BIOCHEMICAL & SUGAR FERMENTATION TEST

Indole Test

Some bacteria have the ability to break down tryptophan for nutritional needs using the enzyme tryptophanase. When tryptophan is broken down, the presence of indole can be detected by a colorimetric reaction with Kovac's Reagent (p-dimethyl–aminobenzaldehyde).

Kovac's Reagent

Amyl alcohol or iso-amyl alcohol	150ml
p-Dimethy-bezaldehyde	10g
Conc. Hydrochloric acid	50ml

Dissolve the aldehyde in the alcohol and slowly add the acid.

Prepare in small quantities and store in the refrigerator. Shake gently before use .

Method

1.Inoculate one tube of peptone water with bacterial isolate under test.
2.Incubate at 37 ^{O}C for 48 h (Sometimes a period of 96 hr at 37 ^{O}C).
3.Add 0.5 ml Kovac's reagent and shake gently.

Interpretation

Red colour ring in the alcohol layer indicates a positive reaction. Yellow colour ring (colour of Kovac's reagent) indicates negative test.

$$\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole + Pyruvic Acid + Ammonia}$$

Methyl Red Test

Detects the production of acid due to fermentation of glucose.

MR-VP Medium (Glucose phosphate peptone water)

Peptone	5gm
Di-potassium hydrogen phosphateK2HPO4	5g
Distilled Water	1000ml
Glucose 10% solution (sterilized separately)	50ml

Dissolve the peptone and phosphate, adjust the pH to 7.6, filter, dispense in 5 ml amounts and sterilize at 121 ^{O}C for 15 minutes. Sterilize the glucose solution by filtration and add 0.25ml to each tube. (Final concentration 0.5 %)

Methyl red indicator solution

Methyl red	0.1gm
Ethanol	300ml
Distilled water	200ml

Method:

1.Inoculate MR-VP medium lightly from a young agar slope of bacterial isolate under test.
2. Incubate at 37 ^{O}C for 48 h.
3. Add 4 -5 drops of methyl red reagent.
4. Mix and read immediately.

Interpretation

Bright red colour indicates positive test and negative are yellow.

Voges –Proskauer Test

Detects the production of acetoin (acetyl methyl carbinol) which is produced by the fermentation of CHO by many bacteria.

MR-VP Medium (Glucose phosphate peptone water)

Peptone	5gm
Di-potassium hydrogen phosphateK2HPO4	5g
Distilled Water	1000ml
Glucose 10% solution (sterilized separately)	50ml

Dissolve the peptone and phosphate, adjust the pH to 7.6,filter, dispense in 5 ml amounts and sterilize at 121 ^{O}C for 15 minutes. Sterilize the glucose solution by filtration and add 0.25ml to each tube. (Final concentration 0.5 %)

Method

1. Inoculate the MR-VP medium lightly from a young agar slope of bacterial isolate under test.
2.Incubate at 37 ^{O}C for 48 h.
3.Add 1ml of potassium hydroxide and 3ml of 5% solution of *α*- napthol in absolute alcohol.

Interperetation

A positive reaction is indicated by the development of pink colour in 2-5 minutes and crimson in 30 minutes.

[Generally Members of Family Enterobacteraceae are either MR positive and VP negative or MR Negative and VP Positive]

Citrate Utilization Test

Test detects the ability of an organism to utilize citrate as the sole source of carbon and energy for growth and ammonium salt as the sole source of nitrogen.
Koser's Liquid citrate medium or Simmon's citrate agar may be used.

Koser's Medium

Sodium chloride	5.0g
Magnesium sulphate	0.2g
Ammonium di-hydrogen phosphate	1.0g
Potassium di-hydrogen phosphate	1.0g
Sodium citrate	5.0g
Distilled water	1000ml

The pH should be 6.8.
The medium is dispensed &sterilized by autoclaving at 121 ^{O}C for 15 min.

Simmon's Medium

(Modification of Koser's medium with agar and indicator added.)

Koser's medium	1000ml
Agar	20g
Bromothymol blue (0.2%)	40ml

Dispense autoclave at 121 ^{O}C for 15 min and allow to set as slopes.

Method

1.Inoculate the suspension of the organism to be tested.
2. Incubate for 96 hours at 37 ^{O}C
3.Read the results as follows

Interpretation

Koser's medium
Positive = Turbidity i.e., Growth
Negative = No turbidity

Simmon's citrate medium

Positive = Blue colour and streak of growth
Negative = Original green colour and no growth.

Dead Organisms can act as a source of carbon and may produce false positive test .

Oxidase Test

The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzyme.The test is used as an aid for the differentiation of *Neisseria, Moraxella, Campylobacter and*
Pasteurella species (oxidase-positive).

Principle

Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein).Both catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen).The test reagent*, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride* acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue.

Reagent

1% N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride in distilled water or impregnated oxidase test strips

[The test solution auto-oxidises rapidly- use a fresh solution or add 1% ascorbic acid to retard oxidation. Do not use if the solution is blue.]

Method

Direct Plate Method (do not use on colonies intended for sub-culture)

Add 2 drops of reagent to suspect colonies on an agar plate. Do not flood the plate. Examine for blue colour within 10 seconds.

Filter Paper Method

Soak a piece of filter paper in the reagent solution.
Scrape some fresh growth from the plate with a disposable loop or
stick and rub onto the filter paper or touch a colony with edge of paper.
Examine for blue colour within 10 seconds.

Interpretation

Positive result : development of a blue colour indicates oxidase production
Negative result : No blue colour

Do not use nichrome inoculating loops or wires. False positive reactions may occur due to surface oxidation products formed during flame sterilisation .

Nitrate Reduction Test

This test detects the production of enzyme nitrate reductase which reduces nitrate to nitrite e.g.,*Enterobacteriaceae* family members are positive for the test.

Medium

Potassium Nitrate (KNO_3) 0.2 G
Peptone 0.5 G
Distilled Water 1 L
Tube in 5 ml amount and autoclave 121 ^{O}C for 15 minutes.

Reagents

Reagent A Disssolve 8.0 G of Sulphanilic acid in 1 L of 5N acetic acid.
Reagent B Dissolve 5.0G of alpha –Napthylamine in 1L of 5N acetic acid.
Immediately before use mix equal volumes of solution A & B.

Method

Inoculate test organisms in 5ml medium containing potassium nitrate,peptone and distilled water.

Incubate at 37^{O}C for 96 Hrs.
[Ad 0.1 ml test reagent which consists of equal volumes of 0.8% Sulphanilic acid and 0.5 % alpha napthylamine in 5N acetic acid mixed just before use.]

Interpretation

A red colour develops within few minutes indicating the presence of nitrite and indicating the ability of test organism to reduce nitrate to nitrites.

Medium +	Nitrate	Sulphanilic acid	
0.02 % Potassium nitrate & 0.55 peptone	---------- Nitrite Reductase	---------- Diazo Red Dye Alpha Naphtylamine	

If no colour develops this may indicate that either nitrate has not been reduced or that nitrate has been reduced beyond nitrite to nitrogen gas, nitric oxide or nitrous oxide, which the reagents will not be able to detect. To detect this add Zinc dust to the test. Metallic zinc

reduces nitrate to nitrite, and red colour develops following addition of zinc dust means that the organism was unable to reduce the nitrate to nitrite.

Phenylalanine Deaminase Test

This test indicates the ability of an organism to deaminate phenylalanine with the production of phenylpyruvic acid which will react with ferric acid to give a green colour.

Medium

Yeast extract	3g
DL-Phenylalanine	2g
Na2HPO4	1g
Sodium Chloride	5g
Agar	12g
Distilled Water	1L

Adjust the pH to 7.4, distribute and sterilize by autoclaving at 121^{O}C for 15 minutes. Allow to to solidify in tubes as long slopes.

Method

- Inoculate with a fairly heavy inoculum. Incubate for 4 Hrs or if desired for up to 24 Hrs at 37^{O}C.Allow few drops of a 10% solution of ferric chloride to run down over the growth on the slope.
- If the test is positive, a green colour will develop in the fluid and in the slope.
- This broth contains 3 essential ingredients:
- 0.5%-1.0% of the carbohydrate to be tested (e.g. lactose or glucose),
- nutrient broth, and
- the pH indicator phenol red.
- The nutrient broth, which is a light red color, supports the growth of most organisms whether they are able to ferment the sugar or not.
- The test organism is inoculated into a broth containing the test sugar and incubated. A bright yellow color indicates the production of enough acid products from fermentation of the sugar to drop the pH to 6.9 or less.
- Production of gas is determined with a Durham tube ,a small inverted vial filled with the carbohydrate fermentation broth.
- If gas is produced during fermentation of the sugar, it is trapped at the top of the Durham tube and appears as a bubble.
- Slow fermenters may take a week or more to cause color changes detectable by the human eye.

Interpretation

Positive (yellow color or yellow color with gas bubble) and negative results (red color, no gas bubble).

Sugar Fermentation Test

Used to differentiate bacteria on the basis of CHO fermentation abilities.

Ability of an organism to ferment a specific carbohydrate added in basal medium results in the production of acid or acid and gas. This ability has been used to characterize a specific species of bacteria which helps in differentiation between genera and aid in the differentiation between genera and aid in the differentiation of species as well.

Principle

When CHO is added to a culture medium. On incubation, it is fermented by microorganisms, the acid (or acid and gas) produced lowers the pH and the indicator in the basal medium changes the colour e.g., Phenol red changes from red to orange to yellow and the gas produced if any, collects in the Durhams tube.

Media and Reagents

Sugars are used as 1% solutions in peptone water to test fermentative reactions of bacteria. Beef extract is also added to the medium. Small inverted tube (Durhams tube) is placed in the medium to detect the formation of gas and one of the indicators such as Phenol Red, Andrades Indicator etc., as shown in the table given below is added to detect formation of acid.

Interpretation

A positive result for acid is yellow after indicator is added (indicating sugar fermentation)
A positive result for gas is a bubble in the Durhams tube.
A completely negative result has no color change or reddish color & no bubble.

Sugars used

Pentoses Arabinose, Rhamnose, Xylose.
Hexoses Glucose(Dextrose), Fructose(Laevulose), Galactose, Mannose, Trehalose.
Disaccharides Sucrose(Saccharose), Lactose, Maltose, Trehalose.
Trisaccharides Raffinose.
Polysaccharides Starch,Dextrin,Inulin,Glycogen.
Glucosides Salicin,Aesculin
Alcohols Glycerol, Erythritol, Adonitol, Dulcitol, Mannitol, Sorbitol, Inositol.

Reactions of Indicators at different pH ranges

Indicators	Conc. used in the medium	Colour Change	pH Range
Andrade	1 N NaOH in 0.5% acid fuchsin (until colour becomes yellow)	Pink –-Yellow	5.0-8.0
Phenol Red	5% of 0.2% Solution	Yellow –-Red	6.8-8.4
Bromothymol Blue	1% of 0.2% solution	Yellow–-Blue	6.0-7.6
Bromocresol Purple	1% of 0.4% solution	Yellow–-Blue	5.2-6.8

Exercise

Q1. Enlist the steps in identification of the bacteria.

Q2. Write the biochemical tests & Sugar fermentation test results for the bacteria mentioned below:

a.*Escherichia coli*
b.Salmonella Pullorum
c.*Proteus mirabilis*
d.*Klebsiella pneumoniae*
e.*Shigella dysentriae*

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