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DEPARTMENT: BIOCHEMISTRY

ASSIGNMENT

1(i) Urea Enzyme Production: Few bacteria have the ability to breakdown urea into ammonia, CO2 and water. This test is used to specifically differentiate between proteus and non-lactose fermenting enteric bacteria (Salmonella and Shigella).

* Procedure: Streak the surface of a urea agar slant with a portion of well-isolated colony or inoculate slant with 1 to 2 drops from a brain-heart infusion broth culture. Leave the cap on loosely and incubate the tube at 35o-37oC in ambient air for 48 hours to 7days. Examine for the development of a pink color for as long as 7 days.

(ii) Starch Hydrolysis: Some bacteria are capable of using starch as a source of carbohydrates but in order to do this, they must first hydrolyze or break it down first so it may enter the cell. The bacteria secrete an exoenzyme called diastase which hydrolyses the starch by breaking bonds between the glucose molecules. This test uses trypticase soy broth cultures of Bacillus subtilis and Escherichia coli.

* Procedure: Divide the plate in half using a marker, label why one-half B. subtilis and the other half E. coli. Make a single streak mark with the appropriate organism on the corresponding half of the plate. Incubate upside down in a 37oC incubator. After some hours, add iodine to see if all the starch in the agar has been hydrolyzed. If the starch has been hydrolyzed there will be a clear zone around the bacteria growth.

(iii) Protein Hydrolysis: Many bacteria can hydrolyze a variety of proteins into peptides and eventually into individual amino acids. They use these amino acids to synthesize their own proteins and other cellular molecules or to obtain energy. The enzyme involved is called protease.

* Procedure: Divide skim milk agar plate in half and inoculate one half with Bacillus subtilis and the other half with Escherichia coli. Incubate upside down and observe after few hours. If casein (milk protein) is hydrolyzed, there will be a clear zone around bacterial growth.

(iv) Fermentation of Carbohydrates: Facultative anaerobic and anaerobic bacteria are capable of fermentation. A wide variety of carbohydrates may be fermented by various bacteria in order to obtain energy. We can detect whether a specific carbohydrate is fermented by looking for common end products of fermentation. The fermentation end products may either be acid end products or acid and gas end products.

* Procedure: Inoculate and incubate tubes of media containing a single carbohydrate (such as lactose or maltose), a pH indicator and a Durham tube. Label each tube with the name of the sugar in the tube and the name of the bacterium you are growing. If the bacterium ferments that particular carbohydrate producing acid end products alone, the acid will lower the pH, causing the pH indicator to change color. If the bacterium ferments that particular carbohydrates producing both acid and gas, the pH indicator changes color and the gas will collect in the Durham tube. If the carbohydrate is not fermented by the bacterium, no acid or gas will be produced and the indicator won’t change color.

(v) Indole and Hydrogen Sulfide Production: Some bacteria use the enzyme tryptophanase to convert the amino acid tryptophan into molecules of indole, pyruvic acid and ammonia. Since only few bacteria contain tryptophanase, the formation of indole from a tryptophan substrate can be another useful diagnostic tool for the identification of an organism. By adding Kovac’s reagent to the medium after incubation we can determine if indole was produced.

Likewise, some bacteria are capable of breaking down sulfur containing amino acids (cystine, methionine) or reducing inorganic sulfur-containing compounds (such as sulfite, sulfate or thiosulfate) to produce hydrogen sulfide(H2S). This reduced sulfur may be incorporated into other cellular amino acids, or perhaps into coenzymes.

* Procedure: Fill three tubes with SIM (sulfide, indole, motility) medium. Stab each SIM medium with bacteria to be tested. Incubate the tubes. If the bacterium reduces sulfur to hydrogen sulfide, the agar will turn black. After some hours add Kovac’s reagent to each tube to detect indole production. If indole was produced, the Kovac’s reagent will turn red.

(vi) Catalase Activity: Catalase is an enzyme found in most bacteria which initiates the breakdown of hydrogen peroxide(H2O2) into water and free oxygen. This is vital for aerobic respiration.

* Procedure: Make a trypticase soy agar culture of the bacteria to be tested. Add a few drops of 3% hydrogen peroxide to the culture and look for the release of oxygen as a result of hydrogen peroxide breakdown. This appears as foaming.

(vii) Oxidase Test: This test is based on the production of the enzyme indophenol oxidase. This enzyme oxidizes a redox dye in the reagent which results in a color change. Indophenol oxidase, in the presence of atmospheric oxygen, oxidizes the phenylenediamine oxidase reagent to form a dark purple compound, indophenol.

* Procedure: Add a few drops of oxidase test reagent to a strip of filter paper. Streak a loopful of bacteria onto the reagent saturated paper. Positive reactions turn the bacteria violet to purple immediately.

(viii) Motility Test: This test is used to detect the motility of microorganisms. Motility is apparent by the presence of diffuse growth away from the line of inoculation.

* Procedure: Inoculate by stab inoculation with a needle. Incubate at a temperature and duration appropriate for the organism being tested. Examine for growth and signs of motility.

(ix) Fluid Thioglycolate Medium Test: This is used to test the oxygen requirements of different bacteria. It categorizes bacteria according to their oxygen requirements; obligate aerobes, facultative anaerobes and obligate anaerobes. Obligate aerobes grow at the surface of the medium where there is a high concentration of oxygen. Obligate anaerobes grow near the bottom of the broth tube where there is no oxygen. Facultative anaerobes grow best where more oxygen is present, but growth will occur throughout the broth tube.

* Procedure: Dilute your organism in a tube of sterile water. Using a sterile 1mL pipette, place 1mL of organism into the middle of a tube. Cap tightly; do not jostle. Incubate for 24 hours at 37oC.

(x) Citrate Utilization Test: Some bacteria can produce the enzyme citrase which breaks down citrate to oxaloacetate and acetate. This is used to test an organism’s ability to utilize citrate as a source of energy.

* Procedure: Streak the slant back and forth with a light inoculum placed from the center of a well-isolated colony. Incubate aerobically at 35 to 37oC for 4 to 7 days. Look out for color change from green to blue along the slant.

(xi) Coagulase Test: This is used to differentiate between Staphylococcus aureus which is coagulase positive from Staphylococcus epidermes and Staphylococcus saprophyticus which are both coagulase negative. Coagulase is an enzyme that converts soluble fibrinogen in plasma to insoluble fibrin. Staphylococcus aureus produces two forms of coagulase, bound and free.

* Procedure: Emulsify a staphylococcal colony in a drop of water on a clean glass slide. Dip a flamed and cooled straight inoculating wire into the undiluted plasma at room temperature, withdraw and stir the adhering traces of plasma into the staphylococcal suspension on the slide. Observe a coarse clumping of cocci visible to the naked eye within 10 seconds as a positive reaction.

2.(i) 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.

* Procedure: Apply two drops of crystal violet on smear for 30s. Wash with tap water. Add two drops of Gram’s iodine for 30s. Wash with water again. Add 95% ethanol. Wash with water again. Add two drops of safranin. Wash with water again. Observe in microscope under oil immersion. Yeasts are gram-positive, but poorly stained.

(ii) Giemsa Staining: Giemsa stain is a member of the Romansky 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.

* Procedure: Flood the smear with methyl alcohol and leave for 3-5 minutes for fixation. Wash slide thoroughly with running tap water. Blot dry with absorbent paper. Observe under oil immersion. Look for intracellular budding yeast, fungi stain with purplish-blue.

(iii) 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. Eosin ions are negatively charged and stain basic components of cells orange to pink.

* Procedure: Cover the smear with freshly filtered Wright stain and leave for 1-3 minutes. Without removing the stain, pour on buffer solution. Gently mix buffer and stain; upon proper mixing, metallic green sheen (green scum) rises to the surface of the fluid. Leave for 3 minutes or longer. Wash the slide gently with flowing tap water and wipe the bottom of the slide with a clean filter paper. Air-dry the slide and observe under the microscope. Intracellular yeast cells typically stain blue and species of Pneumocystis stain purple.

(iv) Wiegert’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 hemalum, which is a complex formed from aluminum ions and oxidized hematoxylin. This stains nuclei of cells blue. Counter stain eosin Y may also be used to color other structures in various shades of red, pink and orange.

* Procedure: Add staining solution on a smear and leave for 1-2 hour. Rinse with tap water. Add 1% HCl. Add 70% ethanol. Rinse again. Counterstain with eosin Y, if necessary. Dehydrate with ethanol. Clear with xylene and observe under microscope. Yeast cells stain blue-gray to black.

(v) Acridine Orange Staining: Acridine orange is a fluorochromatic 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 neutral pH, fungi remain reddish orange but background material stains greenish yellow.

* Procedure: Add Wiegert’s iron hematoxylin on smear for 5 minutes. Wash well with tap water. Place few drops of acridine orange solution for 2 minutes. Wash again with tap water. Observe smear on the fluorescence microscope. Fungi stain bright orange and the background appear greenish yellow.

(vi) 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.

* Procedure: Place chromic acid on smear for 1 hour. Wash well with tap water. Treat with sodium metabisulfite bleach for 1 minute. Wash again with tap water then rinse with distilled water. Place in Schiff’s reagent for 20 minutes. Wash again with tap water. Rinse with 70% ethanol. Place in aldehyde fuchsin for 30 minutes. Rinse off excess with 95% ethanol. Wash with tap water again. Counterstain with mentanil yellow for 1 minute. Rinse well with distilled water. Dehydrate and observe under fluorescence microscope. Fungi show purple color with yellow background.

(vii) Calcoflour White Staining: Calcoflour White (CFW) stain is used to detect fungal elements. 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 fluorescence microscope is observed when collagen or elastin is present.

* Procedure: On smear add a drop of 15% KOH and a drop of the CFW solution or mix in equal volumes before processing. Mix and place a coverslip over the material. If necessary, allow the KOH preparation to remain at room temperature for a few minutes until the material has been cleared; the slide may be warmed to speed up the clearing process. Observe the slide by UV microscopy. Fungal cell walls fluorescence apple green to blue.

(viii) Mayer’s Mucicarmine Staining: Mucicarmine is a red stain that contains aluminum chloride and carmine. Aluminum is believed to form a chelation complex with the carmine and change the molecule to a positive charge, allowing it to bind with the acid substrates of low densities such as mucins. It will also stain the walls of the spores and the inner surface of the sporangia.

* Procedure: Stain the smear with a working solution of Wiegert’s hematoxylin for 7 minutes. Wash well in tap water. Add metanil yellow for 1 minute. Wash again with tap water. Place in mucicarmine stain 45 minutes. Rinse quickly in distilled water. Dehydrate in 95% ethanol and absolute alcohol. Clear with two changes of xylene. Mount in DPX and view under a microscope. Mucopolysaccharide capsule stain deep rose to red, nuclei are black, and the other debris stain yellow.

(ix) 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 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.

* Procedure: Immerse the smear in ethanol for 1 minute. Place 5% periodic acid for 5minutes. Wash gently in running tap water. Place basic fuchsin for 2 minutes. Wash again with running tap water. Add sodium metabisulphite (0.5%) for 3-5 minutes. Wash again with running tap water. Counterstain with dilute aqueous light green (0.2%) for 2 minutes. Observe under microscope. Fungi stain bright pink-magenta or purple against green background when light green is used as a counterstain.