1. **BIOCHEMICAL REACTION OF BACTERIA**

**SUGAR FERMENTATION TEST**

Aim: To determine the ability of microbes to ferment carbohydrates with the production of an acid and/or gas.

Principle: Sugars are metabolized through different metabolic pathways depending on types of microbial species and aerobic or anaerobic environment .If fermenting bacteria are grown in a liquid culture medium containing the carbohydrate, they may produce organic acids as by-products of the fermentation. These acids are released into the medium and so lower pH of medium. If a pH indicator such as phenol red or bromo cresol blue is included in the medium, the acid production will change the medium from its original color to yellow. Gases produced during the fermentation process can be detected by using a small, inverted tube, called a Durham tube, within the liquid culture medium. If gas is produced, the liquid medium inside the Durham tube will be replaced; by the gas in the form of a bubble.

Interpretation: If the medium changes from colorless to yellow and gas bubble is found in Durham’s tube then it indicates acid and gas production. In some cases gas may not be evolved during the process. If no change observed in the colour of medium then sugar is not degraded by the organism.

**INDOLE PRODUCTION TEST**

Aim: To determine the ability of microbe to degrade the amino acid tryptophan.

Principle:

Tryptophan Tryptophanase Indole + Pyruvic acid + Ammonia

P – dimethylaminobenzaldehyde HCl, alcohol Quinoidal red – violet compound + Indole Dehydration reaction

Interpretation:

Development of cherry red colour at the interface of the reagent and the broth, within seconds after adding the Kovacs’ reagent indicates the presence of indole and the test is positive. If no colour change is observed, then the test is negative and so organisms are not capable of producing tryptophanase.

**METHYL RED TEST**

Aim: To differentiate E.coli and E.aerogen and to determine the ability of microbes to oxidize glucose with production and stabilization of high content of acid end product.

Principle:

E.coli: Glucose + H2O Lactic acid, Acetic acid, Formic acid + Methyl Red CO2 + H2 +

Red colour (positive test) (pH 4)

E. aerogen:

Glucose + H2O Acetic acid 2,3butanediol + CO2 + H2O Methyl Red

Yellow colour (negative test) (pH 6)

**VOGES- PROSKAUER TEST**

Aim: To differentiate the E.coli and E.aerogen by the production of 2,3 – butanediol and acetoin via glucose fermentation.

Principle:

This test determines the capability of some organisms to produce non-acidic or neutralend products, such as acetyl methyl corbinol (acetoin), from the organic acid that results fromglucose metabolism. This test is characterizes E.aerogen. Test identifies bacteria that fermentglucose and leading to 2,3-butanediol accumulation in the medium.

Glucose + ½ O2 2 Pyruvate CO2 α- aceto acetate CO2 Acetoin CO2 2,3-butanediol

Acetoin + α- napthol 40% KOH Diacetyl + Creatine (pink coloured complex)

Absolute alcohol

Interpretation: Development of crimson red colour indicates positive test for E.aerogen. And no colour change indicates negative test.

**CITRATE UTILIZATION TEST**

Aim: To determine the ability of the microbes to ferment citrate as sole carbon source.

Principle:

• Citrate as a sole carbon source for their energy needs.

• Presence of a citrate permease that facilitates transport of citrate into the bacterium.

• Sodium citrate as the carbon source, NH4+ as a nitrogen source.

• pH indicator - bromothymol blue.

• This test is done on slants since O2 is necessary for citrate utilization.

• When bacteria oxidize citrate, they remove it from the medium and liberate CO2.

• CO2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate - an alkaline product.

• This raises the pH, turns the pH indicator to a blue color, and represents a positive citrate test; absence of a color change is a negative citrate test.

• Citrate-negative cultures will also show no growth in the medium and the medium remains green.

**NITRATE REDUCTION TEST**

Aim: To determine the ability of some microbes to reduce nitrate (NO3-) to nitrites (NO2-) or beyond the nitrite stage.

Principle:

• Certain organisms like Chemolithoautotrophic bacteria and many chemoorganoheterotrophscan use nitrate (NO3-) as a terminal electron acceptor during anaerobic respiration.

• In this process, nitrate is reduced to nitrite (NO2-) by nitrate reductase.

• Further reduce the nitrite to either the ammonium ion or molecular nitrogen.

• Nitrate broth medium containing 0.5% potassium nitrate (KNO3).

• Examined for the presence of gas and nitrite ions in the medium.

**UREASE TEST**

Aim: To determine the ability of microbes to degrade urea by urease.

Principle:

• Urea is diamide carbonic acid often referred as carbamide.

• The hydrolysis of urea is catalysed by specific enzyme urease to yield2 moles of ammonia.

• Urease attacks the nitrogen and carbon bond in urea and forms ammonia.

• The presence of urease is detected, when the organisms are grown in urea broth.

• Medium containing the pH indicator phenol red.

• Splitting of urea creates the alkaline condition which turns phenol red to deep pink in colour.

• Mainly used for identification of Proteus spp. from other genus of lactose nonfermenting enteric organisms.

Interpretation:

If urea is present in the medium, then it will be degraded which creates alkaline condition in the medium which result in colour change from reddish pink to deep pink.

**TSI (TRIPLE SUGAR IRON) AGAR TEST**

Aim: To differentiate among and between the members of Enterobacteraceae family.

Principle:

• Study and identify the organisms belonging to Enterobacteraceae family.

• It is also used to distinguish the Enterobacteriaceae from other gram-negative intestinal bacilli (by their ability to catabolize glucose, lactose, or sucrose, and to liberate sulfides from ferrous ammonium sulfate or sodium thiosulfate. )

• TSI agar slants contain a 1% concentration of lactose and sucrose, and0.1% glucose.

• The pH indicator, phenol red, is also incorporated into the medium to detect acid production from carbohydrate fermentation.

• The uninoculated medium is red in colour due to presence of phenol red dye.

•Yeast extract, beef extract and peptone provides nitrogen, sulphur, trace elements and vitamin B complex etc.

• NaCl maintains osmotic equilibrium.

• Lactose, Sucrose and Dextrose are the fermentable carbohydrates.

• Sodium thiosulfate and ferrous sulfate make H2S indicator system.

• Thiosulfate is reduced to H2S by several species of bacteria and H2S combines with and form insoluble black precipitates.FeSO4 present in the medium

• Blackening usually occurs in butt of tube.

• Incubation is for 18 to 24 hours in order to detect the presence of sugar fermentation, gas production, and H2Sproduction.

The indicator is pink at alkaline pH and yellow at acidic pH, at neutral pH it remains red. The following reactions may occur in the TSI tube:

•Yellow butt (A) and red slant (K) due to the fermentation of glucose (phenol red indicator turns yellow due to the persisting acid formation in the butt). The slant remains red (alkaline) (K) because of the limited glucose in the medium and, therefore, limited acid formation, which does not persist.

•A yellow butt (A) and slant (A) due to the fermentation of lactose and/or sucrose (yellow slant and butt due to the high concentration of these sugars) leading to excessive acid formation in the entire medium.

•Gas formation noted by splitting of the agar.

•Gas formation (H2S) seen by blackening of the agar.

•Red butt (K) and slant (K) indicates that none of the sugars were fermented and neither gas nor H2S were produced.

**OXIDASE TEST**

Aim: To determine the ability of microbes to produce Oxidase enzyme

Principle:

• Oxidase enzyme plays a key role in Electron Transport Chain during aerobic respiration.

• Cytochrome Oxidase catalyzes the oxidation of reduced Cytochrome by molecular oxygen (O2), resulting in the formation of H2O and H2O2.

• Aerobic as well as some facultative anaerobes and microaerophillic bacteria shows oxidase activity.

Note the purple to dark purple color after the colonies have been added to filter paper moistened with oxidase reagent.

**CATALASE TEST**

Aim: To determine the ability of an organism to produce catalase.

Principle:

• Certain organisms produce hydrogen peroxide during aerobic respiration and sometimes extremely toxic superoxide radicals.

• These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly.

• A bacterium must be able to protect itself against such O2products or it will be killed.

• Many bacteria possess enzymes that afford protection against toxic O2 products.

• Obligate aerobes and facultative anaerobes usually contain the enzymes superoxide dismutase, which catalyzes the destruction of superoxide

• And either catalase or peroxidase, catalyze the destruction of hydrogen peroxide.

• Catalase production and activity can be detected by adding the substrate H2O2 to an appropriately incubated (18- to 24-hour) tryptic soy agar slant culture.

• If catalase was produced by the bacteria, they will liberate free O2 gas on reaction.

• Bubbles of O2 represent a positive catalase test; the absence of bubble formation is a negative catalase test.

1. **IDENTIFICATION/STAINING TECHNIQUES OF FUNGI**

**KOH Wet Mounts**

**Principle:**

KOH softens most tissues, dissolves fat droplets, bleaches many pigments and dissolves the “cement” that holds keratinized cells together; glycerine clears tissue debris, thus making it easier to demonstrate presence of fungal elements.

**Reagents:**

10 – 20 % KOH:

* KOH pellets 10 – 20 grams
* Glycerine (optional) 10 ml
* Distilled water 90 ml

**Procedure:**

◦ Place a small amount of specimen on a clean glass slide

◦ place 1-2 drops of KOH on the specimen and overlay a cover slip

◦ Allow the preparation to stand for 10-30 minutes in a wet chamber.

* You can gently heat preparation to hasten the action of KOH
* Do not over heat for it may crystallize the KOH

◦ Examine preparation under low then high magnification. Take note for the presence of fungal elements (hyphae and/or spores)

**Fungal staining methods**

**Stains used:**

**Lactophenol Cotton Blue** - very popular for quick evaluation of fungal structures; stains chitin in cell walls of fungi.

**Periodic Acid - Schiff Stain (PAS)** - stains polysaccharide in the cell wall of fungi. Fungi stain pink-red with blue nuclei.

**Gomori Methenamine Silver Stain** - silver nitrate outlines fungi in black due to the silver precipitating on the fungi cell wall.

**Lactophenol Cotton Blue**

**Principle:**

◦ The morphology of fungal elements are preserved and stained better.

Reagents:

◦ Lactic acid & Phenol

* Kills the organism

◦ Glycerin

* Prevents easy dehydration

◦ Cotton blue

* Dye or stain

Gridley Stain - Hyphae and yeast stain dark blue or rose. Tissues stain deep blue and background is yellow.

Mayer Mucicarmine Stain - will stain capsules of Cryptococcus neoformans deep rose.

Fluorescent Antibody Stain - extremely specific method of detecting fungi in tissues or fluids.

Papanicolaou Stain - good for initial differentiation of dimorphic fungi. Works well on sputum smears.

Gram Stain - generally fungi are gram positive; but Actinomyces and Nocardia are gram variable.

Acid-Fast Stain - used to differentiate the acid-fast Nocardia from other aerobic Actinomyces.

Giemsa Stain - used for blood and bone marrow specimens.

India Ink - demonstrates the capsule of Cryptococcus neoformans in CSF specimens

**EYE SCRAPINGS & ASPIRATE for KERATOMYCOSIS**

KOH & LPCB, look for

◦ Septate hyaline hyphae

* Aspergillus species
* Fusarium species

◦ Coenocytic hyaline hyphae

* Mucor species

◦ Pseudohyphae and yeasts

* Candida species

**Calcofluor White Stain**

A fluorescent stain for rapid detection of yeasts, fungi and parasitic organisms. Calcofluor White is a non- specific fluorochrom that binds to cellucose and chitin in cell walls

**Composition:**

* Calcofluor White M2R 1g/l
* Evans blue 0.5g/l

**Storage:**

* Store at room temperature and protected from light.

**Directions:**

* Put the sample to be examined onto a clean glass slide.
* Add one drop of Calcofluor White Stain and one drop of 10% Potassium Hydroxide
* Place a coverslip over the specimen and let stand for 1 minute.
* Examine the slide under UV light at x100 to x400 magnification

**Principle/ Interpretation:**

* Calcofluor White Stain is a non-specific fluorochrome that binds with cellulose and chitin contained in the cell walls of fungi and other organisms.
* The staining procedure with Calcofluor White Stain is a rapid method for the detection of many yeasts, pathogenic fungi and Microsporidium, Acanthamoeba, Pneumocystis, Naegleria, and Balamuthia species.
* Evans blue present in the stain act as a counterstain and diminishes background fluorescence of tissues and cells when using blue light excitation (not UV).
* A range of 300 to 440 nm (Emmax 433nm; 0.1 M phosphate pH 7.0; cellulose) can be taken for emission wave length and the excitation occurs around 355nm.
* Fungal or parasitic organisms appear fluorescent bright green to blue, while
* Attention cotton fibers will fluoresce strongly and must therefore be differentiated from fungal hyphae.
* As well amebic cysts are fluorescent but trophozites will not stain or fluoresce, Background fluorescence can be diminished by examining under blue light or by using different filter combinations (emission and excitation filters).
* One drop of 10% potassium hydroxide solution can be added for better visualization of fungal elements

**Calcoflour stain**

Calcoflour mounts for systemic mycoses, look for (flourescence)

◦ Pseudohyphae and yeasts (blood)

* Candida species

◦ Septate, hyaline at right degrees angle (bronchial lavage)

* Aspergillus species