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**COURSE TITLE: GENERAL MICROBIOLOGY.**

**ASSIGNMENT ON BIOLOGICAL AND BIOCHEMICAL REACTIONS OF MICROORGANISIMS.**

**ANSWER.**

1. **Biochemical reactions of bacteria**

Enzymes catalyse all the various Chemical reactions of which the organism is capable. So, different species of bacteria must carry out different and unique sets of biochemical reactions.

These biochemical reactions are used in identification of different genera and species of bacteria (eg; differentiate gram positive bacteria and gram negative). Some of these reactions include;

1. **Mannitol Salt Agar (MSA) test:** 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. This is in contrast to *Streptococcus* species, whose growth is selected against by this high salt agar. The differential ingredient in MSA is the sugar mannitol. Organisms capable of using mannitol as a food source will produce acidic by-products 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. So, *Staphylococcus aureus* is capable of fermenting mannitol while *Staphylococcus epidermidis* is not.
2. **Glucose broth with Durham tubes test:** This is a differential medium. It tests an organism's ability to ferment the sugar glucose as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous by products. This is a test commonly used when trying to identify Gram-negative enteric bacteria, all of which are glucose fermenters but only some of which produce gas.

Like MSA, this medium also contains the pH indicator, phenol red. If an organism is capable of fermenting the sugar glucose, then acidic by products are formed and the pH indicator turns yellow. *Escherichia coli* is capable of fermenting glucose as are *Proteus mirabilis* and *Shigella dysenteriae. Pseudomonas aeruginosa* is a non-fermenter.The end product of glycolysis is pyruvate. Organisms that are capable of converting pyruvate to formic acid and formic acid to H2 (g) and CO2 (g), via the action of the enzyme formic hydrogen lyase, emit gas. This gas is trapped in the Durham tube and appears as a bubble at the top of the tube. *Escherichia coli* and *Proteus mirabilis* are both gas producers. *Shigella dysenteriae* ferments glucose but does not produce gas. Broth tubes can be made containing sugars other than glucose (e.g. lactose and mannitol).  Because the same pH indicator (phenol red) is also used in these fermentation tubes, the same results are considered positive (e.g. a lactose broth tube that turns yellow after incubation has been inoculated with an organism that can ferment lactose).

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.
2. **Streak-stab techniques**: Often when inoculating a BAP to observe hemoloysis patterns, investigators will also stab several times through the agar using an inoculating loop. This stab allows for the detection of streptolysin O, a specific hemolysin produced by *Streptococcus pyogenes*. This hemolysin is inactivated by O2 and is only seen subsurface (in an anaerobic environment) around the stab mark. The oval-shaped areas of clearing around the stab marks are caused by streptolysin O.
3. **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 genus *Enterococcus* (*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.
4. **Catalase test:** his test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas.

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The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result. The *Staphylococcus* spp. and the *Micrococcus* spp. arecatalase positive. The *Streptococcus* and *Enterococcus spp. are* catalase negative.

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.

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. *Klebsiella pneumoniae* and *Proteus mirabilis* are examples of citrate positive organisms. *Escherichia coli* and *Shigella dysenteria*e are citrate negative.

10) **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. The Gram-positive rod, *Bacillus subtilis* is lipase positive (pictured on the right) The plate pictured on the left is lipase negative

1. **Identification/staining techniques of fungi.**

Identification of Fungi is a practical aspect involving the use of various biochemical techniques to distinguish one group of Fungi from the other.

Fungi could be identified using staining techniques, some of these techniques include; **Giemsa Staining, Gram Staining, Wright Staining, Weigert’s Iron Hematoxylin Staining, Gridley Staining, Acridine Orange Staining, Calcoflour White Staining, Periodic Acid–Schiff Staining, Fontana-Masson Staining**, **Grocott-Gomori Methenamine Silver Staining.**

**Giemsa Staining.**

A variety of “Romanowsky-type” stains with

mixtures of methylene blue and azure eosin compounds

have been used successfully for many years on diverse fungi with various procedures and modifications. Giemsa stain is a member of the Romanowsky group of stains, which are defined as being the black precipitate formed from the addition of methanol. In this stain, eosin ions are negatively charged and stain basic components of cells orange to pink.

It was also originally designed to incorporate cytoplasmic

(pink) staining with nuclear (blue) staining and fixation as a single step for smears and thin films. This stain has widely been used to examine *Pneumocystis jiroveci, Rhinosporidium seeberi* and *Histoplasma capsulatum*. The steps include;

1. Flood the smear with methyl alcohol and leave for 3–5 min for fixation.

2. Add prepared Giemsa stain and leave for 45 min.

3. Wash slide thoroughly with running tap water.

4. Blot dry with absorbent paper.

5. Observe under oil immersion.

6. Look for intracellular budding yeasts; fungi stain with purplish-blue.

**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). The steps include;

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. Wash with tap water.

5. Add 95% ethanol.

6. Wash with tap water.

7. Add two drops of safranin.

8. Wash with tap water.

9. Observe in microscope under oil immersion.

10. Yeasts are gram-positive, but poorly stained; *Cryptococcus neoformans* is a notable exception (gram-negative).

**Wright Staining.**

The Wright stain is an alcoholic solution of methylene blue, azure A, thionin, and eosin Y. Methyl groups are activated and react with charged components of the cell to produce coloration. It is used to detect blood parasites, viral and chlamydial inclusion bodies, yeast cells, and species of *Pneumocystis*. Eosin ions are negatively charged and stain basic components of cells orange to pink, whereas other dyes stain acidic cell structures to various shades of blue to purple.

The steps include;

1. Cover the smear with freshly filtered Wright stain and leave for 1–3 min.

2. Without removing the stain, pour on buffer solution (pH 6.4).

3. Gently mix buffer and stain; upon proper mixing, metallic green sheen (green scum) rises to the surface of the fluid.

4. Leave for 3 min or longer.

5. Wash the slide gently with flowing tap water and wipe the bottom of the slide with a clean filter paper.

6. Air-dry the slide and observe under the microscope.

7. Intracellular yeast cells are typically stain blue and species of *Pneumocystis* stain purple.

**Weigert’s Iron Hematoxylin Staining**

This stain can be used with fixatives that include polyvinyl alcohol, sodium acetate, and formalin. The staining method involves application of haemalum, which is a complex formed from aluminium ions and oxidized hematoxylin. This stains nuclei of cells blue. Counterstain eosin Y may also be used to color other structures in various shades of red, pink, and orange.

The steps include;

1. Add staining solution on a smear and leave for 1–2 hours.

2. Rinse with tap water.

3. Add 1% HCl.

4. Add 70% ethanol.

5. Rinse with tap water

6. Counterstain with eosin Y, if necessary.

7. Dehydrate with ethanol.

8. Clear with xylene and observe under microscope.

9. Yeast cells stain blue–gray to black.

**Gridley Staining**

Gridley staining method is used to identify fungi, based on Bauer chromic acid leucofuchsin stain with the addition of Gomori’s aldehyde fuchsin stain and metanil yellow as counterstains. Against a yellow background, hyphae, conidia, yeast capsules, elastin, and mucin appear in different shades of blue to purple. It can be used to identify *Rhiosporidium seeberi* and *Histoplasma capsulatum*.

The steps include;

1. Place chromic acid on smear for 1 hour.

2. Wash well with tap water.

3. Treat with sodium metabisulphite bleach for 1 min.

4. Wash well with tap water.

5. Rinse with distilled water.

6. Place in Schiff’s reagent for 20 min.

7. Wash well with tap water.

8. Rinse with 70% ethanol.

9. Place in aldehyde fuchsin for 30 min.

10. Rinse off excess with 95% ethanol.

11. Wash well with tap water.

12. Counterstain with metanil yellow for 1 min.

13. Rinse well with distilled water.

14. Dehydrate and observe under fluorescence microscope as Fungi will show purple color with yellow background.

**Acridine Orange Staining**

Acridine orange is a fluoro-chromatic dye that binds to nucleic acids of fungi. Under UV light, acridine orange stains RNA and single-stranded DNA orange, while double-stranded DNA appears green. At neutral pH, fungi and cellular materials stain reddish orange. At acid pH, fungi remain reddish orange but background material stains greenish yellow.

The steps include;

1. Add Weigert’s iron hematoxylin on smear for 5 min.

2. Wash well with tap water.

3. Place few drops of acridine orange solution for 2 min.

4. Wash well with tap water.

5. Observe smear on the fluorescence microscope as fungi stain bright orange and the background appears greenish yellow.

**Calcoflour White Staining**

Calcoflour White (CFW) stain is used to detect fungal elements, particularly *Pneumocystis* species. The fluorophore shows a high affinity for chitin-forming hydrogen bonds with free hydroxyl groups and stains fungal cell walls blue. The use of CFW staining requires the addition of KOH, which helps to dissolve keratinized particles and emulsify solid, viscous material and enhance the visualization of fungal elements in microscopic examination. Positive results are indicated by a bright green to blue fluorescence using a fluorescence microscope. A bright yellow-green fluorescence is observed when collagen or elastin is present. KOH-CFW preparations may be preserved for several days at 4 °C. This stain can be used to identify *Fusarium solani* , *Aspergillus fumigatus,* and *Candida albicans*.

The steps include;

1. On smear add a drop of 15% KOH and a drop of the CFW solution or mix in equal volumes before processing.

2. Mix and place a coverslip over the material.

3. If necessary, allow the KOH preparation to remain at room temperature (25 °C) for a few minutes until the material has been cleared; the slide may be warmed to speed up the clearing process.

4. Observe the slide by UV microscopy as Fungal cell walls fluorescence apple green to blue.

**Periodic Acid–Schiff Staining**

Periodic acid–Schiff (PAS) reactions are effective stains for demonstrating fungal elements of essentially all fungi. Periodic acid attacks some carbohydrates containing 1,2-glycol or OH group with the conversion of this group to 1,2-aldehydes, which then react with the fuchsin-sulfurous acid to form the magenta color. Identification of fungal elements can be enhanced if a counterstain such as light green is used. Species of *Coccidioides* , *Cryptococcus* , *Histoplasma* , *Candida, Malassezia* , and *Aspergillus* can be stained with this stain.

The steps include;

1. Immerse the smear in ethanol for 1 min.

2. Place 5% periodic acid for 5 min.

3. Wash gently in running tap water.

4. Place basic fuchsin for 2 min.

5. Wash well with tap water

6. Add sodium metabisulphite (0.5%) for 3–5 min.

7. Wash well with tap water

8. Counterstain with dilute aqueous light green (0.2%) for 2 min.

9. Dehydrate with 70%, 80%, 95%, 100% ethanol and xylene, each for 2 min.

10. Observe under microscope as Fungi stain bright pink-magenta or purple against green background when light green is used as a counterstain.

**Fontana-Masson Staining**

The Fontana-Masson (FM) stain can be used to detect the presence of melanin in cell walls of dematiaceous fungi such as species of *Bipolaris* , *Curvularia* , *Exophiala,* and *Phialophora* . FM stain is often believed to be a diagnostic tool to differentiate dematiaceous fungi from *Aspergillus* sp. And some Zygomycetes. Also, it is particularly useful for distinguishing capsule deficient *Cryptococcus neoforamans* from *Histoplasma capsulatum* and *Blastomyces dermatitis.*

Melanin has the ability to reduce solutions of ammonical silver nitrate to metallic silver without the use of an external reducing agent. The intensity and amount of staining may reflect differences in melanin deposition owing to growth rate, age, availability of precursors, or loss of pigment staining associated with hyphal death and destruction. Extent of stain intensity and its distribution in fungal elements in tissue were evaluated by means of intensity; for example, dark brown (strong intensity), medium brown (moderate intensity), and pale brown (weak intensity).

The steps include;

1. Treat smear with ammonical silver nitrate solution for 20 min at 60°C.

2. Check microscopically after 15 min and repeat step 1 if necessary.

3. Wash well in distilled water.

4. Tone with 0.1% gold chloride for 2 min.

5. Wash well in distilled water.

6. Fix in 2% aqueous sodium thiosulphate for 2 min.

7. Wash well in distilled water.

8. Counterstain with neutral red stain for 1 min.

9. Wash well in distilled water.

10. Rapidly dehydrate well in absolute alcohol, clear, and mount.

11. Observe under microscope as all dematiaceous fungi show strong intensity (black); species such as *Bipolaris, Exophiala, Fonsecaea,* and *Phialophora* are darkly pigmented because of melanin.

**Grocott-Gomori Methenamine Silver Staining**

Grocott-Gomori methenamine silver (GMS) staining is preferred for screening degenerated and nonviable fungi because it provides better contrast. The fungal cell wall contains mucopolysaccharides that are oxidized by GMS to release aldehyde groups, which later react with silver nitrate. Silver nitrate is converted to metallic silver, which becomes visible in the silver stains; this is useful in detecting fungal elements. Fungi stain in black against a pale-green background.

*Pneumocystis jiroveci* , *Cryptococcus neoformans, Coccidiodes immitis, Histoplasma capsulatum, Aspergillus fumigatus,* and *Candida albicans* can be detected by this staining technique.

The steps include;

1. Add two drops of absolute ethanol for 5 min.

2. Wash in distilled water.

3. Flood the smear with 4% chromic acid for 45 min.

4. Wash in distilled water.

5. Add 1% sodium metabisulphite for 1–2 min.

6. Wash in distilled water.

7. Add working solution of hexamine (smear becomes dark brown).

8. Wash with distilled water or if smear turns black, wash with 0.1% ferric chloride.

9. Add 5% sodium thiosulphate for 2 min.

10. Wash in distilled water.

11. Wash with 1% light green solution for 1 min.

12. Dry and view under oil immersion as the slide with fungal elements stains black; inner part of micelle or hyphae stains pink with background in pale green.