LAB:4 **Practical Pathogenic bacteria**

Bacterial diagnosis (Gram negative bacteria)

1- IMViC Test

I= Indole ring production

M= Methyl red

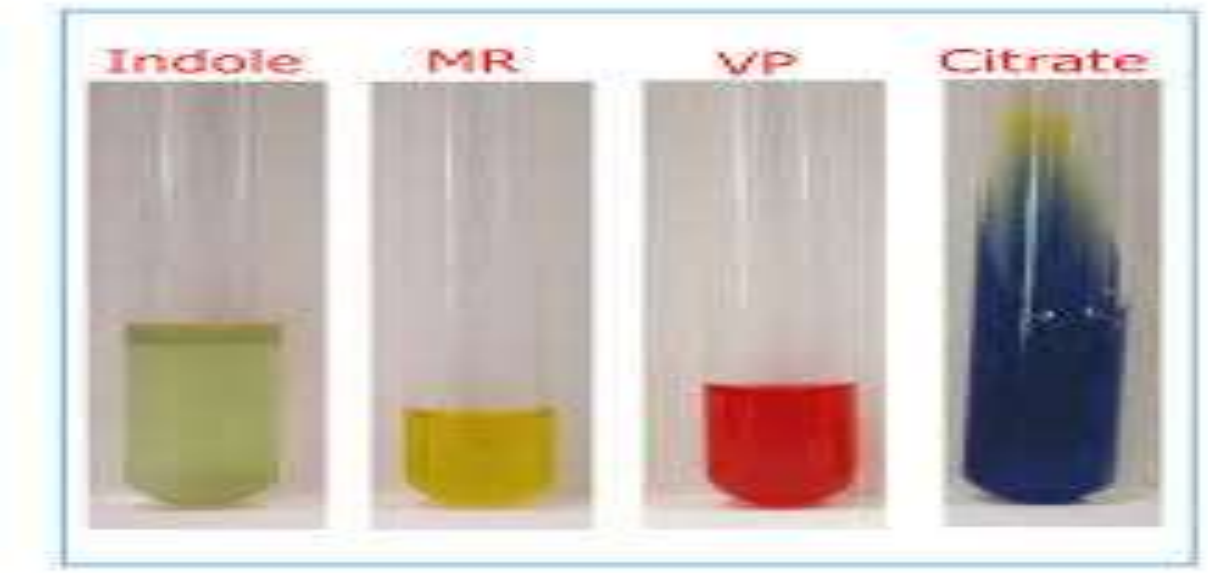
V=Voges Proskauer

C=Citrate utilization

The IMViC tests are a group of individual tests used in microbiology lab testing to identify an organism in the coliform group. A coliform is a gram negative, aerobic, or facultative anaerobic rod, which produces gas from lactose within 48 hours. The presence of some coliforms indicate fecal contamination.

The term "IMViC" is an acronym for each of these tests. "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test. The lower case "i" is merely for "in" as the Citrate test requires coliform samples to be placed "in Citrate".

These tests are useful in distinguishing members of Enterobacteriaceae.



IMViC series = Klebsiella & Enterobacter

Indole test

In this test, the organism under consideration is grown in peptone water broth. It contains tryptophan, which under the action of enzyme Tryptophanase is converted to an Indole molecule, pyruvate and ammonium. The indole is then extracted from the broth by means of xylene. The broth is sterilized for 15 minutes at around 121°c. To test the broth for indole production, Kovac's reagent . Kovac's reagent consist of amyl alcohol and para-dimethylaminobenzaldehyde and concentrated hydrochloric acid. Kovac's reagent is actually used to determine ability of an organism to separate indole from amino acid tryptophan and it is added after incubation. A positive result is indicated by a pink/red layer forming on top of the liquid.

Indole test Method:

Inoculate Tryptone water with the tested microorganism Incubate at 37°C for 24 hours After incubation interval, add 1 ml Kovacs reagent, shake the tube gently and read immediately

- **Kovac's reagent**

Dissolve 10g of p-dimethylaminobenzalaldehyde in 150 ml of amyl, isoamyl or butyl alcohol. Heat it in a 56◦C water bath until dissolved. Cool. Slowly add 50 ml of conc. HCL. Store it in a glass-stoppered brown bottle in the refrigerator. This reagent should be light yellow in color.

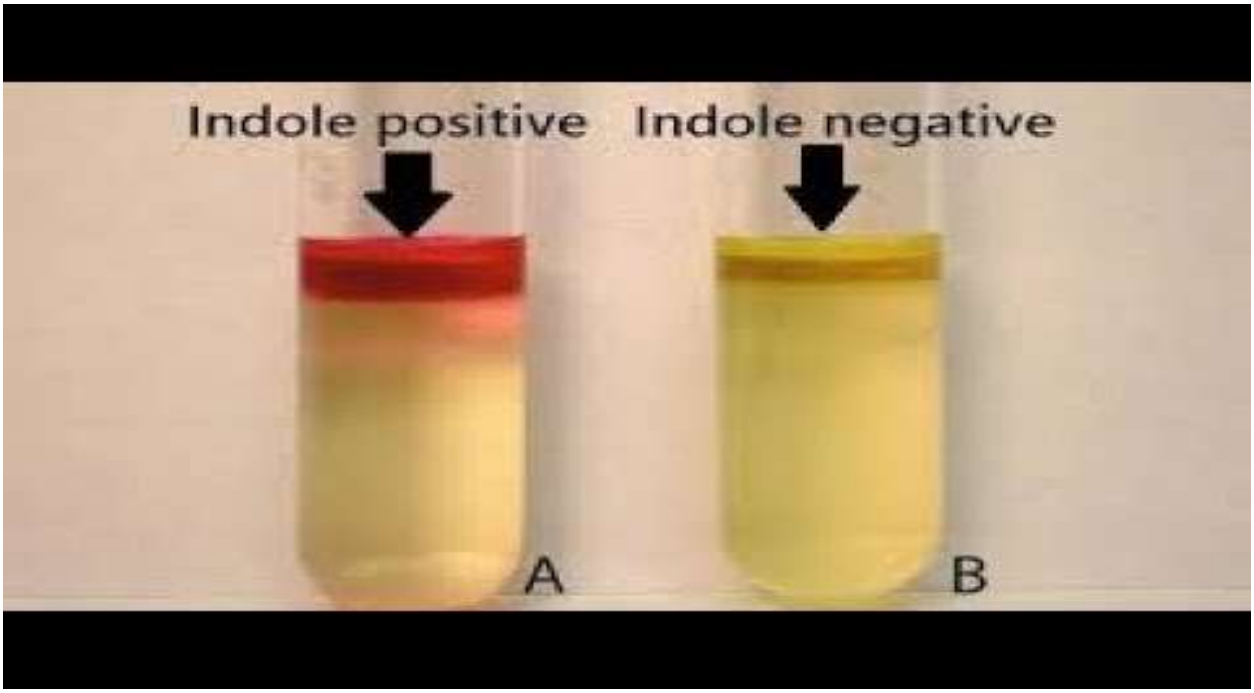
- **Ehrlich's reagent**

Dissolve 1 g of p-dimethylaminobenzalaldehyde in 95 ml of 95% ethyl alcohol. Slowly add 20 ml of conc. HCL acid. Store in a glass stoppered brown bottle in the refrigerator. This reagent should be light yellow in color.

Indole test Negative test e.g. Klebsiella Positive test e.g. E. coli

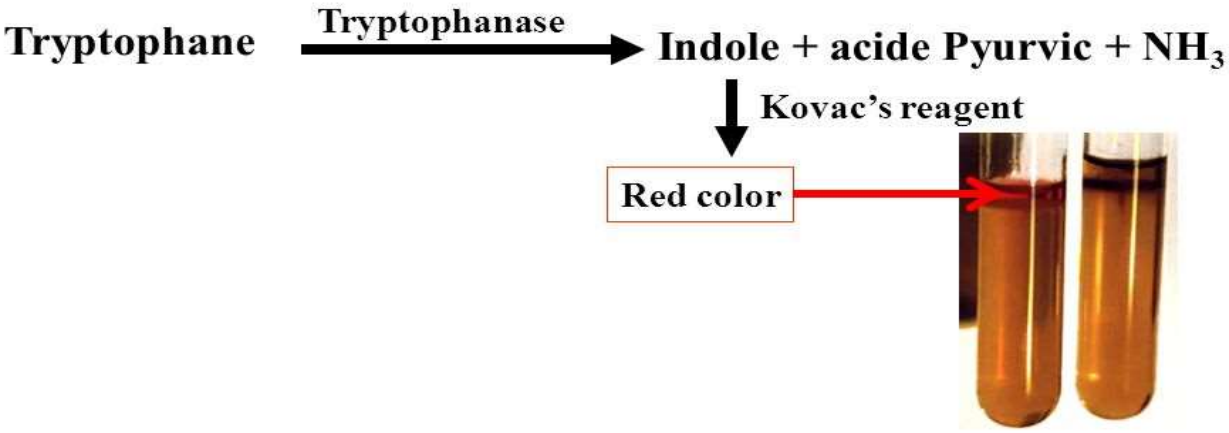
Result: A bright pink color in the top layer indicates the presence of indole the absence of color means that indole was not produced i.e. indole is negative Special Features: Used in the differentiation of genera and species. e.g. E. coli (+) from Klebsiella (-).

- **Indole test**
- **Media culture :peptone water**
- **Reagent ;kovacs**
- **Enzymes: Tryptophanase**
- **Substrate :Tryptophan**
- **Positive result: red ring**
- **Negative result :no change (yellow ring)**



IMViC: Indole Test

- Principal
  - Some microorganisms can metabolize tryptophane by the tryptophanase



Methyl red and Voges–Proskauer test

These tests both use the same broth for bacterial growth. The broth is called MR-VP broth. After growth, the broth is separated into two different tubes, one for the methyl red (MR) test and one for the Voges-Proskauer (VP) test.

The methyl red test detects production of acids formed during metabolism using mixed acid fermentation pathway using pyruvate as a substrate. The pH indicator Methyl Red is added to one tube and a red color appears at pH's lower than 4.2, indicating a positive test (mixed acid fermentation is

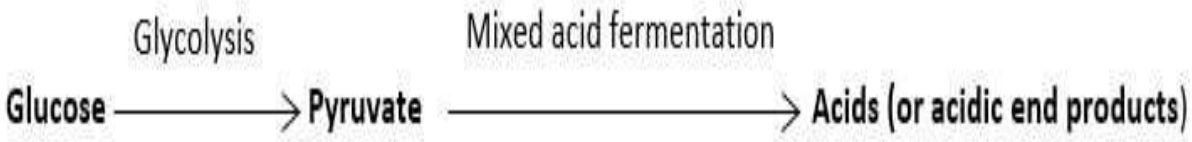
used). The solution remaining yellow (pH = 6.2 or above) indicates a negative test, meaning the butanediol fermentation is used.

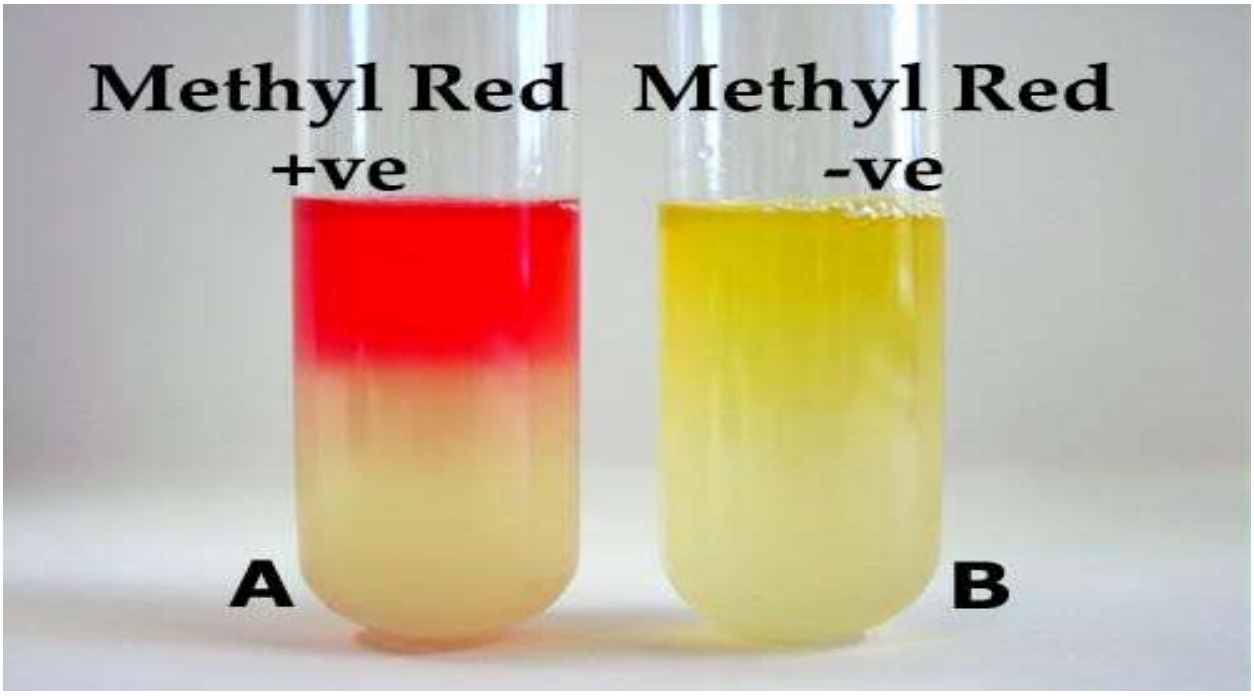
The VP test uses alpha-naphthol and potassium hydroxide to test for the presence of acetylmethylcarbinol (acetoin), an intermediate of the 2,3-butanediol fermentation pathway. After adding both reagents, the tube is shaken vigorously then allowed to sit for 5-10 minutes. A pinkish-red color indicates a positive test, meaning the 2,3-butanediol fermentation pathway is used.

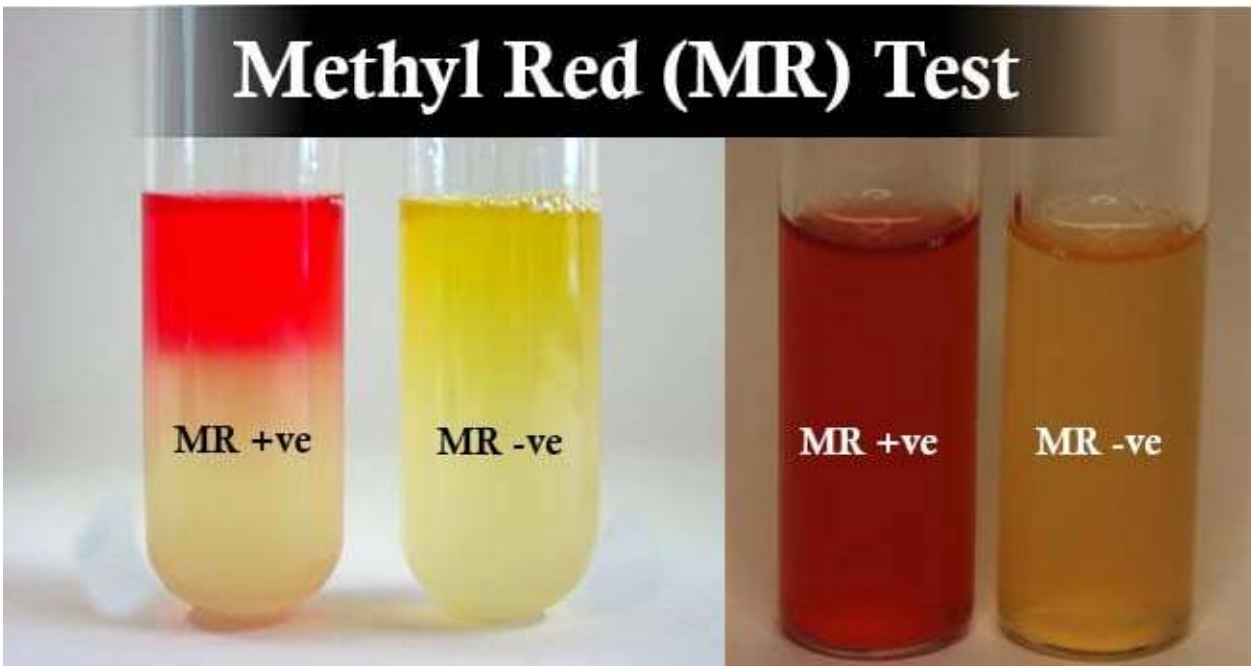
Principle of Methyl Red Test

The principle of the test is based on the ability of bacteria to produce organic acids during glucose fermentation, which lowers the pH of the medium.

In the methyl red test, a pH indicator called methyl red is added to the culture medium. Methyl red is yellow at a pH above 6.0, but it turns red at a pH below 4.4. After inoculating the bacteria into the medium, they ferment glucose and produce organic acids. If the bacteria produce enough acid to lower the pH of the medium below 4.4, the methyl red indicator will turn red.




Methyl Red
+ve
Methyl Red
-ve
A
B


Methyl Red (MR) Test
MR +ve
MR -ve
MR +ve
MR -ve

Requirements of MR Test

1. Sterile glucose phosphate broth **(GPB) medium** (0.5ml in each tube)
2. Glucose phosphate broth (GPB)
3. Test culture suspension
4. Methyl red indicator
5. Dissolve 0.1 g methyl red in **300 ml 95% ethanol**. Add distilled water to make up the volume to 500ml.

Procedure of MR Test

1. Inoculate the GPB medium with culture suspension.
2. Incubate at 37°C for 24 hours.
3. Add 5-6 drops of methyl red indicator.
4. Positive test is indicated by a bright red color of the medium.
5. A negative test indicated by the medium remaining yellow or turning orange.

Uses of MR Test

1. **Identification of bacteria: The methyl red test is used to differentiate between different types of bacteria based on their ability to produce stable acid end products during glucose fermentation. It is commonly used in the identification of enteric bacteria, such as Escherichia coli and Enterobacter aerogenes.**
2. **Quality control: The methyl red test is also used as a quality control measure for microbiological media. It is a simple and reliable method to ensure that the culture media is free from contamination and that the pH of the medium is within the acceptable range for growth of microorganisms.**
3. **Research: The methyl red test can be used in research to study bacterial metabolism and the pathways of glucose fermentation. It is a useful tool to investigate the effects of various treatments or mutations on bacterial metabolism.**

Principle of VP Test

Pyruvate can be metabolized into a neutral intermediate product called 'acetyl methyl carbinol', commonly called the 'acetoin' during the butanediol pathway of 2,3-butanediol production.

If acetoin is present in the media, it is oxidized readily to diacetyl in presence of air and KOH. Thus produced diacetyl, in the presence of ∝ – naphthol, will react with the guanidine component of peptone forming a pink to a red colored product.

Following the 48-hour aerobic incubation on MR-VP broth, VP reagents I and II are added and the color change is observed within 30 minutes. A positive result is indicated by the development of pink – red color at the top of the broth immediately or within 30 minutes but not more than 1 hour. No change in color represents a negative VP test.

VP Positive Bacteria:

Klebsiella spp., Enterobacter spp., Viridans Streptococci (except S. mitis, and S. vestibularis), Proteus mirabilis, Hafnia spp., Serratia spp., Staphylococcus aureus,

VP Negative Bacteria:

Escherichia spp., Proteus vulgaris, Citrobacter freundii.

Requirements VP Test

1. **Sterile glucose phosphate broth (GPB) medium (0.5-1.0ml in each tube).**
2. **Test culture suspension (Enterobacter / Klebsiella).**
3. **5%- α-naphthol in absolute ethanol.**
4. **40% KOH solution (containing 0.5% creatinine, if desired).**

Procedure of VP Test

1. **Inoculate GPB medium with the culture suspension.**
2. **Incubate at 37°C for 24 hours.**
3. **Add 0.6 ml of 5% α-naphthol and mix well.**
4. **Add 0.2 ml of 40% KOH solution, shake well.**
5. **Positive VP test is indicated by a red color of the medium, within 5 minutes. A negative VP test is indicated by the medium remaining brown.**

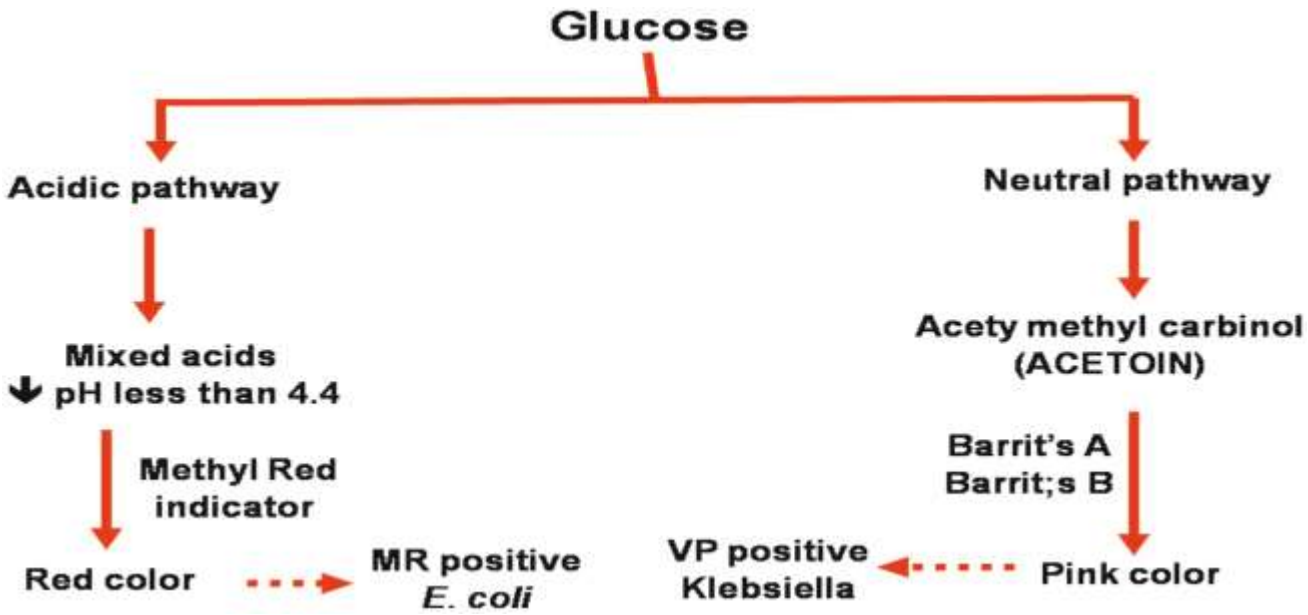
Uses of VP Test

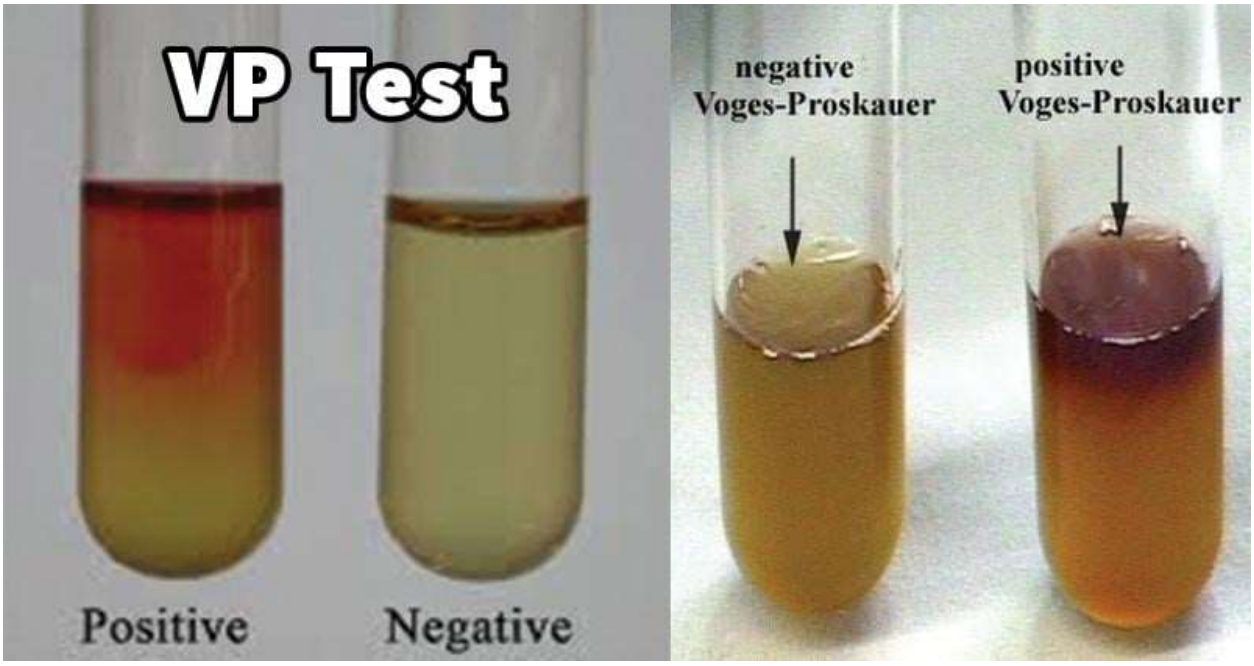
1. **The VP test is often used to** differentiate between members of the Enterobacteriaceae family**, which includes important human pathogens such as *Escherichia coli*, *Salmonella*, and *Shigella*. This test helps in the identification of these organisms in clinical settings.**
2. **The VP test is used in the production of fermented beverages**, **such as beer and wine, to determine the presence of acetoin-producing bacteria. It is also used in the food industry to detect certain spoilage organisms.**

3. **The VP test can be used to identify acetoin-producing bacteria in environmental samples, such as soil and water. This can be useful in monitoring the quality of water and soil for potential bacterial contamination.**
4. **The VP test is commonly used in microbiological research to investigate the metabolic pathways and physiology of bacteria. It is often used in conjunction with other tests to identify bacterial strains and study their characteristics.**

▶ Methyl red – Voges proskauer test

- ▶ **Media culture :Glucose phosphate**
- ▶ **Reagent : Methyl red & Barrits**
- ▶ **Enzymes:**
- ▶ **Substrate : Glucose**
- ▶ **Positive result :M=red color ..V= Red – pinkish color**
- ▶ **Negative result :yellow color**





Citrate test

In the 1930's, S.A. Koser conducted experiments that were used to study bacterial catabolism of organic acids. Koser found that citrate metabolism could be an indicator for bacteria found in natural environments. Additionally, citrate could be used to distinguish bacterial coilforms found in soil, and aquatic environments, such as *Enterobacteriaceae*, and coliforms with fecal contamination. It was found that coilforms without fecal contamination grew, while the coilforms with fecal contamination did not grow.

This test uses Simmon's citrate agar to determine the ability of a microorganism to use citrate as its sole carbon and energy source. The agar contains citrate and ammonium ions (nitrogen source) and Bromothymol blue (BTB) as a pH indicator. Bromothymol blue was added in order to reduce false positives. The citrate agar is green before inoculation, and turns blue, because of BTB as a positive test indicator, meaning citrate is utilized. The test is also prepared on a slant to maximize bacterial growth for an even better indication of the use of citrate.

Principle of Citrate Utilization Test

Some bacteria can utilize 'citrate' as their sole source of carbon. Such bacteria produce citrase enzymes which will break the citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid will then be decarboxylated to produce pyruvate and CO_2.

Released CO_2 will combine with H_2O and excess sodium from sodium citrate to produce alkaline 'sodium carbonate'. The sodium carbonate will increase the pH of the medium.

CO_2 + H_2O + excess sodium from sodium citrate → Na_2CO_3 (alkaline)

Additionally, the released CO_2 will trigger the metabolism of ammonium salts. Utilization of the ammonium salts as a source of nitrogen will cause the production of ammonia (or ammonium hydroxide).

Ammonium salt → Ammonium hydroxide (alkaline)

The combined effect of ammonium hydroxide and sodium carbonate will increase the pH of the media above 7.6. This increase in pH will turn the pH indicator bromothymol blue in the medium from deep forest green (at neutral pH) to Prussian blue.

Requirements of citrate utilization Test

1. **Test culture suspension.**
2. **Sterile Koser's citrate medium (1.0 ml in each tube) / Simmon's citrate medium (agar slant)**

Citrate Utilization Test Procedure

1. **Inoculate the medium (Koser's broth or Simmon's agar) with the culture suspension.**
2. **Incubate at 37°C for 24 hours.**
3. **Check for turbidity (indicating positive test) in Koser's medium; growth and change in colour of indicator to blue on Simmon's citrate agar (positive test).**

Citrate utilization test

Media culture: Simmon citrate agar

PH indicator: Bromothymol blue

Enzymes: Citrase

Substrate: Na-citrate

Positive result : Blue color

Negative result: NO growth

Fecal Coliforms

Indole positive

MR positive

VP negative

Citrate negative

Nonfecal Coliforms

Indole negative

MR negative

VP positive

These IMViC tests are useful for differentiating the family Enterobacteriaceae, especially when used alongside the Urease test.

The IMViC results of some important species are shown below.

Species	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	**Positive**	**Positive**	**Negative**	**Negative**
Staphylococcus aureus	**Negative**	**Positive**	**Positive**	**Negative**
Shigella spp.	**Negative**	**Positive**	**Negative**	**Negative**
Salmonella spp.	**Negative**	**Positive**	**Negative**	**Positive**
Klebsiella spp.	**Negative**	**Negative**	**Positive**	**Positive**
Proteus vulgaris	**Positive**	**Positive**	**Negative**	**Negative**
Proteus mirabilis	**Negative**	**Positive**	**Negative**	**Positive**
Citrobacter freundii	**Negative**	**Positive**	**Negative**	**Positive**
Enterobacter aerogenes	**Negative**	**Negative**	**Positive**	**Positive**

Differentiation of enteric bacteria by IMViC tests:

Genus	Indole	M.R.	V.P.	Citrate
Escherichia	+	+	-	-
Enterobacter	-	-	+	+
Klebsiella	-	-	+	+
Salmonella	-	+	-	+
Proteus	+	+	-	+

2- TSI agar (Triple Sugar Iron Agar) Test , Sugar fermentation , CO2 & H2S Pr0duction Test .

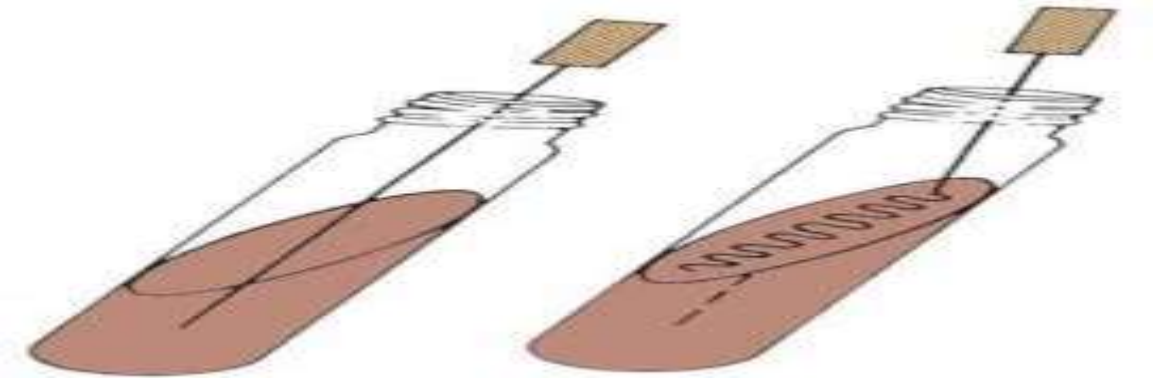
T= Triple , S= Sugar , I= Iron , A=Agar

Sugar = Glucose 0.1% , Lactose 1% , Sucrose 1%

PH indicator = Phenol red

Iron= Ferric Ammonium Citrate

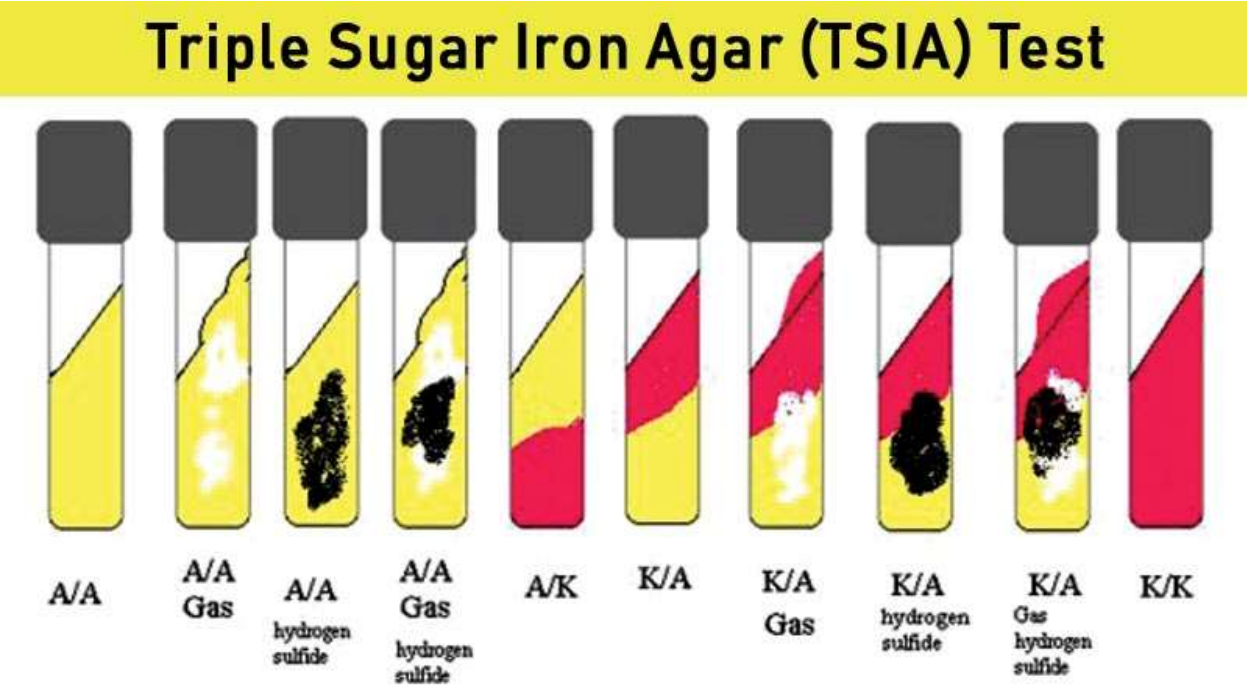
H2S indicator = Sodium Thiosulfate



Objectives of TSIA (Triple Sugar Iron Agar) Test

1- To determine whether a gram negative bacilli ferments glucose and lactose or sucrose and forms hydrogen sulfide (H2S).

2-To differentiate members of the Enterobacteriaceae family from other gram-negative rods.

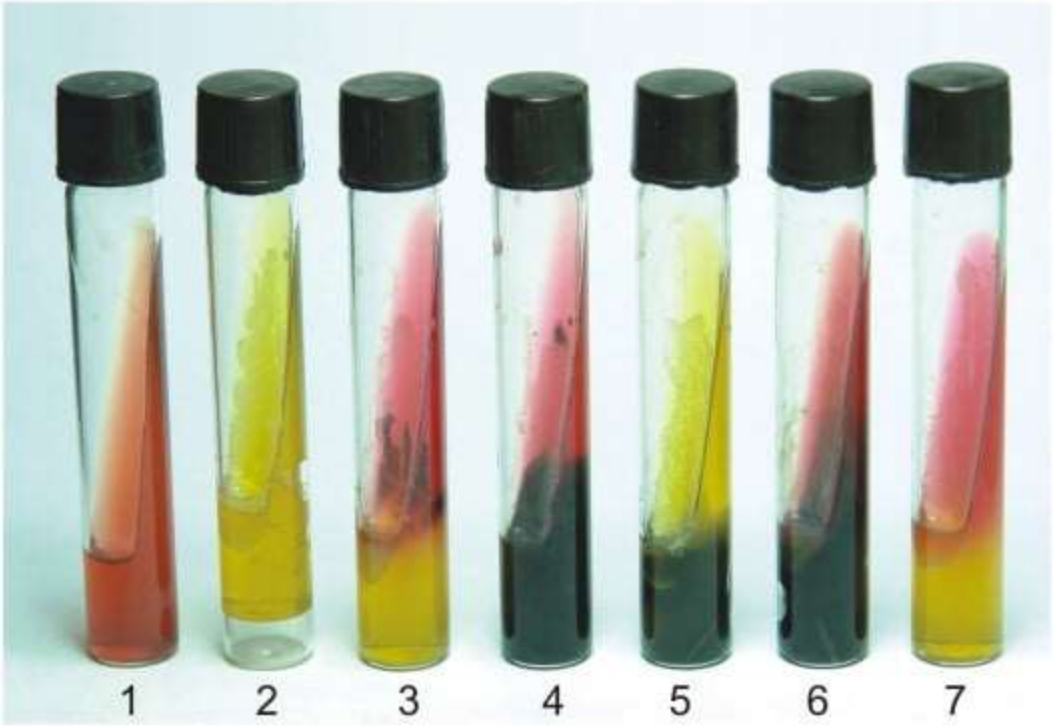


Principle of TSIA (Triple Sugar Iron Agar) Test

The Triple Sugar Iron agar (TSIA) test is designed to differentiate among the different groups or genera of the Enterobacteriaceae, which are all gram-negative bacilli capable of fermenting glucose with the production of acid and to distinguish them from other gram-negative intestinal bacilli. The differentiation is based on fermentation of glucose and lactose or sucrose and hydrogen sulfide (H2S) production. TSIA medium contains 10 parts of lactose: 10 parts of sucrose: 1 part of glucose and peptone. Phenol red and ferrous sulfate serve as indicators of acidification and H2S formation, respectively. The acid-base indicator phenol red incorporated for detecting carbohydrate fermentation is indicated by the change in color of the carbohydrate medium from orange-red to yellow in the presence of acids. In the case of oxidative decarboxylation of peptone, alkaline products are built and the pH rises. This is indicated by the change in color of the medium from orange-red to deep red. Sodium thiosulfate and ferrous ammonium sulfate present in the medium detect the production of hydrogen sulfide and are indicated by the black color in the butt of the tube.

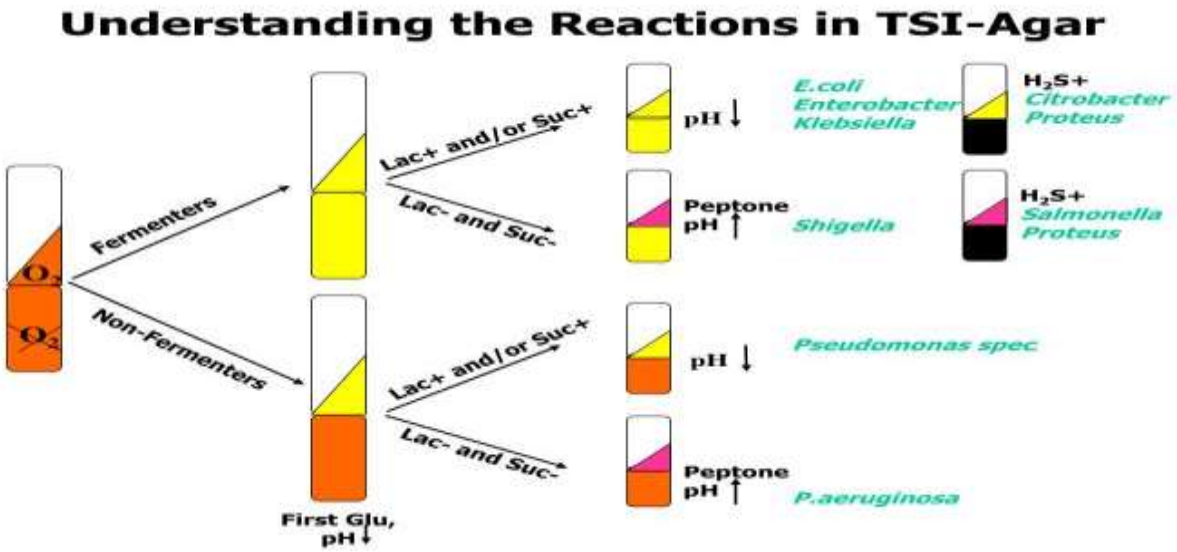
Glucose is utilized first by a fermentative organism and the entire medium becomes acidic (yellow) in 8 to 12 hours. The butt remains acidic even after an 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the tube. The slant, however, reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions on the slant. This change is a result of

the formation of CO_2 and H_2O and the oxidation of peptones in the medium to alkaline amines. When, in addition to glucose, lactose, and/or sucrose are fermented, the large amount of fermentation products formed on the slant neutralizes the alkaline amines and renders the slant acidic (yellow), provided the reaction is read in 18 to 24 hours. If the slant and butt become alkaline, glucose has not been fermented. Organisms showing this reaction are defined as non-fermenters and derive their nutrients from the peptones present in the medium. The formation of CO_2 and hydrogen gas (H_2) is indicated by the presence of bubbles or cracks in the agar or by the separation of the agar from the sides or bottom of the tube. The production of H_2S (sodium thiosulfate reduced to H_2S) requires an acidic environment, and reaction with the ferric ammonium citrate produces a blackening of the agar butt in the tube.



Triple Sugar Iron Agar (M021)

1. Control
2. Escherichia coli ATCC 25922
3. Salmonella Typhi ATCC 6539
4. Proteus vulgaris ATCC 13315
5. Citrobacter freundii ATCC 8090
6. Salmonella Typhimurium ATCC 14028
7. Shigella flexneri ATCC 12022



Result = Alkaline\ Acid, no CO2, no H2S \ Shigella. spp

Media Used in TSIA (Triple Sugar Iron Agar) Test

Triple sugar iron agar

Pancreatic Digest of Casein	
Lactose	
Sucrose	
Sodium Chloride	
Peptic Digest of Animal Tissue	
Yeast Extract	
Beef Extract	
Dextrose	
Ferric Ammonium Citrate	
Sodium Thiosulfate	
Phenol Red	
Agar	

Final pH 7.3 +/- 0.2 at 25ºC.

Procedure of TSIA (Triple Sugar Iron Agar) Test

- **With a straight inoculating needle, touch the top of a well-isolated colony.**
- **Inoculate TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.**
- **Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 18 to 24 hours.**
- **Following incubation, examine for color change in slant and butt, blackening and cracks in the medium.**

S. N.	Result (slant/butt)	Symbol	Interpretation
1	Red/Yellow	K/A	Glucose fermentation only, peptone catabolized.
2	Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation.
3	Red/Red	K/K	No fermentation, Peptone catabolized under aerobic and/or anaerobic conditions.
4	Yellow/Yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation, Gas produced.
5	Red/Yellow with bubbles	K/A,G	Glucose fermentation only, Gas produced.
6	Red/Yellow with bubbles and black precipitate	K/A,G,H_2S	Glucose fermentation only, Gas produced, H_2S produced.
7	Yellow/Yellow with bubbles and black precipitate	A/A,G,H_2S	Glucose and lactose and/or sucrose fermentation, Gas produced, H_2S produced.
8	Red/Yellow with black precipitate	K/A,H_2S	Glucose fermentation only, H_2S produced.
9	Yellow/Yellow with black precipitate	A/A,H_2S	Glucose and lactose and/or sucrose fermentation, H_2S produced.

Limitations of TSIA (Triple Sugar Iron Agar) Test

- **It is important to stab the butt of the medium. Failure to stab the butt invalidates this test. The integrity of the agar must be maintained when stabbing.**
- **Caps must be loosened during this test or erroneous results will occur.**
- **Reactions in TSI should not be read beyond 24 hours of incubation, because aerobic oxidation of the fermentation products from lactose and/or sucrose proceeds, and the slant eventually reverts to the alkaline state.**
- **An organism that produces hydrogen sulfide may mask acid production in the butt of the medium. However, hydrogen sulfide production requires an acid environment, thus the butt portion should be considered acid.**
- **TSI is not as sensitive in detecting hydrogen sulfide in comparison to other iron containing mediums, such as Sulfide Indole Motility (SIM) Medium. Thus, organisms that have weak hydrogen sulfide production may show only trace hydrogen sulfide activity, or none at all.**
- **Certain species or strains may give delayed reactions or completely fail to ferment the carbohydrate in the stated manner. However, if the organism fails to ferment glucose within 48 hours, it most likely is not in the Enterobacteriaceae family.**
- **A pure culture is essential when inoculating Triple Sugar Iron Agar. If inoculated with a mixed culture, irregular observations may occur.**

- **Further biochemical tests and serological typing must be performed for definite identification and confirmation of organisms.**

Quality Control of TSIA Test

Test organism	Slant	Butt	Gas production	H2S production
Escherichia coli ATCC25922	Yellow	Yellow	+	–
Pseudomonas aeruginosa ATCC27853	Red	Red	–	–
Salmonella enterica ATCC14028	Red	Yellow	+	+
Shigella sonnei ATCC9290	Red	Yellow	–	–

3-Urease Test

Urease Test: Principle, Procedure, Results

Urease is a constitutively expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia. Many organisms especially those that infect the urinary tract, have a urease enzyme that 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.

Medium used for urease test:

Any urea medium, agar (Christensen's urea agar), or broth (Stuart's urea broth). Urease test medium can be a sole medium or part of a panel like motility indole urease (MIU) test. The composition and preparation of these media are given at the end of this blog post.

Microorganisms Tested

- A. The urea test is part of the battery of tests to identify the following.
- Gram-negative enteric pathogens, including *Yersinia* spp.
- Fastidious Gram-negative rods—*Brucella,*
- *H. pylori,* and *Pasteurella*
- Gram-positive rod:
  Corynebacterium and *Rhodococcus* spp.
- Yeasts—*Cryptococcus* spp.
- B. Directly, this test is performed on gastric biopsy samples to detect the presence of *H. pylori*

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.**

Result and Interpretation

Organisms that hydrolyze urea rapidly (*Proteus* spp., *Morganella morganii,* and some *Providencia stuartii* strains) will produce strong positive reactions within 1 or 6 hours of incubation; delayed positive organisms (e.g. *Klebsiella* spp and *Enterobacter* species) will produce weak positive reactions in the slant in 6 hours of incubation which will be intense during further incubation. The culture medium will remain a yellowish color if the organism is urease negative e.g. *Escherichia coli.*

In routine diagnostic laboratories the urease test result is read within 24 hours.

- **If organism produces urease enzyme, the color of the slant changes from light orange to magenta.**
- **If organism does not produce urease the agar slant and butt remain light orange (medium retains original color).**

If Stuart's Urea Broth is used; **rapidly urease positive organisms (*Proteus* spp., *Morganella morganii*) will produce a strong positive reaction within 8-24 hours of incubation but delayed positive organisms (e.g., *Enterobacter)* will not produce a positive reaction due to high buffering capacity of this medium.**

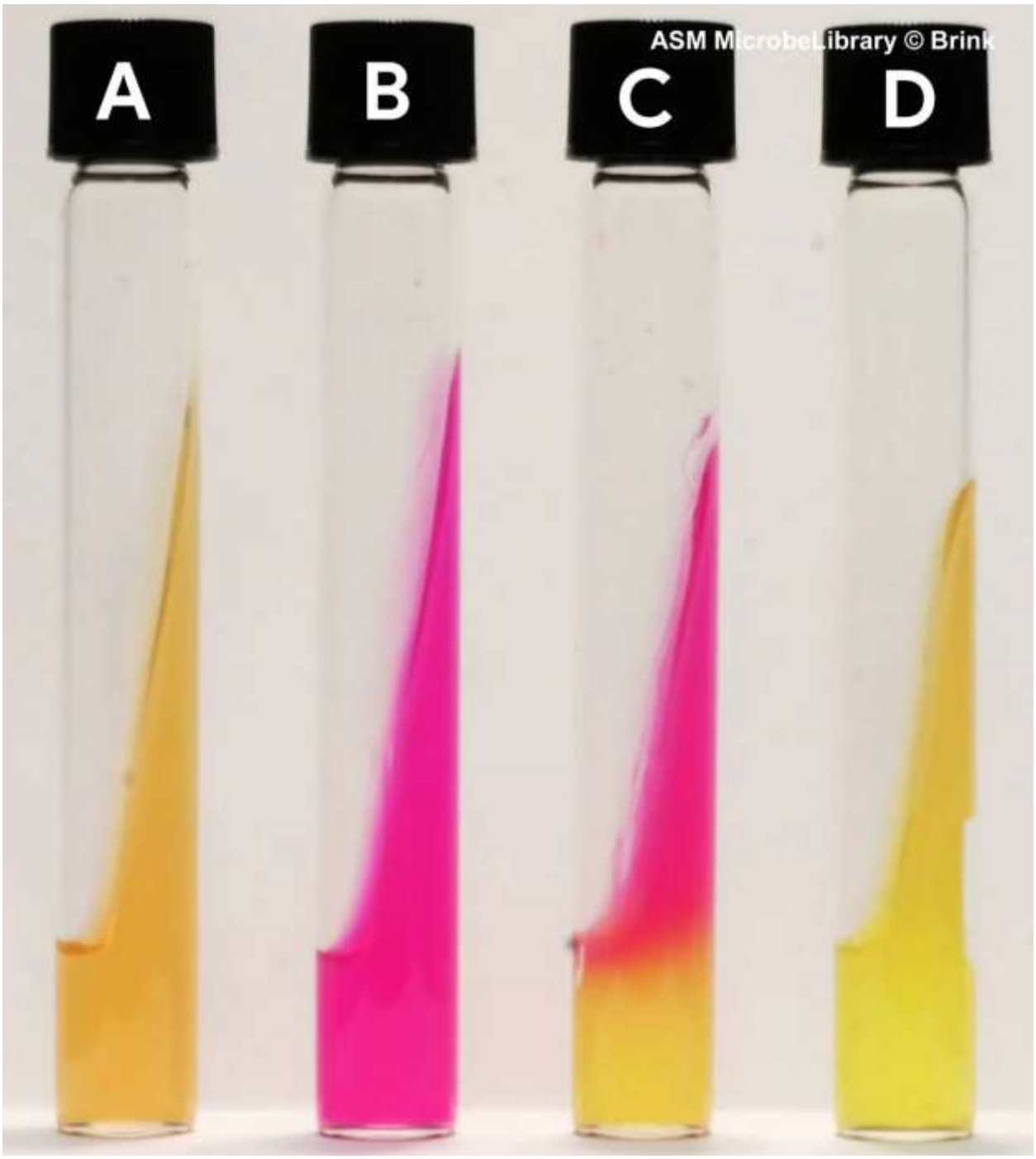


Figure: Urea agar test (a) uninoculated, (b) *Proteus mirabilis* (rapidly urease positive), (c) *Klebsiella pneumoniae* (delayed urease positive), (d) *Escherichia coli* urease negative).

Figure: positive result pink color (Urease positive)

Diagnostic utility of Urease test

1. **Urease test can be used as part of the identification of several genera and species of *Enterobacteriaceae* including *Proteus and Klebsiella*. It is also useful to identify *Cryptococcus* species, *Brucella*, *Helicobacter pylori.***
2. **Urease test helps for the identification of *Proteus* species (urease positive) and to differentiate it from other non-lactose fermenting members of the Enterobacteriaceae family.**
3. **Urease test is used for the presumptive evidence of the presence of *Helicobacter pylori* in tissue biopsy material. This is done by placing a portion of crushed tissue biopsy material directly into urease broth. A positive urease test is considered the presence of *Helicobacter pylori.* Commercially available urease agar kits are also available.**
4. **Rapid urease test is can be used to differentiate between the yeasts, *Candida albicans*, and *Cryptococcus neoformans*. Presumptive identification of *C. neoformans* may be based on rapid urease production, whereas *Candida albicans* do not.**
5. **Urea breath test: A common noninvasive test to detect *Helicobacter pylori* also based on urease activity. This is a highly sensitive and specific test.**

Name of urease positive organisms

1- *Proteus. spp*

2-*Klebsiella .spp*

Limitations

- **Some organisms rapidly split urea (*Brucella* and *H. pylori*), while others react slowly.**
- **When performing overnight tests from medium that contains peptone, the alkaline reaction may be due not to urease but to hydrolysis of peptone.**
- **Urea is light sensitive and can undergo auto hydrolysis. Store at 2 to 8°C in the dark.**
- **The test is less sensitive if the medium is not buffered.**

Urea agar

1- Urea

2- PH- indicator = phenol red

3- Enzyme = Urease