MEDICAL MICROBIOLOGY BIOCHEMICAL TEST 1

PROFESSOR DOCTOR

OSAMA NADHOM NIJRIS

UNIVERSITY OF SAMARRA, COLLEGE OF APPLIED SCIENCE

BIOCHEMICAL TESTS

- Group of qualitative tests, used to differentiate bacterial genera/species.
- **Biochemical tests classified as:**
  1. Enzymatic tests
  2. Metabolic pathways tests
     a) Carbohydrate oxidation and fermentation tests
     b) Amino acids degradation tests
     c) Single substrate utilization tests
  3. Antibiotic inhibition tests
  4. Specific tests

ENZYMATIC TESTS

Enzymatic tests includes:

1. Catalase test
2. Coagulase test
3. Oxidase test
4. PYRase test
5. DNase test
6. Hippurate test
7. Indole test
8. Urease test
9. Nitrate reduction test
10. ONPG (β-galactosidase) test

CATALASE TEST

- **Catalase:** is an enzyme used which is produced by aerobic microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites H_2O_2.

- **Principle:**

$$2H_2O_2 \xrightarrow{\text{Catalase}} \underbrace{2H_2O + O_2 \uparrow}_{\text{Bubbles}}$$

- **There are two forms of test:**

1. Slide catalase test
2. Tube catalase test

TOOLS, CONSUMABLES AND REAGENTS

Wooden stick

Glass slide

3% H_2O_2

Glass tube

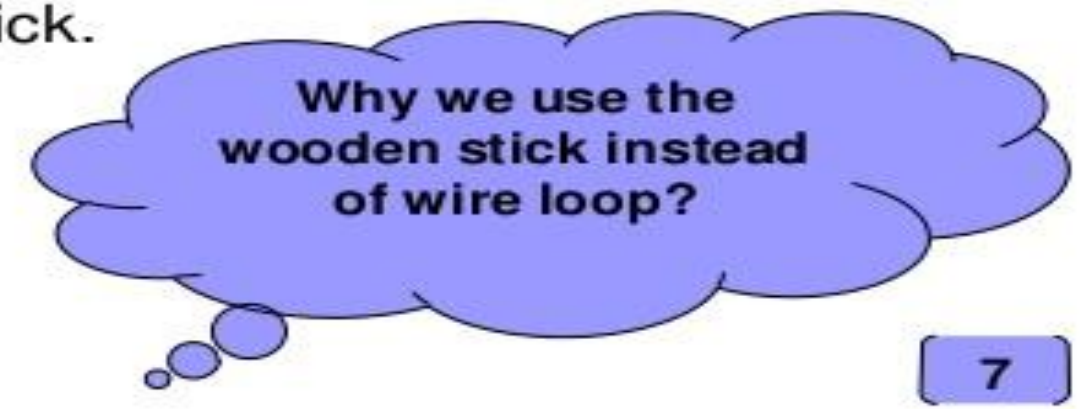
Bacteria to be tested

PROCEDURE (slide method)

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% H_2O_2 on to the slide and mix.
3. A **positive** result is the rapid evolution of oxygen (within 5-10 seconds) as evidenced by bubbling.
4. A **negative** result is no bubbles or only a few scattered bubbles.

PROCEDURE (tube method)

1. Add 4 to 5 drops of 3% H_2O_2 to in a test tube.
2. Using a wooden applicator stick, collect a small amount of organism from a well isolated 18- to 24-hour colony and place into the test tube **(Note: Be careful not to pick up any agar, especially if using Blood Agar).**
3. Place the tube against a dark background and observe for immediate bubble formation (O_2 + water = bubbles) at the end of the wooden applicator stick.



RESULTS

The results:

- If the bubbles appear, the test is positive (+ve).
- If there is no bubbles, the test is negative (–ve).

PRECAUTIONS AND NOTES

- The test organisms should not be taken from blood agar culture. Red Blood cells contain catalase and their presence will give a false positive test.
- Culture should be 18 to 24 hours old.
- Hydrogen peroxide must be fresh as it is very unstable.
- Iron wire loop should not be used, iron containing loops will cause false positive test results if exposed to hydrogen peroxide.
- Some bacteria produce a peroxidase that catalyzes a breakdown of hydrogen peroxide causing the reaction to be weakly positive; (a few bubbles elaborated slowly). This should not be confused with a truly positive reaction.

COAGULASE TEST

- **Coagulase:** is an enzyme produced by *Staphylococcus aureus* that converts (soluble) fibrinogen in plasma to (insoluble) fibrin. *S. aureus* produces two forms of coagulase, bound and free.

- **Principle:**

$$\text{Fibrinogen}_{(\text{soluble})} \xrightarrow{\text{Coagulase}} \text{Fibrin}_{(\text{insoluble})}$$

- **Forms of test:**

1. Slide coagulase test: to detect bound coagulase (clumping factor)
2. Tube coagulase test: to detect free coagulase

TOOLS, CONSUMABLES AND REAGENTS

Wire loop

Glass slide

Rabbit plasma

Glass tube

Bacteria to be tested

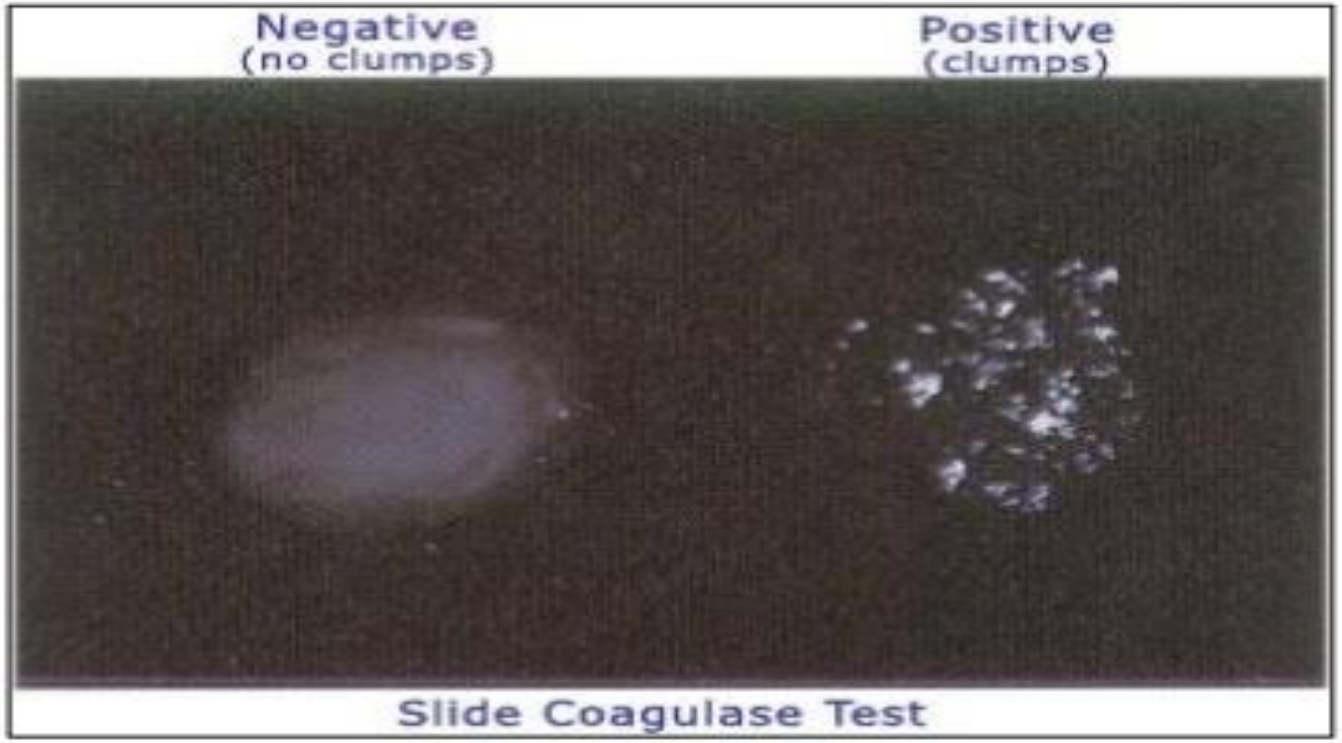
PROCEDURE (slide method)

1. Suspend a staphylococcal colony in a drop of water on a clean and glass slide.
2. Make similar suspensions of control positive and negative strains to confirm the proper reactivity of the plasma.
3. Dip a flamed and cooled inoculating wire into the undiluted plasma at room temperature, withdraw, and stir the adhering traces of plasma (not a loopful) into the staphylococcal suspension on the slide. Flame the wire and repeat for the control suspensions.
4. Read as **positive** a coarse clumping of cocci visible to the naked eye within 10 seconds.
5. Read as **negative** the absence of clumping or any reaction taking more than 10 seconds to develop, *but re-examine any slow reacting strains by the tube coagulase test.*

PROCEDURE (tube method)

1. Prepare a 1 in 6 dilution of the plasma in saline (0.85% NaCl) and place 1 mL volumes of the diluted plasma in small tubes.
2. Emulsify several isolated colonies of test organism in 1 mL of diluted rabbit plasma to give a milky suspension.
3. Incubate tube at 35 °C in ambient air or in water bath for 4 hours.
4. Examine at 1, 2 and 4 hour for clot formation by tilting the tube through 90°. **(Clots may liquefy after their formation)**
5. Leave negative tubes at room temperature overnight and re-examine. (This step is essential, for some strains of *S. aureus*, including many MRSA, produce a delayed clot which is rapidly lysed at 37 °C by the organism's staphylokinase.

RESULTS



The results:

- **If the a clot appears (at any degree), the test is positive (+ve).**
- **If there is no clot, the test is negative (–ve).**

NOTES

- Rabbit plasma is preferable, as it gives better clotting, is free from inhibitors and is safe.
- Human plasma contains sodium citrate as anticoagulant, and some citrate utilizing bacteria such as *Enterococcus faecalis* can destroy the anticoagulant and cause clotting.
- False positive or false negative results can occur if the plasma is not sterile.

OXIDASE TEST

- **Oxidase:** an enzyme of the bacterial electron transport chain.
- When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product.
- When the enzyme is absent, the reagent remains reduced and is colorless.

- **Principle:**

2 oxidized cytochrome C + p-aminodimethylalanine oxalate ⟶ **2 reduced cytochrome C + Wurster's blue (purple color)**

TOOLS, CONSUMABLES AND REAGENTS

Wooden stick

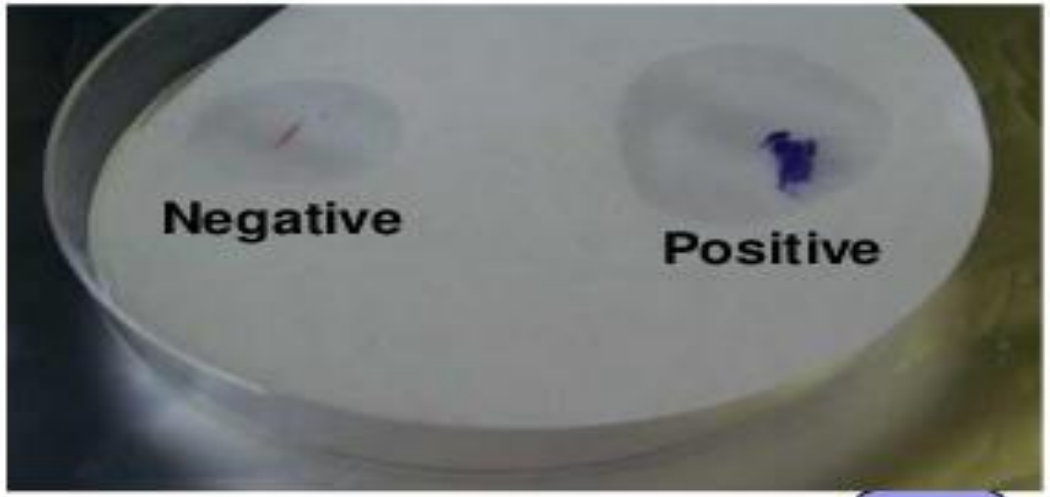
Filter paper

Oxidase reagent

Bacteria to be tested

PROCEDURE

1. Moisten the paper with an oxidase reagent.
2. Pick the colony to be tested with wooden or platinum loop and smear on the filter paper.
3. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.
4. **Positive:** deep purple or blue
5. **Negative:** no change



NOTES

- Do not use Nickel-base alloy wires containing chromium and iron (nichrome) to pick the colony and make smear as this may give false positive results.
- Interpret the results within 10 seconds, timing is critical.
- **The oxidase test must be performed from 5% Sheep blood agar or another medium without a fermentable sugar.**
- Fermentation of a carbohydrate results in acidification of the medium (e.g., lactose in MacConkey Agar or Sucrose in TCBS), and a false negative oxidase test may result if the surrounding pH is below 5.1.

PYRASE TEST

- Used for the detection of **pyrolidonyl arylamidase** (also called pyrrolidonyl aminopeptidase) activity in *Streptococcus pyogenes*, *Enterococcus* spp., some coagulase-negative staphylococci, and some Enterobacteriaceae.
- It is also known as PYR (L-pyrrolidonyl-β-naphthylamide) which serve as a substrate for the detection of pyrrolidonyl peptidase.
- **Principle:**

β–naphthylamide + p-dimethylaminocinnamaldehyde $\xrightarrow{\text{PYRase}}$ **Pink color**

TOOLS, CONSUMABLES AND REAGENTS

Wire loop

PYR disc

PYR broth

Bacteria to be tested

PROCEDURE (broth method)

1. Inoculate PYR broth with 3-5 colonies from 18-24 hours pure culture.
2. Incubate the tube aerobically at 35-37 °C for 4 hours.
3. Add 2-3 drop of PYR reagent and observe for color change.
4. Observe for the red color development within 1-2 minutes.

PROCEDURE (disc method)

1. Wet the PYR test disc on the strip with 10 µL sterile distilled water or deionized water, **(Note: Do not flood the disk).**
2. Put 5-10 colonies of the tested strain from 18-24 hours culture on the surface of the disc with a loop and smear them lightly on it.
3. Incubate the disc for 1-2 minutes at room temperature.
4. After incubation, add 1 drop of N, N-dimethylamino-cinnamaldehyde.
5. Observe for red color development within 1-2 minutes.

RESULTS

LIMITATION OF TEST

1. **A false-negative test can result if the disk or filter paper are too moist.**
2. **False-negative tests can result if selective media or tube biochemical agars are used to provide inocula.**
3. The *Escherichia coli* and indole-positive *Proteus* obtained from media containing a high tryptophan content may yield a blue-green color development. This is a negative result.
4. **Some less commonly encountered isolates of lactococci and aerococci may be PYRase positive.**
5. **Non-specific colour reactions may occur if results are read after 20 seconds.**

DNASE TEST

- Used to determine the ability of an organism to hydrolyze DNA and utilize it as a source of carbon and energy for growth.
- **DNase agar, a differential medium is used to test the ability of an organism to produce deoxyribonuclease or DNase.**
- This medium is pale green in color because of DNA-methyl green (indicator) complex (Note: Methyl green is a cation which binds to the negatively-charged DNA). It also contains nutrients for the bacteria.
- If the organism that grows in the medium produces deoxyribonuclease, it breaks down DNA into smaller fragments. When the DNA is broken down, it no longer binds to the methyl green, and green color fades and the colony is surrounded by a colorless zone.

TOOLS, CONSUMABLES AND REAGENTS

Wire loop

Flask

Petri-dish

DNase agar base

Graduated cylinder

Bacteria to be tested

Bunsen burner

Cotton

1N HCl

PROCEDURE

1. Prepare DNase agar.

If the DNase agar with indicator:

2. Inoculate the test agar medium: There are two types of inoculation that can be done.

   - **Spot Inoculation**
     - Touch a colony of the organism under test with a loop and inoculate it onto a small area of the DNase test agar plate, in the middle of one of the marked sections to form a thick plaque of growth 5-10 mm in diameter after incubation.
     - Incubate the plate at 37 °C for 18-24hr.
   - **Band or line streak inoculation**
     - Use a heavy inoculum and draw a line 3-4 cm long from the rim to the centre of the DNase test agar plate
     - Incubate the plate at 37 °C for 18-24hr.

PROCEDURE

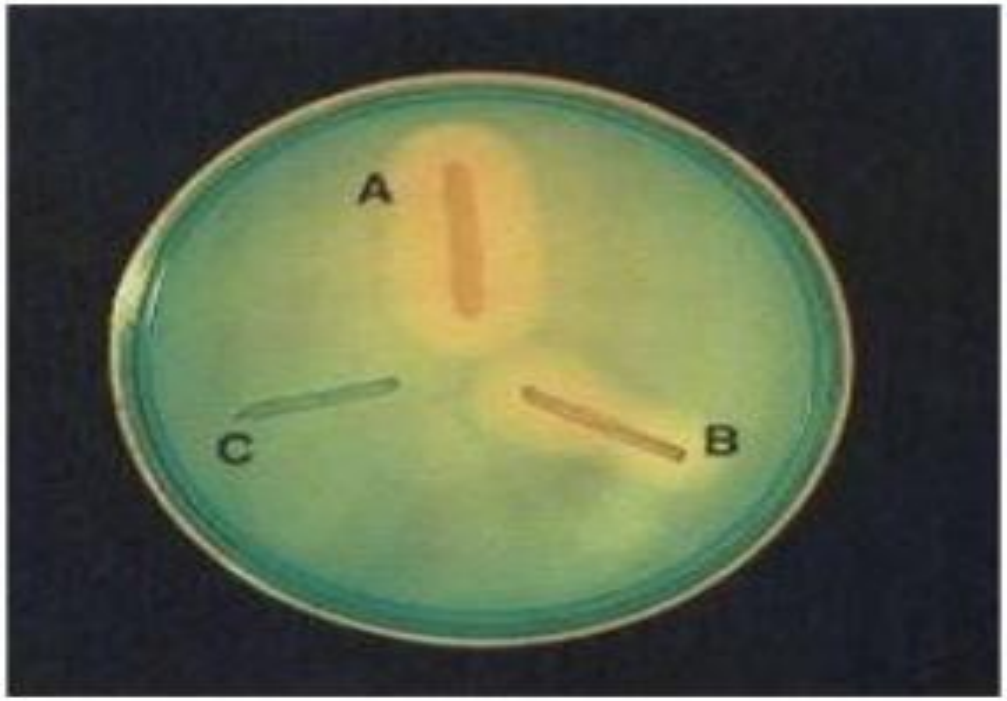
1. Prepare DNase agar.

If DNase agar without indicator:

2. Flood the plate with 1N Hydrochloric (HCl) acid.
3. Leave the plate to stand for a few minutes to allow the reagent to absorb into the plate.
4. Decant excess hydrochloric acid and then examine the plate within 5 minutes against a dark background.

RESULTS

RESULTS



DNA Hydrolysis (DNase) test:

A. Positive; *Staphylococcus aureus*

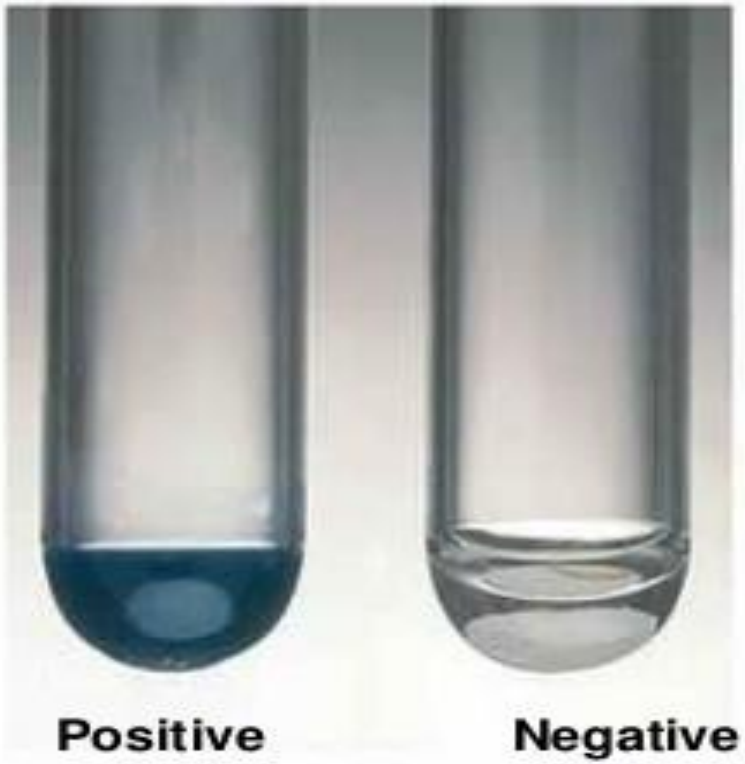
B. Positive; *Serratia marcescens*

C. Negative: *Staphylococcus epidermidis*

HIPPURATE TEST

- Hippuric acid is hydrolyzed to benzoic acid and glycine by the enzymatic action of **hippuricase**.

- The glycine end product is detected by the addition of ninhydrin reagent.



TOOLS, CONSUMABLES AND REAGENTS

Wire loop

Ferric chloride

Hippurate broth

Ninhydrin indicator solution

Distilled water

Bacteria to be tested

PROCEDURE (classical method)

1. Take or prepare Sterile Sodium Hippurate broth and inoculate with test organism.
2. Incubate overnight at 35 °C.
3. Centrifuge the broth and remove the precipitate.
4. Add Ferric chloride reagent in the supernatant.
5. If the precipitate remains after 10 minutes, benzoic acid is present and the test is **positive** for hippurate hydrolysis.

PROCEDURE (Ninhydrin method)

1. Add 0.2 mL of distilled water in the test tube.
2. Make a heavy suspension of the organism in the Hippurate reagent with a standard inoculating loop.
3. Incubate the tube for two hours at 35-37 °C.
4. During the incubation period, reconstitute the Ninhydrin indicator solution in the dropper bottle by adding 2 mL of distilled water. Replace the cap tip and cap, and vigorously shake for one minute. Let stand at room temperature for 30 minutes or until all the substrate has dissolved.
5. After the two hour incubation period, add two drops of the Ninhydrin indicator solution to the hippurate reagent and organism mixture.
6. Re-incubate at 35-37 °C for 30 minutes. Observe the tubes at 10 minute intervals for the appearance of a deep blue color, which is a positive test. The color change will usually appear in 10 to 15 minutes after the Ninhydrin indicator solution has been added.

INDOLE TEST

- Used to determine the ability of an organism to split amino acid tryptophan to form the compound indole.
- Tryptophan is hydrolysed by **tryptophanase** to produce three possible end products – one of which is indole.
- Indole production is detected by **Kovac's** or **Ehrlich's reagent** which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red colored compound.
- **Principle:**

TOOLS, CONSUMABLES AND REAGENTS

Wire loop

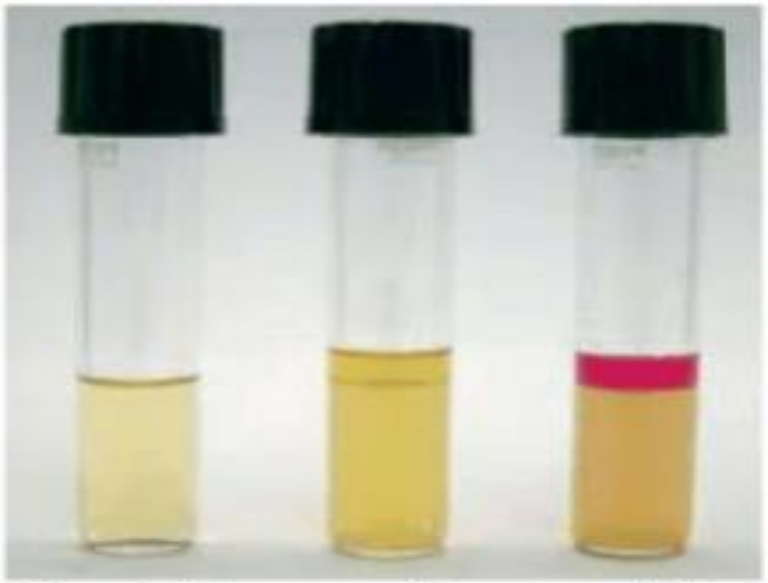
Kovac's Reagent

Tryptophan broth

Bacteria to be tested

PROCEDURE

1. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.
2. Incubate at 37 °C for 24-28 hours in ambient air.
3. Add 0.5 mL of Kovac's reagent to the broth culture.



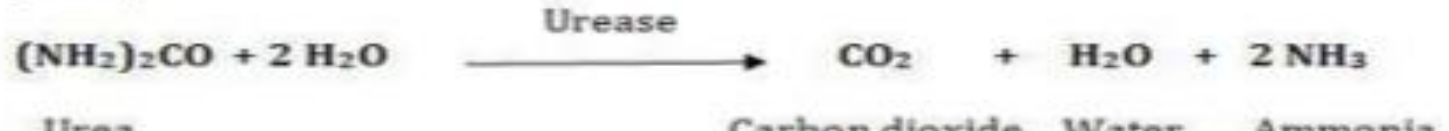
Control negative positive

Results:

- **Positive:** Pink ring after addition of Kovac's reagent
- **Negative:** No color change after addition of Kovac's reagent.

UREASE TEST

- Urea is a diamide of carbonic acid, it is hydrolyzed with the release of ammonia and carbon dioxide.
- Many organisms especially those that infect the urinary tract, have an **urease** enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide.
- The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.
- **Principle:**



TOOLS, CONSUMABLES AND REAGENTS

Wire loop

Bacteria to be tested

Urea broth (urea agar may be used)

PROCEDURE

1. The broth medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.
2. Leave the cap on loosely and incubate the test tube at 35 °C in ambient air for 18 to 24 hours; unless specified for longer incubation.

- If the color is changed from light orange to magenta, the test is **positive**.
- If the color isn't change, the test is **negative.**

NITRATE REDUCTION TEST

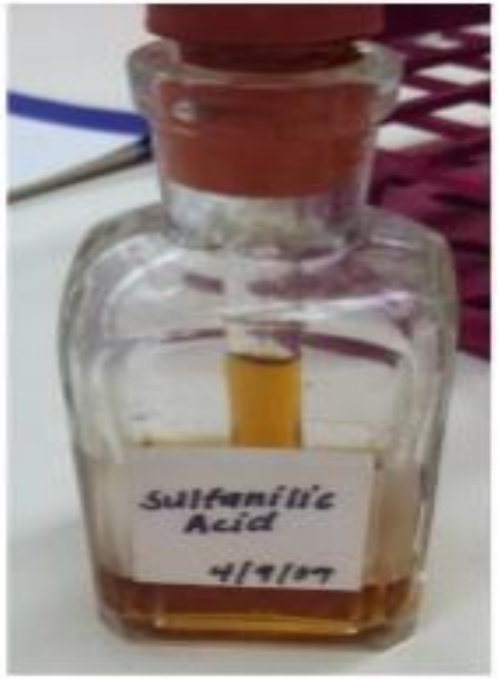
- Used for the differentiation of members of Enterobacteriaceae on the basis of their ability to produce **nitrate reductase** enzyme that hydrolyze nitrate (NO_3^-) to nitrite (NO_2^-) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3) depending on the enzyme system of the organism and the atmosphere in which it is growing.
- **Principle:**

$$NO_3^- \xrightarrow{\text{Nitrate reductase}} NO_2^- \text{ (red color)}$$

TOOLS, CONSUMABLES AND REAGENTS

Wire loop

Bacteria to be tested



sulphanalic acid

α-naphthylamine

Nitrate broth

PROCEDURE

1. Inoculate nitrate broth with a test organism using aseptic technique.
2. Incubate at an appropriate temperature for 24 to 48 hours.
3. Add one dropperfull of sulphanalic acid and one dropperfull of a α-naphthylamine to each broth.
   A. At this point, a color change to **RED** indicates a **POSITIVE** nitrate reduction test. If you get a red color, then you can stop at this point.
   B. No color change indicates the absence of nitrite. This can happen either because nitrate was not reduced or because nitrate was reduced to nitrite, then nitrite was further reduced to some other molecule. If you DO NOT get a red color, then you must proceed to the next step.

PROCEDURE

4. Add a small amount of zinc (a toothpick full) to each broth. Zinc catalyzes the reduction of nitrate to nitrite.

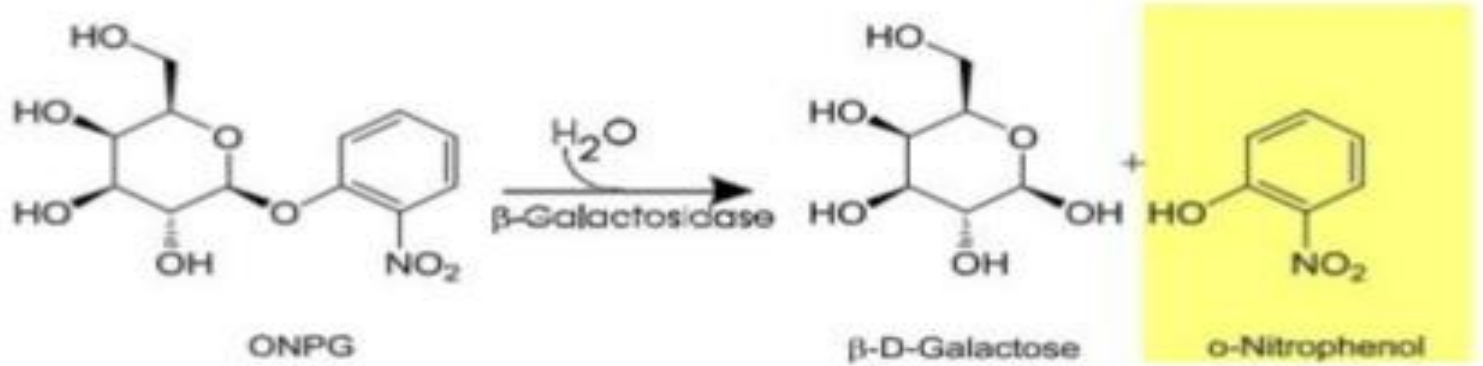
   A. At this point, a color change to RED indicates a NEGATIVE nitrate reduction test because this means that nitrate must have been present and must have been reduced to form nitrite.

   B. No color change means that no nitrate was present. Thus no color change at this point is a **POSITIVE** result.

Positive Negative

ONPG TEST

- O-Nitrophenyl-β-D-galactopyranoside (ONPG) is structurally similar to lactose (i.e. ONPG is an analog of lactose), except that orthonitrophenyl has been substituted for glucose.
- On hydrolysis, through the action of the enzyme **β-galactosidase**, ONPG cleaves into two residues, galactose and O-nitrophenol.
- ONPG is colorless compound, O-nitrophenol is yellow, providing visual evidence of hydrolysis.



TOOLS, CONSUMABLES AND REAGENTS

Wire loop

ONPG tablets

Normal saline

ONPG solution

Bacteria to be tested

PROCEDURE

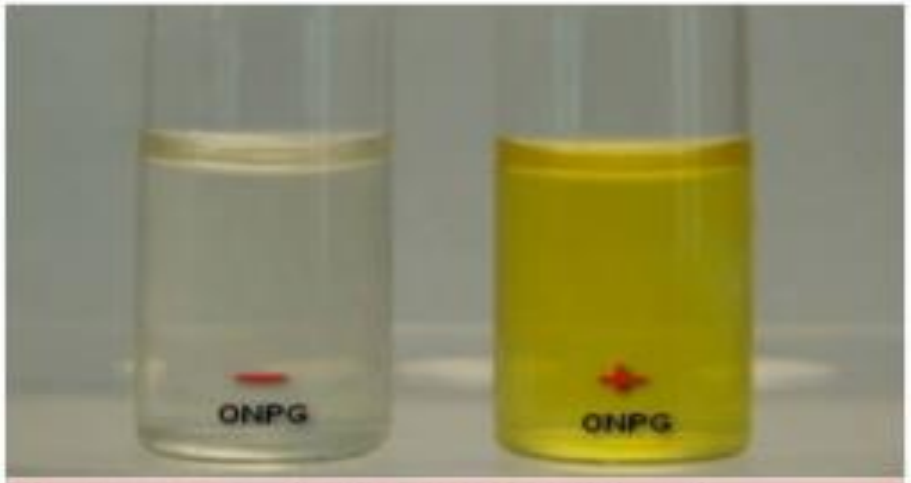
- Bacteria grown in medium containing lactose (to induce the production of the galactosidase enzyme), such as Kligler iron agar (KIA) or Triple sugar Iron (TSI) agar, produces optimal results in ONPG Test. (Note: β-galactosidase enzyme (inducible enzyme) is made ONLY in the presence of the lactose substrate)

1. A loopful of bacterial growth is emulsified in 0.05 mL of physiologic saline to produce a heavy suspension
2. One drop of toluene is added to the suspension and vigorously mixed for a few seconds to release the enzyme for bacterial cells.
3. An equal quantity of buffered ONPG solution is added to the suspension.
4. The mixture is placed in a 37 ºC water bath

PROCEDURE

- **When using ONPG tablets:**

1. A loopful of bacterial suspension is added directly to the ONPG substrate resulting from adding 1 mL of distilled water to a tablet in a test tube.
2. This suspension is also placed in a 37 °C water bath



Proteus vulgaris (Left): ONPG Negative
Escherichia coli (Right): ONPG Positive