxygen because of vigorous growth of microorganisms thereby lowering oxidation-reduction potential in the sample. The dye methylene blue, a redox indicator, becomes reduced in an anaerobic environment and loses its color. Thus, there is direct relation between the length of time required for reduction and the number of bacteria present. The speed at which methylene blue becomes colorless following addition to sample indicates quality of milk. The shorter the reduction time, lower the quality of milk. Based on dye reduction test four grades of milk quality is recognized.

Decolonization Time	Approx. no of microorganisms/ml	Quality grade
0- 20 minutes	$> 2 \times 10^7$	Very bad
20- 120 minutes	$> 4 \times 10^6$	Bad
120- 330 minutes	$> 5 \times 10^5$	Fair
> 330 minutes	$< 5 \times 10^5$	good

Materials required

1. Sterile test tubes with stopper
2. Milk sample
3. Methylene blue solution (1: 25,000)
4. Pipettes (10ml, 1ml)

Procedure

To sterile tests containing 10 ml of milk 1 ml methylene blue solution is added, mixed well and incubated in water bath at 35°C. The tubes are observed for color change at 30 minutes time intervals for up to six hours and time taken for turning the tubes colorless recorded.

Indole Test

Indole test is used to determine the ability of microorganisms to degrade the essential amino acid tryptophan to form indole by the enzyme tryptophanase.

The test organism is grown in broth medium containing tryptophan and the presence of indole is detected by adding Kovac's reagent which produces cherry red colour in the form of a ring on the top layer of the medium.

Materials required:

Test bacterial cultures (*E. coli*, *Proteus sp*, *Pseudomonas* etc)

- Tryptone broth
- Kovac's reagent

Procedure:

The test microorganisms are inoculated in to tryptone broth, incubated at 37 °C for 24 h, and 3-4 drops of Kovac's reagent (para-dimethyl aminobenzaldehyde prepared in amyl alcohol) and observed for colour development. The positive reaction is characterized by the development of a pink coloured ring that will float on the surface of the broth.

Tryptone broth

Tryptone : 10.0 g
Sodium chloride : 5.0 g
Distilled water : 1000.0 ml
pH : 7.1 ± 0.1

Disolve the ingredients by boiling , distribute to tubes in 10 ml volume and sterilize at 121°C for 15 minutes

Kovac's reagent

Para-dimethyl aminobenzaldehyde (DMAB) :5.0 g
Iso-amyl alcohol :75.0 ml
Concentrated HCl acid : 25.0 ml

Dissolve DMAB in iso-amyl alcohol (if it does not readily dissolve, warm it at 50 -60°C in water bath) and add slowly concentrated HCl acid by drop by drop. Store the reagent at 4°C.

Methyl Red (MR) Test

This test detects the organisms that do not convert glucose into neutral products, but ferment glucose to acidic end products which can be demonstrated by lower of pH in growth medium. Because of this low pH, the methyl red indicator will turn to red colour indicating positive reaction. Negative reaction is indicated by development of yellow color.

Procedure:

Inoculate the organisms into the Glucose Phosphate Peptone (GPP) broth and incubate at 37 °C for 48 to 96 h. After the incubation, add a few drops (about 5 drops for 2.5 ml of culture) of alcoholic solution of methyl red and read the results immediately. Positive reaction is indicated by the culture broth turning to red colour and yellow colour is a sign of negative reaction.

Glucose Phosphate Peptone (GPP) broth

Peptone : 7.0 g
Glucose : 5.0 g
Sodium chloride : 5.0 g
Dipotassium hydrogen phosphate: 5.0 g
Distilled water : 1000.0 ml
pH : 7.1 ± 0.1

Heat to dissolve the ingredients and distribute in test tubes and sterilize at 110°C for 10 minutes.

Alcoholic Methyl Red

Methyl red : 0.1 g
Alcohol (95 %) : 300.0 ml
Distilled water : 200.0 ml

Dissolve methyl red in alcohol and add distilled water to the alcohol-indicator mixture. Store the reagent at 4°C.

Voges - Proskauer (VP) Test

In this test, bacteria convert the metabolic intermediate product (pyruvic acid) of glucose into neutral products (acetylmethylcarbinol, acetoin) and carbon dioxide instead of accumulating acidic products from the fermentation of glucose. The neutral product in the presence of air gets oxidized to diacetyl which reacting with guanidino groups under alkaline condition forms a complex which gives a port wine colour.

Procedure:

The test organism is grown in Glucose Phosphate Peptone (GPP) broth by incubating at 37°C for 48 to 96 h. The peptone in the medium serves as a source of guanidino group. After the incubation, a pinch of creatine (to intensify the reaction), 0.6 ml of 5 % α -naphthol and 0.2 ml of 40 % potassium hydroxide is added for 1 to 2.5 ml of culture and allowed to stand for 30 minutes to 2 h. A positive reaction is indicated by change of colour from pink to red (port wine colour), which indicates the presence of acetyl methyl carbinol.

Glucose Phosphate Peptone (GPP) broth

Peptone : 7.0 g
Glucose : 5.0 g
Sodium chloride : 5.0 g
Dipotassium hydrogen phosphate: 5.0 g
Distilled water : 1000.0 ml
pH : 7.1 ± 0.1

Heat to dissolve the ingredients and distribute in test tubes and sterilize at 110°C for 10 minutes.

α -naphthol (5%)

α – naphthol : 5.0 g
Ethanol : 100.0 ml

Dissolve α – naphthol in ethanol and store it at 4°C.

Potassium hydroxide (40 %)

Potassium hydroxide : 40.0 g
Distilled water : 100.0 ml

Dissolve Potassium hydroxide in distilled water and store it in a polyethylene or paraffin-lined glass reagent bottle at 4°C. Instead of adding a pinch of creatine separately, a mixture of 0.3 % creatine and 40 % potassium hydroxide solution can be added.

Citrate utilization test

The test is used to determine the ability of bacteria to utilize citrate as a sole source of carbon and energy, and ammonium as a sole source of nitrogen. In the absence of fermentable glucose or lactose, some microorganisms use citrate as carbon source. Pyruvic acid and carbon dioxide formed during the process of citrate utilization turns the medium alkaline. The increase in pH is due to the reaction of carbon dioxide with sodium resulting in alkaline sodium carbonate. Under alkaline condition the bromothymol blue indicator in the medium changes to green to deep Prussian blue colour.

Procedure:

The Simmon's citrate agar slant is inoculated with the test organism and incubated at 37°C for 24- 48 h. The positive reaction is indicated by blue colour (alkaline) and the negative result by yellowish green colour (acidic). If it is negative, there will be no growth in the slant itself.

Simmon's citrate medium or Citrate agar

Sodium chloride : 5.0 g
Magnesium sulphate : 0.2 g
Ammonium dihydrogen phosphate: 1.0 g
Dipotassium hydrogen phosphate: 1.0 g
Sodium citrate : 2.0 g
Agar : 15.0 g
Distilled water : 1000.0 ml
Bromothymol blue : 0.1 g
pH : 7.0 ± 0.2

Dissolve all the ingredients by boiling, distribute to test tubes and sterilize at 121°C for 15 minutes. Place the tubes in sloped condition to make slants. The agar slant should be in green colour.

Urease test

This test is used to determine the ability of microorganisms to degrade urea into ammonia and carbon dioxide by means of the enzyme, urease. This hydrolytic enzyme attacks nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia which can be detected by change in pH.

Procedure

The Christensen's medium or urea agar slants containing the pH indicator phenol red is inoculated with young culture of test organism, incubated for 24 hr at 37°C. A positive reaction is indicated by slants turning purplish pink or red colour due to increase in pH. The uninoculated slants serving as control remain yellow in colour.

Christensen's urease medium

Peptone : 1.0 g
Sodium chloride : 5.0 g
Glucose : 1.0 g
Potassium dihydrogen phosphate: 2.0 g
Agar : 15.0 g
Distilled water : 900.0 ml
Phenol red : 0.012 g
pH : 6.8 ± 0.1

Heat to dissolve the ingredients and sterilize at 115°C for 15 minutes. Mix 100 ml of filter-sterilized urea solution with the sterile medium. Distribute it in sterile test tubes (approximately 3 ml each) and place the tubes in sloped condition for making slants.

Urea solution

Urea - 20.0 g
Distilled water - 100.0 ml

Dissolve urea in distilled water and filter-sterilize it.

Coagulase test

This test is done to confirm the identification of enterotoxin producing virulent strains of *Staphylococcus aureus*. Rabbit plasma, which contains coagulase reacting factor (CRF) is made to react with bacteria producing coagulase enzyme which results in clotting or coagulation of blood plasma. Any degree of coagulation, from a loose clot suspended in plasma to a solid immovable clot, is considered as positive result. Coagulase test can be done by two methods: Slide method and Tube method.

A. Slide method: Mark a slide into two sections. Place a drop of sterile saline in both the sections and emulsify a loopful of 18 – 24 h old culture with saline placed in one of the sections, and treat the other section as control. Add one or two drops of rabbit plasma to both the sections and mix well for 5 seconds. If it coagulates, the test is positive. Slide method is mainly used for detecting the presence of free coagulase.

B. Tube method: To a test tube containing 0.5 ml of rabbit plasma add 0.5 ml of 18 – 24 h old culture, incubate at 37°C and examine for clump formation in 2, 3, 6 and 24 h. If clump formation occurs, the test is considered as positive. A control to be maintained without adding bacterial culture. This method is mainly used for detecting the presence of bound coagulase.

Oxidase test

This test helps to determine the ability of bacteria to produce cytochrome oxidase enzyme involved in electron transport chain in aerobic respiration. Aerobic bacteria including some microaerophilic and facultative anaerobes exhibit oxidase activity by catalyzing the oxidation of a reduced cytochrome by molecular oxygen, resulting in the formation of water or hydrogen peroxide.

Procedure:

A small portion of the test cultures grown on nutrient agar plates are picked using sterile platinum loops or sterile tooth picks and placed on pre-moistened filter paper strips containing test reagent (1% solution of dimethyl- para –phenylenediamine hydrochloride) and observed for 10-15 seconds. The test reagent serving as artificial substrate donates electrons and gets oxidized to a blackish compound in the presence of oxidase and free oxygen. Appearance of black colour is indicative of positive test.

Oxidase

reagent

Oxidase reagent is prepared by dissolving 1.0 g of N, N, N', N'-tetramethyl-p-phenylene diamine dihydrochloride in 100 ml distilled water.

Preparation of oxidase filter paper strips: Whatman filter paper No.1 strips cut to size of 2-5 x 1.0 cm, sterilized in hot air oven at 140°C for 1 hour, then dipped in oxidase reagent, dried and stored in dark bottle at 4°C.

Catalase test

This test is useful in differentiating certain groups of bacteria based on their ability to grow in presence of oxygen. Most aerobes and facultative anaerobes during aerobic respiration produce hydrogen peroxide which is extremely toxic to their enzyme systems. But organisms producing catalase rapidly degrade hydrogen peroxide to oxygen and water enabling their survival.

Procedure

To the trypticase soy agar slants containing test culture, a few drops of 3% hydrogen peroxide is added covering the culture surface and observed for release of free oxygen as bubbles or effervescence. Absence of effervescence is negative for catalase.

Triple sugar iron agar (TSI) test

The TSI test is designed to differentiate among different groups/genera of enterobacteriaceae which ferment glucose with the production of acid, and also distinguish enterobacteriaceae from other Gram negative intestinal bacilli. This differentiation is based on the differences in carbohydrate fermentation pattern and hydrogen sulphide production by various groups of intestinal bacteria. The TSI agar slant contains three sugars (1% each of glucose and lactose, and 0.1% sucrose), and phenol red indicator which indicates the acid production due to carbohydrate fermentation by change in colour from orange red to yellow. Upon incubation both the butt and slant are observed for colour change (fermentative activities), gas production and hydrogen sulphide production

1. Alkaline slant (red) and acid butt (yellow) with or without gas production:

This reaction indicates fermentation of only glucose. The organism has preferentially utilized glucose present in small concentration and produce small amount of acid on slant surface, lowering the pH initially (indicated by indicator turning yellow). But the utilization of peptone increases pH making the slant turn to red colour. In the butt, the initial acid reaction is maintained because of reduced oxygen and slow growth, and this makes butt appear yellow.

2. Acid slant (yellow) and acid butt (yellow) with or without gas production:

This reaction is indicative of utilization of lactose and/or sucrose, after initial exhaustion of glucose, which serve as substrates for continued fermentation and maintenance of acid reaction both in slant and butt.

3. Alkaline slant (red) and alkaline butt (red) or no change (orange red) butt.:

This reaction is indicative of inability of test organisms to ferment carbohydrate. But utilization of peptones under aerobic and/or anaerobic condition results in ammonia production resulting in alkaline condition both in slant and butt. The degradation of peptone only by aerobic condition results in alkaline pH in slant.

Procedure:

The TSI slants are stab-streak inoculated with test bacterial culture using sterile inoculation loop, incubated for 18-24 hours at 37°C and observed for colour change both in slant and butt.

Triple sugar iron agar

Beef extract : 3.0 g
Yeast extract : 3.0 g
Peptone : 15.0 g
Proteose peptone : 5.0 g
Lactose : 10.0 g
Saccharose : 10.0 g
Dextrose : 1.0 g
Ferrous sulphate : 0.2 g
Sodium chloride : 5.0 g
Sodium thiosulphate : 0.3 g
Phenol red (15 aqueous solution) : 2.5 ml
Agar : 15.0 g
Distilled water : 1 l
pH : 7.4

Dissolve the ingredients by boiling and transfer 5 ml volume to test tubes, autoclave at 121°C for 15 minutes, and allow setting in a slope to get a butt and slant.

Sugar fermentation test

This test is carried to determine the ability of microorganisms to utilize different carbohydrate as carbon source with resultant production of acid and gas. The bromothymol blue indicator used in the medium indicates acidic pH due to sugar fermentation by change in colour from purple to yellow.

Procedure:

To the test tubes containing different sugars and Durham's tube to collect gas are inoculated with test cultures and incubated at 37°C for 24 hours. The change in colour from purple to yellow is indicative of positive result.

Sugar fermentation medium

Soya peptone : 1.0 g
Sodium chloride : 1.0 g
Sugars : 1.0 g
Bromocresol purple : 0.0016%
Distilled water : 100 ml
pH : 7.2 ± 0.2

The media containing individual sugars dissolved and dispensed in 4 ml volumes in test tubes containing inverted Durham's tube and autoclaved at 11°C for 10 minutes.

Aminoacid decarboxylase test

The ability of the microorganisms to decarboxylate aminoacids such as lysine, ornithine and arginine is tested by inoculating test cultures to media containing individual aminoacids and observing increase in pH. Under anaerobic condition microorganism initially ferment glucose resulting in lowering of pH and subsequent decarboxylation of aminoacids results in ammonia production which

causes increase in pH. The pH changes are indicated by the bromocresol purple incorporated to the medium.

Procedure:

To test tubes containing aminoacids are inoculated with test culture, overlayed with sterile liquid paraffin and incubated at 30°C for 24 to 48 hours. The change in colour of the medium from purple to yellow and back to purple is indicative of positive result.

Basal medium for aminoacid decarboxylase test

Soya peoptone : 5.0 g
Yeast extract : 3.0 g
Sodium chloride : 10. 0 g
Glucose : 1.0 g
Bromocresol purple : 0.016 g
Distilled water : 1 L
pH : 7.2 ± 0.2

The basal medium is divided in to four equal parts and aminoacids lysine, ornithine and arginine added at 0.5 %, 0.5 % and 4.0 % concentration respectively, transferred to test tubes in 3 ml volumes and autoclaved at 110°C for 15 minutes. The aminoacid free medium is used as control.