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**Review Article**

# A REVIEW ON COMMONLY USED BIOCHEMICAL TEST FOR BACTERIA

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**ABSTRACT**

Bacteria are meant to be omnipresent and are mostly involved in lots of human microbial infections. Such bacterial infections can be identified by the different properties of this microorganism. Bacteria are bearing several inherent properties. by using these properties we can differentiate, can check there presence and absence, can check their gram negative and gram positive nature and many more. The present review is therefore focused to combine different biochemical test in one article.

**Keywords:** Microorganism, Bacteria, omnipresent, biochemical test.

## INTRODUCTION

Different biochemicals tests are are performed to check and differentiate the bacteria. Different biochemical test has been mentioned below-

1. IMViC,
2. Sulphate reduction test.
3. Catalase production test
4. Urease test
5. Fermentation of carbohydrates
6. Starch hydrolysis test
7. Casein hydrolysis
8. Gelatin liquefaction test
9. Oxidae test
10. Microbial assay of antibiotics.

### IMViC-

This test is important for coliform group of bacteria. The coliform group of bacteria includes all the aerobic and facultatively aerobic bacteria, gram-negative nonsporulating bacilli that produce acid and gas from the fermentation of lactose. The classical species of this group are *Escherichia coli* and *Enterobacter* aerogene. The relationship of these organisms to others of the enteric group is with salmonella, shigella, Klebsiella, proteus, Serratia and other genera which are Gram negative[p]. The IMViC name stands for the first letter of each test in the series with the lower 'i' for case for pronunciation the IMViC test is designated to differentiate *E.coli* from *Enterobacter aerogenes*[1].

As far back as 1889, the indole test was used as a means to distinguish between *Escherichia coli*and *Enterobacter aerogenes* [2]. The numerous variations of the indole test alone and in combination with other biochemical tests attest to the central role this test has played in the characterization of coliforms (gram-negative nonsporulating bacilli that ferment lactose, producing acid and gas)[3]. The indole test is still used as a classic test to distinguish . Indole – positive E coli/from enterobactor and klebsiella[4]. The methyl red test (MR) was developed by Clark and Lubes in 1915 who rather optimistically laid that simple condition have been found under which metabolism can be so controlled that the hydrogen ion concentration of culture of one group can be made to diverge widely from those of the other group.

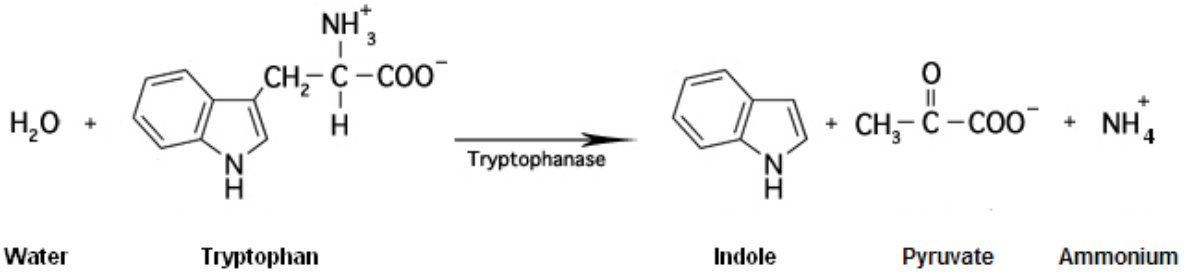
### INDOL TEST

#### Purpose

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobacteriaceae.

#### Principle

Tryptophan is an essential amino acid, which is oxidized by some bacteria resulting in the formation of indole, pynivic acid and ammonia. The indole test is done by inoculating the test organism into tryptophan broth, which contain tryptophan. The indole which is produced is detected by adding KOVAC's reagent which produced cherry red colored ring.



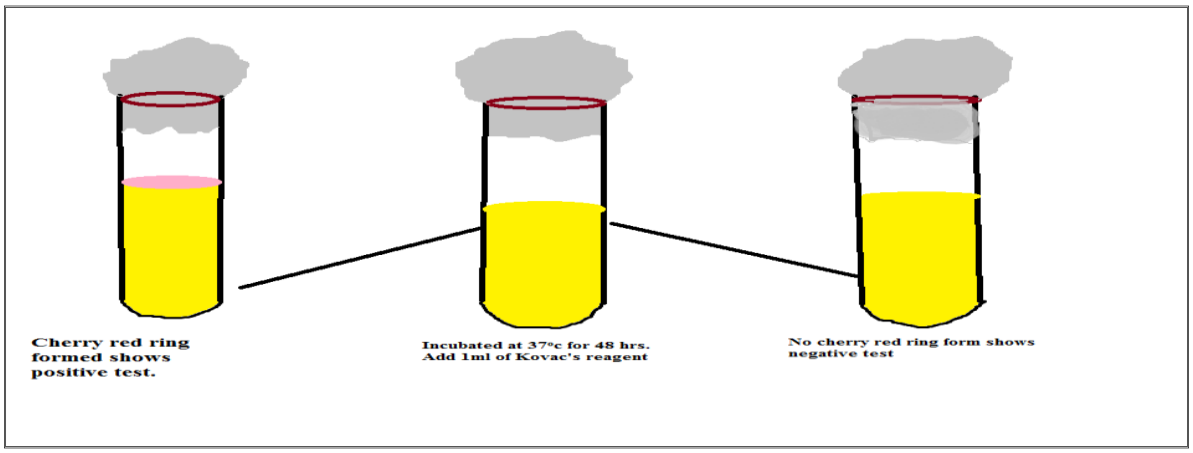



**Figure.1: Shows Methyl red test**

Dissolve the ingredients in 1 liter of sterile water. Dispense 4 ml per tube. Cap tube and autoclave at 121°C under 15 psi pressure for 15 minutes. Store the tubes in the refrigerator at 4 to 10°C.

**Ingredients of KOVACS reagent[5-6].**

| Ingredient | Amount |
|---|---|
| Amyl or isoamyl,reagent grade (Butyl alcohol may be substituted. | 150.0ml |
| P-dimethylaminobenzaldehyde (DMAB) | 10.0ml |
| HCL (concentrated) | 50.0ml |

Dissolve DMAB in the alcohol. Gentle heating might be required to get the aldehyde into solution. Slowly add the acid to the aldehyde-alcohol mixture. The solution should be a pale yellow color and is only stable for a short time. Store the mixture in a brown glass bottle in the refrigerator.

**Procedure to perform the test-**

Trypton broth was prepared according to the given composition. The broth was dispensed into the tubes and sterilized. The test organisms were inoculated into the tubes and one was left uninoculated as control. The tubes were inoculated at 37°c for 48h After incubation 1ml of KOVAC's reagent was added to all the tubes including control. The tubes were shaken gently and allowed to stand for 1-2 min. The tubes were observed for formation of cherry red ring.

Alternate Methods of Detecting Indole Production Tryptophan peptone broth

**Tryptophan peptone broth [7-8]**

| Ingredients | Amount |
|---|---|
| Casein peptone | 10.0 g |
| Sodium chloride | 5.0 g |
| Tryptophan | 100 g |

The tryptophan concentration can be varied; the amount used here will result in a 1% final concentration. Dissolve ingredients in 1 liter of distilled water. Dispense 4 ml per tube. Cap tube and autoclave at 121°C under 15 psi pressure for 15 minutes.Store tubes in the refrigerator at 4 to 10°C. Inoculate, incubate, and perform test as described above for tryptone broth.

**Sulfide-indole-motility (SIM) medium**[7-9] The SIM medium is a multitest agar used to test for indole production while simultaneously determining other characteristics of the Bacterium .

**SIM medium**

| Ingredients | Amount |
|---|---|
| Peptone | 30.0 g |
| Beef extract | 3.0 g |
| | 0.2 g |
| Ferrous ammonium sulfate | |
| Sodium thiosulfate | 0.025 g |
| Agar | 3 g |

**MIO medium**

| Ingredients | Amount |
|---|---|
| Yeast extract | 3.0 g |
| Peptone | 10.0 g |
| Tryptone | 10.0 g |
| L-ornithine HCl | 5.0 g |
| Dextrose | 1.0 g |
| Agar | 2.0 g |
| Bromcresol purple | 0.02 g |

Dissolve ingredients, except agar, in 1 liter of distilled water. Adjust pH to 7.3. Add agar and heat mixture to boiling to dissolve agar. Cool to 50°C. Dispense in 4.0 to 5.0 ml aliquots in 16-mm test tubes. Cap tubes and autoclave at 121°C under 15 psi pressure for 15 minutes. After autoclaving, allow tubes to cool in an upright position to form the agar deep. Tubes can be stored at 4 to 8°C for several months. SIM medium is commercially available both as a premixed powder and as premade deep tubes. To inoculate SIM medium, pick an isolated colony with a needle. Stab the needle approximately two-thirds of the way into the deep and then remove it following the same path as the entry. Incubate at 35°C (+/-2°C) for 24 to 48 hours or until growth is evident. To test for the presence of indole, a by-product of tryptophan metabolism, 5 drops of Kovács reagent should be added to the top of the deep. A positive indole test is indicated by the formation of a red color in the reagent layer on top of the agar deep within seconds of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.See Indole Atlas images—Alternative Methods for Determining Indole Production for SIM result images.

**Motility-indole-ornithine (MIO) medium**[5, 7] The MIO medium is a multitest agar used to test for indole production while simultaneously determining other characteristics of the bacterium (see Comments and Tips section).

Bring to 1 liter with distilled water. Heat mixture to boiling to

dissolve agar. Cool to 50°C. Dispense in 4.0 to 5.0 ml aliquots in 16-mm test tubes. Cap tubes and autoclave at 121°C under 15 psi pressure for 15 minutes. After autoclaving, allow tubes to cool in an upright position to form the agar deep. Tubes can be stored at 4 to 8°C for several months. MIO medium is commercially available both

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**Ehrlich's reagent**[9]

Ehrlich's reagent, an alternative to Kovács reagent, also contains DMAB, which reacts with indole to produce a red product. The Ehrlich formulation is more sensitive but contains additional toxic or flammable solvents; it is recommended when testing bacterial groups that produce little indole such as nonfermentative bacilli or anaerobes. Kovács reagent is apparently more stable and the absence of the additional organic extraction (required with Ehrlich's) makes Kovács formulation more suitable for undergraduate laboratories.

**Ehrlich's reagent**

| Ingredients | Amount |
|---|---|
| Ethyl alcohol (absolute) | 95.0 ml |
| *p*-dimethylaminobenzaldehyde (DMAB) | 1.0 g |
| HCl (concentrated) | 20.0 ml |

**METHYL RED TEST [10-12]**

**Theory-**The test measures the final acidity of a culture in buffered medium containing glucose and peptose, but the pH value recorded at the time of the test is not necessarily the lowest value attained during growth. In the early stage of glucose fermentation sufficient acid is produced by both *E.coli* and *Klebsiella aurogens* to turn methyl red indicator orange and red (+ve) but on further incubation *Klebsiella aurogens* breaks down the Pyruvic acid and other acids and produces a reversion in the reaction of the culture towards neutrality; methyl red turns yellow (-ve).Timing of the sequence

Starting pH (c.7) → Low pH (c.5) → higher pH (6 or more)

Will be affected by the medium, particularly the buffering power of the peptone and the phosphate by the temperature and duration of incubation, and by the strain. It is surprising therefore, that a greater attempt has not been made to standardize the test.

Some bacteria perform mixed-acid fermentation. The by-products are mixtures of large amounts of stable acids. Other fermentative organisms produce smaller amounts of less stable acids.

The Methyl-Red test tests for the ability to perform mixed-acid fermentation. MR-VP broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. Organisms that perform other kinds of fermentation cannot overcome the buffering capacity of the broth.

After incubation, the pH indicator Methyl Red is added to the broth. Methyl Red is red at pH below 4.4 (this would be a positive result) and yellow at pH above 6.0. An orange color indicates an intermediate pH and would be considered a negative result.

This test is among a suite of tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate) that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae.

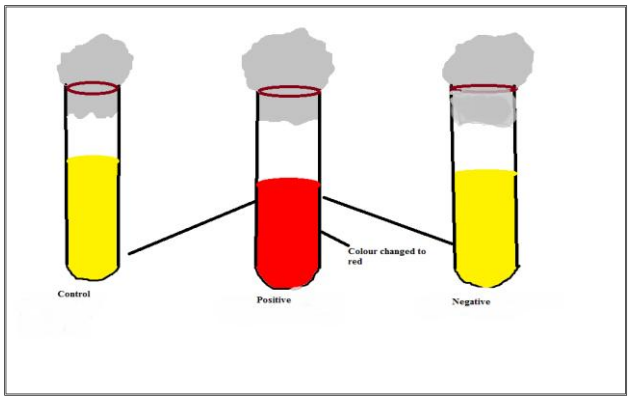
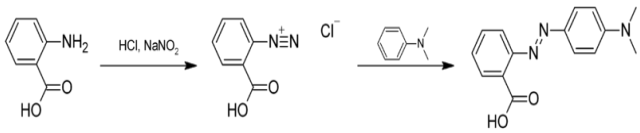



**Figure.2: Shows methyl red test**

Results




**VOGES-PROSKAUER TEST**[13-15]

**Purpose**

**Voges–Proskauer** or **VP** is a test used to detect acetoin in a bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which has been inoculated with bacteria. A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result.

The test depends on the digestion of glucose to acetylmethylcarbinol. If glucose is being broken down, it will react with alpha-naphthol (VP reagent #1) and potassium hydroxide (VP reagent #2) to form a red color. Alpha-naphthol and potassium hydroxide are chemicals that detect acetoin.

Procedure: First, add the alpha-naphthol; then, add the potassium hydroxide. A reversal in the order of the reagents being added may result in a weak-positive or false-negative reaction.

VP positive organisms include *Enterobacter*, *Klebsiella*, *Serratia marcescens*, *Hafnia alvei*, *Vibrio damsela*, and *Vibrio alginolyticus*.

**History-** The reaction was developed by Daniel Wilhelm Otto Voges and Bernhard Proskauer — German bacteriologist in 1898 at the Institute of infectious Disease.

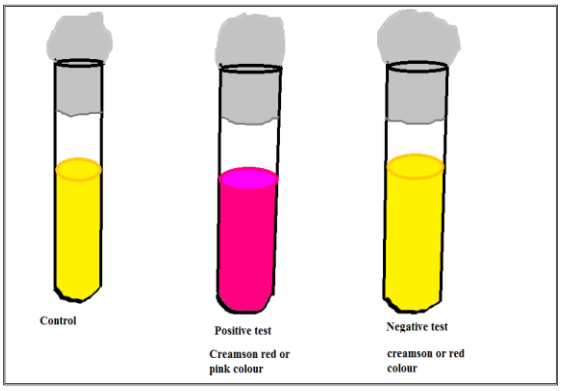



**Figure.3: Shows Voges Proskauer test**

**CITRATE UTILIZATION TEST [17-18]**

**Purpose**-The test is used to check the ability of microorganisms for utilization of citrate.

**Principle**-Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize as their soul carbon source. The utilization of citrate depends upon the presence of enzyme "*Citrate Permease*" produced by organism that helps its transports into the cell.

The citrate test is performed by inoculating microorganism into in an organic synthetic media, "Simons Citrate broth" when sodium citrate is only source of Carbon and energy.Bromothymol blue is used as an indicator when the citric acid is metabolized ,carbondioxide is generated which combines withsodium and water to form sodium carbonate which is an alkaline product which is responsible for change in colour from green to blue and this constitute positive test.

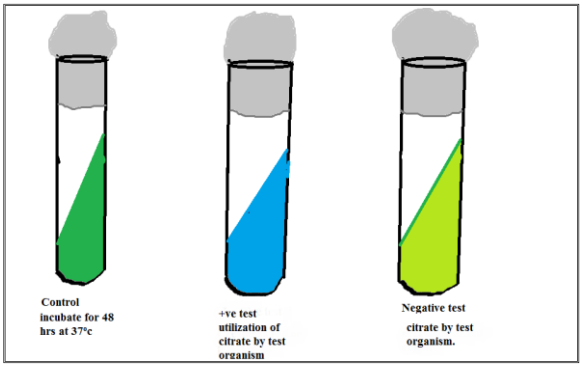



**Figure 4: Shows Citrate utilization test.**

**Procedure-**Prepare the citrate slant according to the given composition and dispensed into the the tes tube and autoclaved at 121°c for 15lbs.inocciulate the tubes with the given culture and then inoculate at 37°c for 48 hours. Observe the tubes for colour change from green to blue.

**SULPHATE REDUCTIOIN** TEST[19]**-**

**Purpose-** The test is done to show the presence of hydrogen sulphide by sulphide reducing bacteria.

Principle- $H_2S$ is formed by some bacteria by reduction of sulphure containing amino acids (Cystein), Cysteine and metheonine or through reduction of inorganic sulphure compounds like thiosulphates ($S_2O_3^-$)or sulphates ($SO_4^{--}$) or sulphite ($SO_3^{--}$ ).The $H_2S$ production can be detected by incorporating a heavy metal salt containing ion ($Fe^{++}$) or lead ($Pb^{++}$) ion as a $H_2S$ indicator to neutrient culture medium containing cystein and sodiumthiosulphate as the sulphure substance.$H_2S$ a colourless gas when produced react with metal salts ($FeSO_4$) forming visible insoluble black Ferrous Sulphide precipitates. Production of $H_2S$ from systein and $Na_2S_2O_3$.

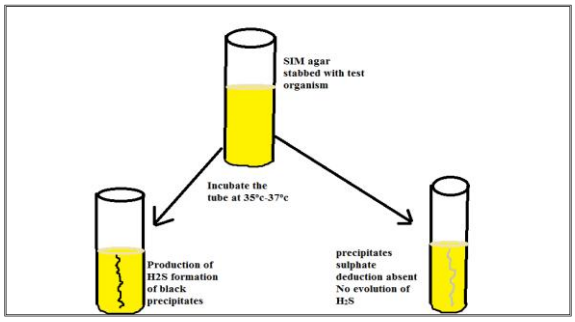



**Figure.5: Shows Sulphate reduction test**

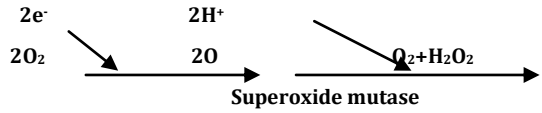
**Procedure-** Prepare the sulphate agar according to given composition . sterilize the Sulphate agar by autoclaving at 121oc at 15 lbs pressure for 20 minutes. Dispense the sterilized agar into sterilized test tubes and labeled with respective organism. Stab inocculate the test organism after solidification of the medium and incubate the tubes at 37°c for 24 hrs and observed for black coloration at the point of stab.

**CATALASE PRODUCTION TEST**[20]

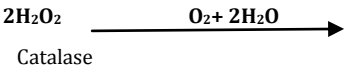
**Purpose-** The test is done to check weather the test organism produce Catalase or not.

**Principle-**The oxidation of flavoproteins invariably result in the formation of Hydrogen peroxide as one major product. In addition this oxidation (and other oxygenation) produce small quantities of and even more toxic radical-The superoxide

In aerobes and aerotolerant aerobes the potentially leathal accumulation of oxygen is prevented by the enzyme superoxide dismutase which catalysis it to hydrogen peroxide and oxygen.




$H_2O_2$ is produced ois lethal to the cells and Catalase breaks it to water and oxygen .




In this test the organism is subjected to 3% H2O2 solution and Catalase enzyme acts on it just as it could $H_2O_2$ is produced inside the cell. Catalase lies close to the cell membrane.A positive result is detected by the formation of air bubbles.

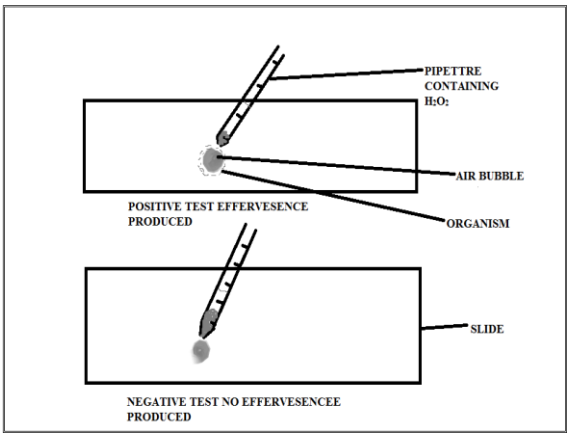


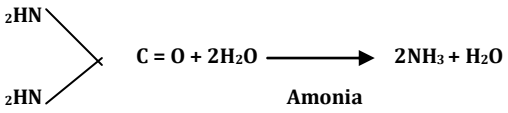

**Figure.6: Shows Catalase production test**

**Procedure-** Take a drop of reagent on a clean slide and transfer bacterial colonies into it. Observe the effervescence formation.

**UREASE TEST**[21-22]**-**

**Purpose-**the test is done if the given organism produces enzyme Urease or not.

**Pinciple-Urea** is a major organic waste production of protein digestion by most vertebrates and is excreted in urine.Some microorganisms have the ability to produce the enzyme urease. Urease is a hydrolytic enzyme, which attack the amide linkage liberating Ammonia.



This test distinguishes members of genus *Proteus* from other lactose non fermenting enteric microbes. Urease test is performed by growing test organisms on urea broth or agar medium containing $P^H$ 6.8 indicator phenol red. During incubation microorganism processing urease will produce ammonia that raises the $P^H$ of the medium ($P^H$ 8.1 or >)

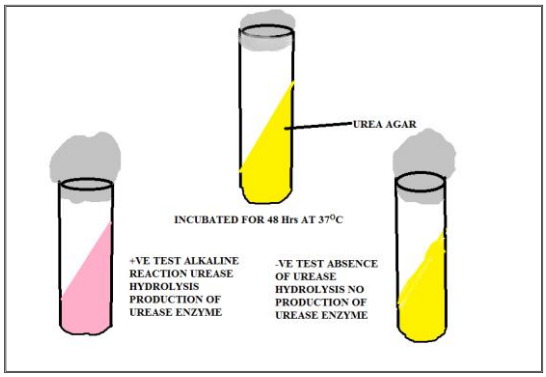



**Figure.7**: **Showing Urease test**

**Procedure-** Prepare and dispense Urea agar ( basal medium) into tubes and sterilized.Glucose and phenol red is added to the basal medium and steamed for 1 Hr.Add filtered sterilized urea solution and mix all contents well and dispense into sterile test tubes.The test organisms are inoculated and incubated at 37°c for 24-48 Hrs. Colour change is observed.

**FERMENTATION OF CARBOHYDRATE[23]:**

**Purpose-** This test I performed to check the ability of bacteria to check its ability to ferment sugar.

**Introduction**-: Morphological and cultural characteristics are essential in identifying an organism in the case of bacteria. Physiological information (through physiological reactions) in an organism contribute informations for specific identification as all the biochemical reactions are catalysed by enzyme and enzyme specific to species the bio-oxidation reaction could be oxidative or fermentative oxidizing organic substance s for energy result in the production of $CO_2$ and water it is oxidative reaction.Aldehydes or alcohols produced with various type of gases such as $CO_2$,$H_2$,$CH_{4s}$ called fermentive reactions.Fermentation of sugars can be easily demonstrated and the end products of particular fermentation depends on the nature of the organism characteristics of substrate and environmental conditions such as temperature and $P^H$.

**Principle**- Fermentation can be defined as an energy yielding process that does not involve an electron transport chain and exogenous terminal electrons acceptors instead it relies on substrate level. Phasphorylation and an endogenously generated electron acceptor.

Fermentation of sugar is carried out anaerobically with the indicator production incompletely oxidized acidic or alkaline and products with gas production, to detect fermentation in labs. A fermentation tube is used with a Durham's tube for the detection of gas as the end product of carbohydrate metabolism.

Fermentation broth contains a nutrient broth with specific carbohydrates may be altered during autoclaving by heat in the pressure of other ingredients so they are sterilized separately. Peptone water base is also autoclaved separately at 121°c. for 15 minutes.

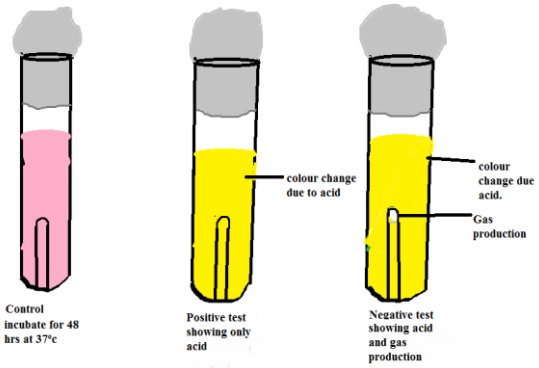



**Figure.8: Shows fermentation of carbohydrates**

**Procedure-**

- The fermentation medium is prepared and sterilized with the indicator and Durham's tube has no air bubbles in them.
- The sugar solution is autoclaved at 10 lbs/Sq inch pressure for 10 minutes and 0.5 ml of the sugar is added to sterile peptone water.
- The fermentation tubes are inoculated with the test organism.
- Negative control is maintained for all the sugars.
- The tubes are inoculated at 37°c for 24 hrs.

**STARCH HYDROLYSIS [24]-**

**Purpose-**This test is performed to test the utilization of starch by Bacteria by producing the enzyme Amylase.

**Principle:** Amylase is an exoenzyme that hydrolysis starch, a polysaccharides into monosaccharide and disaccharides such as glucose.these mono and disaccharide enters into cell cytoplasm of bacteria through the semi-permeable membrane and their by attacked by endoenzymes.Starch is a complex carbohydrate composed of glucose molecules linked together by α-1,4 and α-1,6 glycosidic bonds.

Amylase production is known in some bacteria while well known known in some fungi.

The ability to degrade starch is used as the criteria for determination of amylase production by a microbe. In laboratory it is tested by performing the agar test to determine the absence and presence of iodine produces a dark blue coloration and a yellow zone around the colony in an otherwise blue media indicates amylolytic activity.

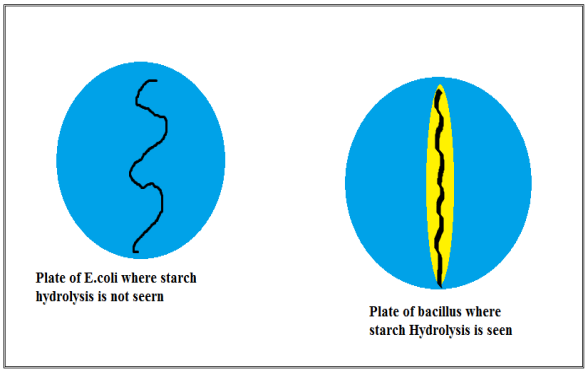



**Figure.9: Shows starch hydrolysis**

**Procedure**

- Sterile starch agar medium is poured on to the sterile petriplates and allowed to solidify.
- The test organism is streaked on the plate and incubated for 48 hrs. at 37°c.
- The plates are flooded with gram's iodine and excess iodine is drained off.
- Plate are examined for the zone of clearance around the growth for each organism.

**CASEIN HYDROLYSIS [25-26]**

**Purpose-** This test is done to check the ability of microorganism to hydrolyse casein .

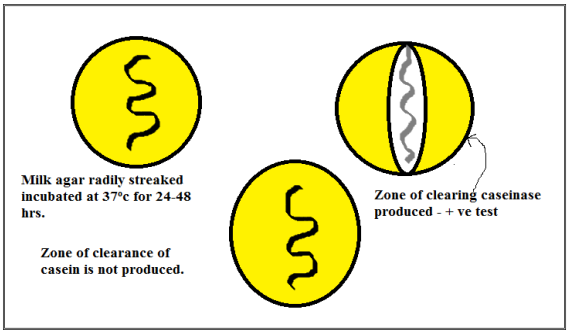



**Figure 10. Shows casein hydrolysis**

**Principle-**Casein is a major protein found in milk. It is a macromolecule composed of amino acid linked together by peptide bond. Some microorganism have the ability to decrease the protein casein by producing proteolytic exo-enzymes called protease. The process breake down the peptide bond by introducing water into the molecule liberating the soluble amino acid pool for use in the synthesis of structural and functional cellular protein.

Casein hydrolysis can be demonstrated by supplementing nutrient agar media with milk .The medium is opaque due to the casein is colloidal suspension formation of a clear zone surrounded the bacterial growth. after inoculation and incubation of agar plate culture is due to hydrolysis of casein by protease activity. The medium surrounding the growth of the organism remains opaque since it defects light rays rather than transmitting which indicates a negative reaction.

**Procedure**

1. Prepared the Casein media.
2. Sterilize the media by autoclaving.
3. Then dispense the media in sterilized petriplates and after solidification streake the test organism at the centre of the plate.
4. incubate the plate at 37°c for 24 hrs.
5. After incubation the plates are observed for hollow and clear zone around the colonies.

**GELATIN LIQUIFICATION TEST [27-33]**

**Purpose-**The test is performed to check the ability of the microorganism to produce the enzyme gelatinase.

**Principle-**Gelatin is a protein derived form collagen,which is insoluble in cold water but insoluble in cold water but soluble in hot water and form gel on cooling it. gelatin is liquid at room temperature that solidify on cooling up to the temperature of -4°c for bacteriological use and edible grade of gelatin is used since it is free of preservatives and inhibitory amount of heavy metals.

The proteolytic organisms digest protein and may liquefy gelatin. the liquification of gelatin is index of proteolytic activity of the organism produce the enzyme gelatinase.

**Procedure**

- Prepare the media according to the given composition and sterilize after pouring into the test tubes.
- Inoculate the test organism into the sterilized test tubes and left one uninocculated as control .
- Incubate the tube at 37°c for 24-48 hrs.
- After incubation kept the tubes in an ice bath for 30 min.
- note down the results depending on the condition wheather gelatin is in the liquid state or in the solid form

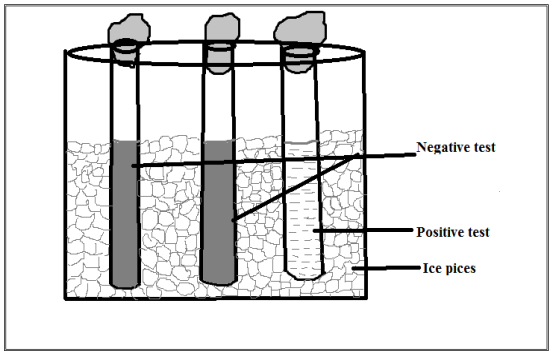



**Figure.11: Showing Gelatin liquification test**

**OXIDASE TEST-**

**Purpose-**The test is done to detect the presence of cytochrome C and hence the production of oxidase enzyme by given test organism.

**Principle-**Cytochrome are heam containing catalytic enzyme which are tightly bound to (prokaryotic cells) cells of plasma membrane.they are concerned with later stages of biochemical oxidation.They act as electron or $H_2$ carries of biological oxidation by virtue of their availability valence by heam ions i.e., they can undergo reduction and oxidation . During period of great activity they are reduced. Cytochrome C is more abundant and freely soluble in water. Cytochrome C does not react directly with $O_2$ but reduced form will be oxidized by cytooxidase with which it is closely associated. Cyto $a,a_3$ is called cytooxidase. or indophenolase or endophenol oxidase or ferro cytochrome C or oxygen oxido reductase.

Cyt. $a.a_3$ is the terminal codon in electron transport chain hence called Cytooxidase.This test tests for lytic and not cytooxidase.

In this test an oxidisable amine namely tetramethyl-p-phenylene diamine dihydrochloride is used to detect cytochrome C as wel as oxidase enzyme on flooding of bacterial cell with reagent, a purple colour is formed due to oxidation of reagent. Eletrones are transferred to cytochrome C and from cytochrome c to molecular oxygen via cytochrome $a,a_3$ .The enzyme catalyses the transfer of electrons from reagent to molecular oxygen.

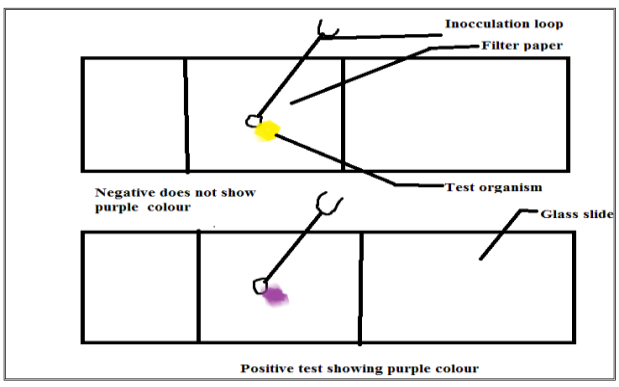



**Figure. 12 : Showing Oxidase test**

**PROCEDURE**

- Place 1-2 drops of 1 % oxidase reagent on 6 cm square piece of whatman filter paper.
- Transferr a small colony of test organism using a loop onto soaked filter paper and observe for purple colour development.
- A positive test is indicated by development of purple colour in 5-10 seconds.

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