1a**. Oxidative fermentative test**: The test is used to determine if gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are nonsacchrolytic (have no ability to use the carbohydrate in the media).

**Procedure**

1. Inoculate two tubes of OF test medium with the test organism using a straight wire by stabbing “half way to the bottom” of the tube.
2. Cover one tube of each pair with 1 cm layer of **sterile mineral oil or liquid paraffin** (it creates anaerobic condition in the tube by preventing diffusion of oxygen), leaving the other tube open to the air.
3. Incubate both tubes at 35°C for 48 hours (Slow growing bacteria may take 3 to 4 days before results can be observed)

**Imvic test**: Differentiation of the principal groups of enterobacteriaceae can be accomplished on the basis of their biochemical properties and enzymeatic reactions in the presence of specific substrates. The IMVIC series of test indole, methyl red, Voges-Proskauer, and citrate utilization can be used.

**Indole test**

**Procedure**

1. Preparation of tryptone broth:  
   Ingredients: Tryptone – 10 gms  
                        Distilled water – 1000 mL  
   Distribute 5 mL of the broth into the test tubes and plug with cotton plugs.  
   Sterilize it at 121°C for 15 minutes.
2. Preparation of Kovac’s reagent:  
   N-amyl alcohol – 75 mL  
   Concentrated HCl – 25 mL  
   P-dimethylaminebenzaldehyde – 5 g.
3. Inoculate the tubes with the test bacterial culture.
4. Incubate all the tubes for 48 hours at 37°C.
5. Test for indole – Add 0.3 mL of Kovac’s reagent to each test tube. Mix well by rotating the tubes between your hands. The formation of a red layer at the top of the culture indicates a positive test.

**Methyl red**

**Procedure**

1. Preparation of methyl red indicator—Dissolve about 0.2 gm of methyl red in 500 mL of 95% ethyl alcohol and add 500 mL of distilled water and filter.
2. Preparation of MRVP media – (glucose phosphate broth)  
   Dipotassium hydrogen phosphate (K2HPO4) – 5 gms  
   Peptone – 5 gms Glucose – 5 gms  
   Distilled water – 1000 mL  
   Suspend all the ingredients in distilled water and gently warm. Do not alter the pH. 5 mL of media that is distributed in plugged test tubes. Sterilize at 121°C for 15 minutes.
3. Innoculate the tubes with the test bacterial culture (except for the control tube.)
4. Incubate all the tubes at 37°C for 48 hrs. 5. Add 3-4 drops of MR indicator into each tube. A distinct red color indicates the positive test; yellow color indicates a negative test.

**Vogues proskauer**

**Procedure**

1. (a) Preparation of MRVP medium or glucose phosphate broth:  
   Dipotassium hydrogen phosphate (K2HPO4) - 5 gms  
   - Peptone - 5 gms  
   - Glucose - 5 gms  
   - Distilled water - 1000 mL  
   Suspend all the ingredients in distilled water and gently warm. Do not alter the pH. 3 mL of the media are distributed into test tubes, which are plugged. Sterilized at 121°C.  
   (b) Preparation of Barrit’s reagent:  
   Barrit’s reagent consists of 2 solutions, i.e., solutions A and B.  
   Solution A is prepared by dissolving 6 gms of alpha naphthol in 100 mL of 95% ethyl alcohol.  
   Solution B is prepared by dissolving 16 gms of potassium hydroxide in 100 mL of water.
2. Inoculate the tubes with the bacterial culture.
3. Incubate for 48 hours at 37°C.
4. Pipette 1 mL from each culture tube into clean separate tubes. Use separate pipettes for each tubes.
5. Add 18 drops (0.5 mL) of Barrit’s solution A (a-naphthol) to each tube that contains the media. Add an equal amount of solution B into the same tube.
6. Shake the tubes vigorously every 30 seconds. A positive reaction is indicated by the development of a pink color, which turns red in 1–2 hours, after vigorous shaking. It is a very important step to achieve complete aeration.

**Coagulase Test**: Coagulase test is used to differentiate *Staphylococcus aureus* (positive) which produce the enzyme coagulase, from *S. epidermis*and*S. saprophyticus* (negative) which do not produce coagulase. i.e Coagulase Negative *Staphylococcus* (CONS).

**Procedure and Types of Coagulase Test**

Slide Test (to detect bound coagulase)

1. Place a drop of physiological saline on each end of a slide, or on two separate slides.
2. With the loop, straight wire or wodden stick, emulsify a portion of the isolated colony in each drops to make two thick suspensions.
3. Add a drop of human or rabbit plasma to one of the suspensions, and mix gently.
4. Look for clumping of the organisms within 10 seconds.
5. No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

Tube Test (to detect free coagulase)

1. Dilute the plasma 1 in 10 in physiological saline ( mix 0.2 ml of plasma with 1.8 ml of saline).
2. Take 3 small test tubes and label as T (Test), P (Positive Control) and N (Negative Control). Test is 18-24 hour broth culture, Positive control is 18-24 hr S. aureus broth culture and Negative control is sterile broth.
3. Pipette 0.5 ml of the diluted plasma into each tube.
4. Add 5 drops (0.1 ml) of the Test organisms to the tube labelled “T”, 5 drops of S. aureus culture to the tube labelled “P” and 5 drops of sterile broth to the tube labelled “N”.
5. After mixing, incubate the three tubes at 35-37 Degree Celsius.
6. Examine for clotting after 1 hours. If no clotting has occurred, examine at 30 minutes intervals for up to 6 hours.

**Oxidase test**: The oxidase test is designed for specifically detecting the presence of the terminal enzyme system in aerobic respiration called cytochrome C oxidase or cytochrome a3. Cytochrome C oxidase is the terminal or last H2 electron acceptor in aerobic respiratory mechanism which is composed of a number of enzymes which alternatively oxidize and reduce each other by donating or accepting electrons derived from H2.

**Procedure of Oxidase Test**

1. Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride.
2. Moisten the paper with a sterile distilled water.
3. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper.
4. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 second

**Catalase test**: The catalase test is primarily used to distinguish among Gram-positive cocci: members of the genus [***Staphylococcus***](https://microbeonline.com/staphylococcus-aureusdisease-properties-pathogenesis-and-laboratory-diagnosis/)**are catalase-positive**, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative.

**Procedure of Catalase test (Slide Test)**

1. Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick
2. Place a drop of 3% H2O2 on to the slide and mix.
3. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.
4. A negative result is no bubbles or only a few scattered bubbles.\*
5. Dispose of your slide in the biohazard glass disposal container.

**Triple sugar iron test**: To determine the ability of an organism to ferment glucose, lactose, and sucrose, and their ability to produce hydrogen sulfide.

**Procedure for Triple Sugar Iron Agar (TSI) Test**

1. With a sterilized straight inoculation needle touch the top of a well-isolated colony
2. Inoculate TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant.
3. Leave the cap on loosely and incubate the tube at 35°C in ambient air for 18 to 24 hours.

**Carbohydrate fermentative test**: The carbohydrate fermentation test is used to determine whether or not bacteria can ferment a specific carbohydrate. Carbohydrate fermentation patterns are useful in differentiating among bacterial groups.

Allow medium to warm to room temperature prior to inoculation.

1. Inoculate the Purple Broth (with carbohydrate of choice) with isolated colonies from an 18-24 hour pure culture of the organism.
2. Inoculate a control tube of Purple Broth Base in parallel with the carbohydrate based media.
3. Incubate inoculated media aerobically at 35-37ºC. for 3-5 days.

Note: Increased incubation up to 30 days may be necessary for some microorganisms.

1. Observe daily for development of a yellow color in the medium.

**Urease test**: Urease broth is a differential medium that **tests** the ability of an organism to produce an exoenzyme, called **urease** that hydrolyzes urea to ammonia and carbon dioxide. The broth contains two pH buffers, urea, a very small amount of nutrients for the bacteria, and the pH indicator phenol red.

**Procedure for urease test**

**For Christensen’s Urea Agar**

1. Streak the entire slant surface with a heavy inoculum from an 18-24 hour pure culture (Do not stab the butt as it will serve as a color control).
2. Incubate tubes with loosened caps at 35°C.
3. Observe the slant for a color change at 6 hours and 24 hours unless specified for longer incubation.

**For Stuart’s Urea Broth**

1. Inoculate the broth with a heavy inoculum from an 18-24 hour pure culture
2. Shake the tube gently to suspend the bacteria
3. Incubate the tubes with loosened caps at 35°C.
4. Observe the broth for a color change at 8, 12, 24 hours.

**Citrate test**: This is used to test organisms ability to utilize citrate as a source of energy.

**Procedure**

1. Streak the slant back and forth with a light inoculum packed from the center of a well isolated colony.
2. Incubate aerobically at 35 to 37°C for 4 to 7 days
3. Observe a colour change from green to blue along the slant

**Protein hydrolysis:** Many bacteria can hydrolyze a variety of proteins into peptides and eventually into individual amino acids. They can then use these amino acids to synthesize their own proteins and offer celluar molecules or to obtain energy. The process is termed Proteolysis

**Procedures**

1. Innoculate the organism on the plate either on a straight line or zigzag
2. Innoculate the plate 25°C or37°C.
3. Following incubation examine the plate for zone of hydrolysis.

### Types of Staining Techniques[[7]](https://en.wikipedia.org/wiki/Staining" \l "cite_note-7)[[edit](https://en.wikipedia.org/w/index.php?title=Staining&action=edit&section=8)]

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sr. No.** | **Staining Technique** | **Preparation** | **Application** | **Result** |
| 1. | Simple (Monochrome) | Smear stain with single dye .  eg. Methylene blue , Safranin etc | Used to highlight microbes and illustrate cellular  shapes and arrangements . | Organisms are stained in the color of applied stain |
| 2. | Negative (Relief) | Smear mixed with Nigrosin and spread  into thin film | Study cell morphology | Organism is stained, the background is black |
| 3 | Gram | Primary stain: Crystal violet applied to film then treated with iodine (mordant), alcohol (decolourizer) and counter stained with safranin | Characterizes bacteria in one of two groups, Gram positive or Gram negative | Gram positive appears purple in color  Grams negative appears pink in color |
| 4 | Acid fast (Ziehl-Neelsen technique) | Film stained with hot Z.N.C.F. decolourized (acid-alcohol) and counter stain with methylene blue | Separate non-decolorized acid fast bacteria that are not decolorized from colorized non-acid fast bacteria | Acid fast bacteria:Red  Non acid fast: Blue |
| 5 | Endospore (Dornor's method) | Primary stain Malachite green heat fixed to penetrate spores; vegetative cells are counterstained with Safranin | Detects the presence of endospores in six genera of bacteria | Endospores: Green  Vegetative cells: Red |
| 6 | Capsule  A: Hiss method (Positive technique)  B: Manevals's technique (Negative) | Smear stained with Hiss stain following treatment with copper sulphate  Bacterial suspension smeared along with congo red and the Maneval's stain is applied | Capsules can be observed as clear zones surrounding cells of capsulated bacteria and are used to demonstrate the presence of capsules. | Capsule: Light violet/ pale mauve color  Bacteria: Purple capsule, bacterial cell, Stands out against dark background |
| 7 | Cell wall (Dyar's method) | Smear treated with C.P.C. which dissociates to form positively charged cetyl pyridinium and negatively charged chloride ions. Positively charged ions are adsorbed on negatively charged cell wall | Stains cell wall of bacterium | Cell wall: Red Cytoplasm: Blue |
| 8 | Flagella (Leifson's method) | Mordant acts to thicken flagella before staining and increases visibility microscopically when stained with Leifson stain | Demonstrates presence of flagella | Flagella: Red Vegetative cells: Blue |
| 9 | Nuclear material (Feulgen technique) | Smear is treated for hydrolysis to release purines from DNA, purines to cause shift form furanose to aldehyde. Aldehyde groups are available to react with schiff's reagent to form addition compounds. | To demonstarte the presence of DNA in cell. But for detection of the DNA, RNA should be selectively destroyed by acid hydrolysis without affecting DNA | Nuclear material- pinkish purple,  Cytoplasm- colorless |
| 10 | Metachromatic granules (Alberts's method) | The smear is first treated with chloroform to remove fats . Smear applied with Alberts stain which contains cationic dyes such as toluidine blue amd malachite green. Toluidine blue preferentially stains granules while malachite green stains cytoplasm. | The granules show the typical monochromatism nature, this is used to demonstrate granules | Granules: Bluish black, Cytoplasm: Green |
| 11 | Intracellular lipids (Burdon's method) | Lipids are stained with fat soluble dyes like Sudan black. On application of Sudan black-B dyes move into lipids and are retained there while cytoplasm is counter stained with safranin. | To detect the presence of lipids in cell wall, cell membrane or fat globules (PHB in cytoplasm) | Lipid granules: Deep blue,  Cytoplasm: Light pink |
| 12 | Polysaccharide (Hotch kuss method) | Polysaccharide is oxidized with periodate to form polyaldehyde which reacts with Schiff's reagents to red color, while cytoplasm is counter stained with malachite green | Detects the accumulation of polysaccharide granules in the cells | Polysaccharide: Red  Cytoplasm: Green |