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**BIOCHEMICAL REACTIONS OF BACTERIA**

1. MANNITOL SALT AGAR (MSA)

This type of medium is both selective and differential. The MSA will select for organisms such as Staphylococcus species which can live in areas of high salt concentration (plate on the left in the picture below). This is in contrast toStreptococcus species, whose growth is selected against by this high salt agar (plate on the right in the picture below).

The differential ingredient in MSA is the sugar mannitol. Organisms capable of using mannitol as a food source will produce acidic byproducts of fermentation that will lower the pH of the media. The acidity of the media will cause the pH indicator, phenol red, to turn yellow. Staphylococcus aureus is capable of fermenting mannitol (left side of left plate) while Staphylococcus epidermidis is not (right side of left plate).

1. Oxidase Test

This test is used to identify microorganisms containing the enzyme cytochrome oxidase (important in the electron transport chain). It is commonly used to distinguish between oxidase negative Enterobacteriaceae and oxidase positive Pseudomadaceae.

Cytochrome oxidase transfers electrons from the electron transport chain to oxygen (the final electron acceptor) and reduces it to water. In the oxidase test, artificial electron donors and acceptors are provided. When the electron donor is oxidized by cytochrome oxidase it turns a dark purple. This is considered a positive result. In the picture below the organism on the right (Pseudomonas aeruginosa) is oxidase positive.

1. CAMP Test

CAMP factor is a diffusible, heat-stable protein produced by group B streptococci. This is a synergistic test between Staphylococcus aureusand Streptococcus agalactiae. S. agalactiaeproduces CAMP factor. S. aureus produces sphingomyelin C, which binds to red blood cell membranes. The two bacteria are streaked at 90oangles of one another. They do NOT touch. The CAMP factor produced by S. agalactiae enhances the beta-hemolysis of S. aureus by binding to already damaged red blood cells. As a result, an arrow of beta-hemolysis is produced between the two streaks. The test is presumptive for S. agalactiae that produces CAMP factor.

1. Motility agar

This is a differential medium used to determine whether an organism is equipped with flagella and thus capable of swimming away from a stab mark. The results of motility agar are often difficult to interpret. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile). If, however, the stab mark is clearly visible and the rest of the tube is not turbid, the organism is likely nonmotile

1. Spirit Blue agar

This agar is used to identify organisms that are capable of producing the enzyme lipase. This enzyme is secreted and hydrolyzes triglycerides to glycerol and three long chain fatty acids. These compounds are small enough to pass through the bacterial cell wall. Glycerol can be converted into a glycolysis intermediate. The fatty acids can be catabolized and their fragments can eventually enter the Kreb’s cycle. Spirit blue agar contains an emulsion of olive oil and spirit blue dye. Bacteria that produce lipase will hydrolyze the olive oil and produce a halo around the bacterial growth.

1. Taxos P (optochin sensitivity testing)

This is a differential test used to distinguish between organisms sensitive to the antibiotic optochin and those not. This test is used to distinguish Streptococcus pneumoniae (optochin sensitive) from other a-hemolytic streptococci (optochin resistant (Streptococcus mitis is)).

1. Blood Agar Plates (BAP)

This is a differential medium. It is a rich, complex medium that contains 5% sheep red blood cells. BAP tests the ability of an organism to produce hemolysins, enzymes that damage/lyse red blood cells (erythrocytes). The degree of hemolysis by these hemolysins is helpful in differentiating members of the genera Staphylococcus, Streptococcus and Enterococcus.

Beta-hemolysis is complete hemolysis. It is characterized by a clear (transparent) zone surrounding the colonies. Staphylococcus aureus, Streptococcus pyogenes and Streptococcus agalactiae are b-hemolytic.

Partial hemolysis is termed alpha-hemolysis. Colonies typically are surrounded by a green, opaque zone. Streptococcus pneumoniae and Streptococcus mitis are a-hemolytic.

If no hemolysis occurs, this is termed gamma-hemolysis. There are no notable zones around the colonies. Staphylococcus epidermidis is gamma-hemolytic.

1. Bile Esculin Agar

This is a medium that is both selective and differential. It tests the ability of organisms to hydrolyze esculin in the presence of bile. It is commonly used to identify members of the genusEnterococcus (E faecalis and E. faecium).The first selective ingredient in this agar is bile, which inhibits the growth of Gram-positives other than enterococci and some streptococci species. The second selective ingredient is sodium azide. This chemical inhibits the growth of Gram-negatives.

The differential ingredient is esculin. If an organism can hydrolyze esculin in the presence of bile, the product esculetin is formed. Esculetin reacts with ferric citrate (in the medium), forming a phenolic iron complex which turns the entire slant dark brown to black.

1. Coagulase test

Coagulase is an enzyme that clots blood plasma. This test is performed on Gram-positive, catalase positive species to identify the coagulase positive Staphylococcus aureus. Coagulase is a virulence factor of S. aureus. The formation of clot around an infection caused by this bacteria likely protects it from phagocytosis. This test differentiates Staphylococcus aureus from other coagulase negative Staphylococcus species.

1. Simmon’s Citrate Agar

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of Enterobacteriaceae. In organisms capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and CO2. If CO2 is produced, it reacts with components of the medium to produce an alkaline compound (e.g. Na2CO3). The alkaline pH turns the pH indicator (bromthymol blue) from green to blue. This is a positive result (the tube on the right is citrate positive). Klebsiella pneumoniae and Proteus mirabilis are examples of citrate positive organisms. Escherichia coli and Shigella dysenteriae are citrate negative.

**IDENTIFICATION AND STAINING TECHNIQUES OF FUNGI**

In general, fungal identification requires greater visual acuity than bacteria. Unlike other important microorganisms such as bacteria and viruses, the identificcation of fungi heavily relies on morphological criteria. The characteristics of fungal structure are identified by observing colonial growth both macroscopically and microscopically. These morphological features and other classical methods that are routinely used in classification are also useful in fungal identification.

1.Wet mount techniques

2.Potassium hydroxide wet mount

3.India ink wet mount

4.Lactophenol cotton blue wet mount

5.Gram staining

6.Periodic acid Schiff staining

7.Toluidine blue o staining

8.Pour plate Auxanographic method

9.Casein hydrolysis

10.Carbohydrate fermentation

LACTOPHENOL COTTON BLUE WET MOUNT

Lactophenol cotton blue (LCB) is a mounting medium commonly used in microbiology laboratories for preparing mounts of fungal cultures. LCB is used as both mounting fl uid and stain. In this method, phenol will kill the organisms, and the lactic acid preserves fungal structures; chitin in the fungal cell wall is stained by the cotton blue. It can be used alone or in conjunction with KOH. Library slides may be made by allowing the mount to dry for 3 weeks and then sealing with collodion.

1. Put a large drop of LCB with a Pasteur pipette.

2. Transfer a small quantity of the culture to the drop.

3. Tease the culture well with teasing needles, so as to get a uniform spread.

4. Put on a coverslip gently to avoid entrapment of air bubbles.

5. Examine under the 20× and 40× objectives of light microscope.

6. Observe the morphological features carefully.

7. Fungal elements will stain deep blue against a clear pale-blue background.

INDIA INK WET MOUNT

India ink can be added to specimens to provide dark background that will highlight hyaline yeast cells and capsular material. This method is used to detect microorganisms that are surrounded by capsules. The dye is excluded by the capsule, cre-ating a clear halo around the yeast cell. It is a rapid method for the preliminary detection and identification of specimens containing species of Cryptococcus .

1. Add a small drop of India ink on a smear.

2. Place a coverslip over the smear and press it gently to obtain a thin mount.

3. If India ink is too thick (dark), dilute it by 50% with saline.

4. Allow the preparation to stand for few minutes to settle.

5. Scan under low power in reduced light; switch to high power, if necessary.

6. Organisms possessing a capsule appear highly refractile, surrounded by a clear zone against a dark background.

POUR PLATE AUXANOGRAPHIC METHOD

1. Peptone discs can be obtained commercially or prepared manually (steps 2–5).

2. Punch 6-mm diameter discs from Whatman no. 1 fi lter paper.

3. Sterilize the discs by placing them in a hot-air oven for 1 h.

4. Allow to cool, and then add one drop of 3% filter-sterilized potassium nitrate or peptone solution to each disc.

5. Dry the disc in 30 °C in incubator and store at 0 °C.

6. Prepare yeast carbon base (YCB) medium.

7. Prepare a yeast suspension from 24- to 48-hold culture in 2 mL of YCB by adding heavy inoculum.

8. Add this suspension to the 18 mL sterilized molten agar and mix well.

9. Pour the entire medium into Petri dish.

10. Allow the media to solidify at room temperature.

11. Now place the various nitrate-impregnated discs onto the surface of the agar plate.

12. Incubate at 30 °C for 4–7 days.

13. Positive reactions can be noted by growth and color change around the disc. Carbohydrate Fermentation

This method is a powerful tool for definitive characterization and taxonomy of yeasts.

CARBOHYDRATES FERMENTATION

Carbohydrate fermentation tests whether a certain microbe has the capability to ferment different carbohydrates. Fungi able to ferment a particular sugar are also able to assimilate the same sugar; however, the reverse is not always true. To test fermentative abilities, a different basal medium is employed. Normally, 2% sugar solution is added to the basal medium in a test tube that also contains an inverted Durham tube in order to observe production of CO 2 and ethanol as the by-products of sugar fermentation. Because most yeasts are also able to assimilate ethanol as the sole source of carbon, it is necessary to incubate assimilation tests separately from fermentation tests, as ethanol vapor produced by fermentation can dissolve in assimilation tests and cause false-positive results [ 90–98 ] .

1. Prepare basal medium and sterilize at 121 °C for 20 min.

2. Add fi lter-sterilized sugar at the concentration of 2% (w/v) to the medium aseptically.

3. Pour the medium to the test tubes. 4. Insert inverted single sterile Durham’s tube in each and close the lid.

5. Incubate at 20 °C for 7 days.

6. Gas accumulation in Durham’s tube is indicative of a positive result.

CASEIN HYDROLYSIS

Caseinase is an exoenzyme that is secreted outside of the cells into the surrounding media. It has the ability to break down milk protein, called casein, into small peptides and individual amino acids for their energy use or as building material. The hydrolytic reaction creates a clear zone around the cell as the casein protein is converted to soluble and transparent end products, like small chains of amino acids, dipeptides, and polypeptides. This test can be used to identify some species of yeast and fungi like Citeromyces matritensi s, Aspergillus dimorphicus , A. ochraceus , Fusarium illudens, F. moniliforme, F. solani, Penicillium citrinum, P. brevicompactum, P. chrysogenum, P. fellutanum,and P. waksmanii .

1. Prepare Petri plates with autoclaved skim milk agar or casein agar in sterile conditions.

2. Inoculate fungal mycelia onto the center of the plate and incubate at 20 °C for 14 days.

3. Examine for the presence of a clear zone.

4. The appearance of a clear zone around the fungal colony is the positive result.

GRAM STAINING

Gram stain is a key starting point to identify microbial species. The stain differentiates membrane structures between gram-positive and gram-negative microorganisms. Gram-positive microbes have a thick cell wall made up of peptidoglycan (50–90%), which are stained purple by crystal violet, whereas gram-negative microbes have a thinner layer (10% of cell wall), which are stained pink by the counter-stain safranin .

1. Apply two drops of crystal violet on smear for 30 s.

2. Wash with tap water.

3. Add two drops of Gram’s iodine for 30 s.

4. Repeat step 2.

5. Add 95% ethanol.

6. Repeat step 2.

7. Add two drops of safranin.

8. Repeat step 2.

9. Observe in microscope under oil immersion.

10. Yeasts are gram-positive, but poorly stained;

Cryptococcus neoformans is a notable excep-tion (gram-negative).