Al-Anbar University

College of Sciences

Biology department

Subject name: Microbial Identification

Educational level: Master

Lecture title: Complete of Biochemical Test

Subject teacher

Assistant prof. Dr. Jamal A. Rahman

Lab Procedure

INDOLE PRODUCTION

Purpose and Procedure Summary

Indole production is the "I" portion of the four IMViC tests used to characterize enteric bacteria. The amino acid tryptophan can be broken down by the enzyme **tryptophanase** to form indole, pyruvic acid, and ammonia as end-products. Tryptophanase differentiates the indole-positive enterics, such as *Escherichia coli* and *Proteus vulgaris*, from the indole-negative enterics, such as *Serratia marcescens* and *Enterobacter aerogenes*.

SIM medium, also used to detect motility and hydrogen sulfide production, is used to demonstrate indole production. SIM medium contains peptone and beef extract to support growth. Peptone contains the amino acid tryptophan. Agar is added to make the medium semisolid.

An isolate is inoculated into a tube with a sterile transfer needle. The tube is incubated at 35°C for 24–48 hours. After incubation, five drops of Kovac's reagent are added to the agar surface. Kovac's reagent contains amyl alcohol, hydrochloric acid, and para-dimethylaminobenzaldehyde, which reacts with indole to form a red color.

Tips for Success

- *Test the reactivity of the Kovac's reagent* by using it on a known indole-positive organism.
- Watch for color development *in the alcohol layer on the agar surface.*

Expected Results

The aldehyde in Kovac's reagent combines with indole to form a red color on the agar surface (Figure 5.17). A red color represents a positive test for indole production. No red color development represents a negative test. Positive and negative test results are shown in Figure 5.18.

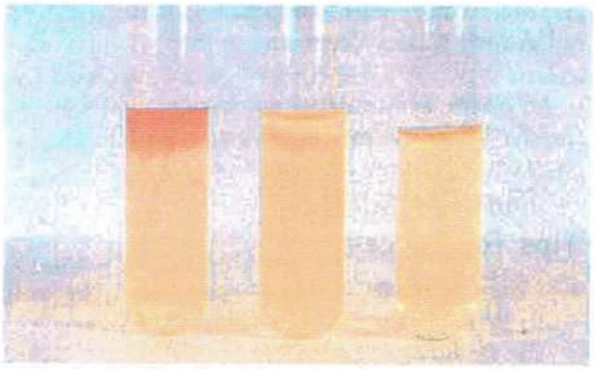

FIGURE 5.18 Results for indole production in SIM medium tubes. Inoculated tubes received five drops of Kovac's reagent after incubation. The reagent appears as a layer on the agar surface. Left: *Escherichia coli* (indole positive); center: *Enterobacter aerogenes* (indole negative); right: uninoculated.

Positive test: tryptophan —(trytophanase)→ NH_3 + pyruvic acid + indole

indole + added Kovac's reagent = **red color**

Example: ***Escherichia coli***

Negative test: tryptophan —(no tryptophanase)→ tryptophan

tryptophan + added Kovac's reagent = **no red color**

Example: ***Enterobacter aerogenes***

FIGURE 5.17 Indole production: Reactions and results for positive and negative tests.

LITMUS MILK REACTIONS

Purpose and Procedure Summary

Bacteria can act on several different substrates in litmus milk, including lactose, casein, and litmus, causing a variety of reactions that are specific for each species of bacteria.

Litmus milk contains skim milk, the source of lactose and casein, and litmus, the pH/oxidation-reduction indicator. Litmus is purplish-blue at the initial pH of 6.8 in the uninoculated medium. Several other colors for litmus are possible after bacterial growth occurs, depending on the action of the isolate.

An isolate is inoculated into a tube with a sterile transfer loop. The tube is incubated at 35°C for 24–48 hours before it is examined for changes.

Tips for Success

- *Compare results to an uninoculated control tube* to determine whether changes have occurred in the medium.
- *An additional incubation of 24–48 hours* may be necessary for some reactions to develop.
- The *blue of the alkaline reaction* is most evident *at the top* of the medium, while the *white of litmus reduction* is most evident *at the bottom.*

Expected Results

The variety of reactions possible in litmus milk, diagrammed in Figure 5.19, are the following:

lactose fermentation (A): Lactose fermentation releases lactic acid, which lowers the pH of the medium to 4.5, causing the litmus to turn pink. A pink color indicates lactose fermentation (Figure 5.21).

alkaline reaction (Alk): The action of bacteria on the nitrogen-containing components of skim milk causes ammonia to be released into the medium. As a result, the pH increases to 8.3 and the litmus

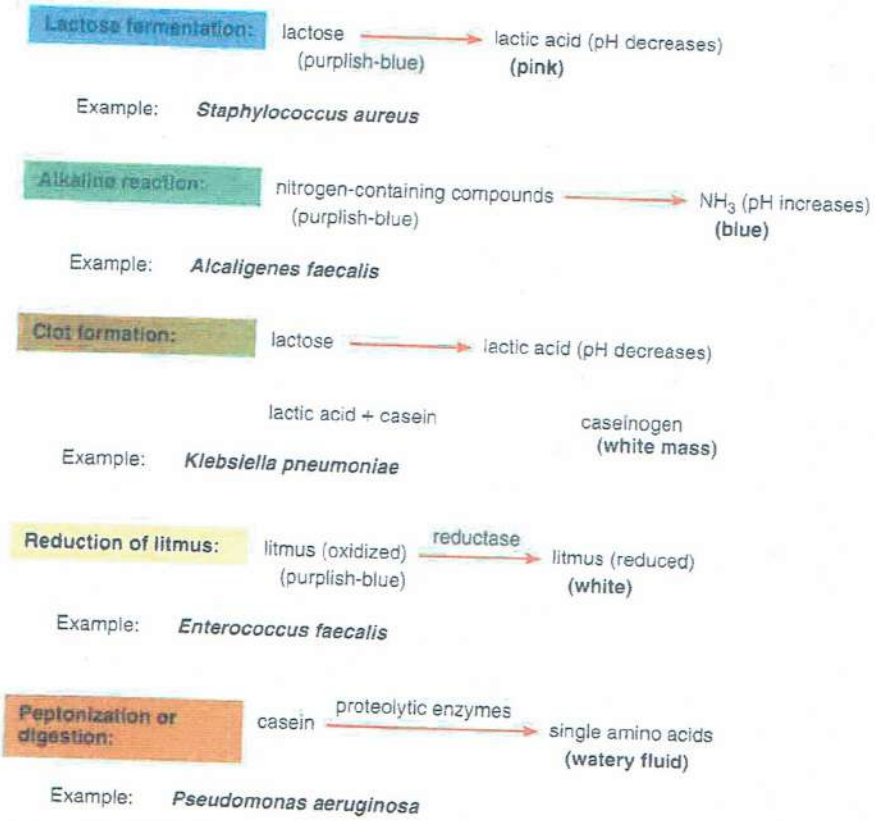

FIGURE 5.19 Possible reactions and results in litmus milk.

turns blue. A blue color indicates an alkaline reaction (Figures 5.20 and 5.21).

clot formation (C): Either the precipitation of casein by lactic acid or the action of the enzyme rennin on casein may cause the formation of a clot, which appears as a white mass in the bottom of the tube (Figure 5.20).

reduction of litmus (R): If litmus is used as an electron acceptor during lactose fermentation, it is reduced and turns white. A white color in the medium below the surface represents litmus reduction (Figure 5.21).

peptonization or digestion (D): Bacteria with proteolytic enzymes digest casein at the surface of the medium, which then takes on a watery appearance there (Figures 5.20 and 5.21). This reaction is called peptonization or digestion.

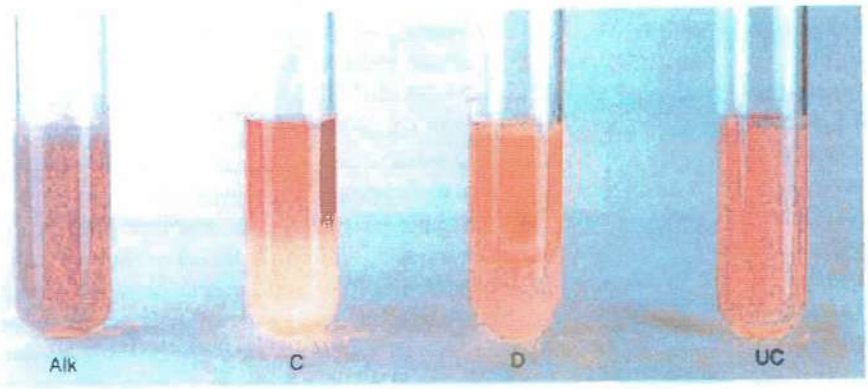

FIGURE 5.20 Results in litmus milk tubes. (Left to right): *Alcaligenes faecalis,* alkaline reaction (Alk); *Klebsiella pneumoniae,* clot formation (C); *Pseudomonas aeruginosa,* peptonization or digestion (D); uninoculated control (UC).

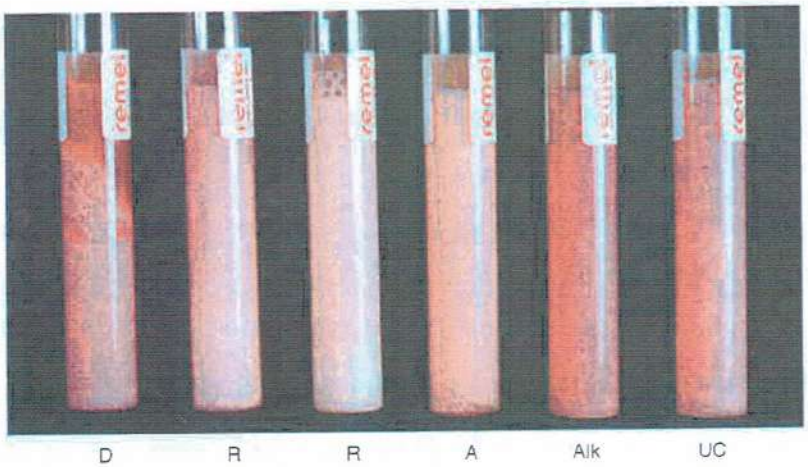

FIGURE 5.21 Results in litmus milk tubes. (Left to right): *Pseudomonas aeruginosa,* peptonization or digestion (D); *Proteus vulgaris,* litmus reduction (R); *Enterococcus faecalis,* litmus reduction (R); *Staphylococcus aureus,* lactose fermentation (A); *Micrococcus luteus,* alkaline reaction (Alk); uninoculated control (UC).

METHYL RED TEST

Purpose and Procedure Summary

The methyl red test is the "M" portion of the four IMViC tests used to characterize enteric bacteria. The methyl red test is used to identify enteric bacteria based on their pattern of glucose metabolism. All enterics initially produce pyruvic acid from glucose metabolism. Some enterics subsequently use the **mixed acid pathway** to metabolize pyruvic acid to other acids, such as lactic, acetic, and formic acids. These bacteria are called methyl-red-positive and include *Escherichia coli* and *Proteus vulgaris*. Other enterics subsequently use the **butylene glycol pathway** to metabolize pyruvic acid to neutral end-products. These bacteria are called methyl-red-negative and include *Serratia marcescens* and *Enterobacter aerogenes*.

Methyl Red-Voges Proskauer (MR-VP) medium, also used for the Voges-Proskauer test, is used for the methyl red test. This medium contains peptone to support growth, and glucose as the fermentable carbohydrate.

An isolate is inoculated into a tube with a sterile transfer loop. The tube is incubated at 35°C for 2–5 days. After incubation, 2.5 ml of the medium is transferred to another tube. Five drops of the pH indicator methyl red is added to this tube. The tube is gently rolled between the palms of the hands to disperse the methyl red.

At this pH, methyl red turns red (Figure 5.22). A red color represents a positive test. Enterics that subsequently metabolize pyruvic acid to neutral end-products lower the pH of the medium to only 6.0. At this pH, methyl red is yellow. A yellow color represents a negative test. Positive and negative test results are shown in Figure 5.23.

Positive test: glucose → pyruvic acid (1 day)

pyruvic acid → lactic, acetic, and formic acids (2-5 days)

many acids (pH 4.2) + added methyl red = **red color**

Example: ***Escherichia coli***

Negative test: glucose → pyruvic acid (1 day)

pyruvic acid → neutral end-products (2-5 days)

neutral end-products (pH 6.0) + added methyl red = **yellow color**

Example: ***Enterobacter aerogenes***

FIGURE 5.22 Reactions and results for a positive and negative methyl red test.

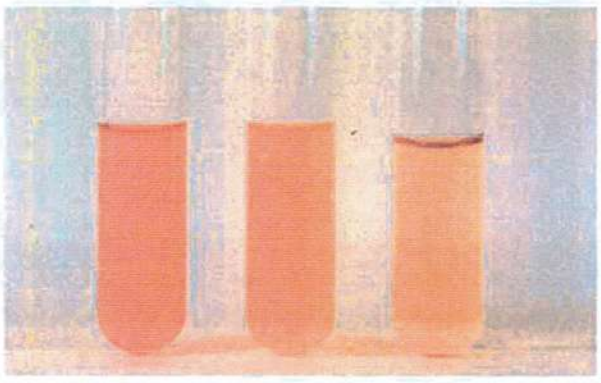

FIGURE 5.23 Methyl red test results in MR-VP medium tubes. The inoculated tubes received five drops of methyl red after incubation. Left: *Escherichia coli* (methyl-red-positive); center: *Enterobacter aerogenes* (methyl-red-negative); right: uninoculated.

Tips for Success

- *Allow a minimum of 2 days incubation;* 3–5 days incubation is recommended to allow the subsequent formation of acids or neutral end-products from pyruvic acid.
- *Use no more than five drops of methyl red;* adding more methyl red may impart a red color to the medium that is unrelated to metabolic end-products.

Expected Results

Enterics that subsequently metabolize pyruvic acid to other acids lower the pH of the medium to 4.2.

Lab Procedure

MOTILITY TEST

Purpose and Procedure Summary

Although the motility test is not a biochemical test, it is included here because it is often used to distinguish certain bacteria. The motility test determines the presence of flagella, external appendages used by bacteria for movement (see Chapter 3). Bacteria with flagella, such as *Citrobacter freundii,* are called **motile,** while bacteria without flagella, such as *Staphylococcus epidermidis,* are called **nonmotile.**

Motility test medium, used to detect bacteria with flagella, contains beef extract and peptone to support growth, and 0.5% agar. The medium is semisolid because of the low concentration of agar, allowing movement of motile organisms through the medium. **SIM medium** is another semisolid medium used to determine bacterial motility.

An isolate is inoculated into a tube with a sterile transfer needle. The needle is inserted and withdrawn in a straight line in the center of the medium. The tube is incubated at 35°C for 24–48 hours before examining the growth along the line.

Tips for Success

- *Hold the tubes up to the light* for better contrast when examining growth.
- *Compare results to an uninoculated tube* held up to the light.

Expected Results

Bacteria with flagella spread away from the line of inoculation. When the tube is held up to the light, growth is seen macroscopically as turbidity extending through the semisolid medium. Growth away from the line of inoculation indicates that the organism is motile (Figure 5.24). Bacteria without flagella do not spread away from the line of inoculation, so their growth does not extend into the medium. Growth along the line of inoculation only indicates that the organism is nonmotile. These results are shown in Figure 5.25.

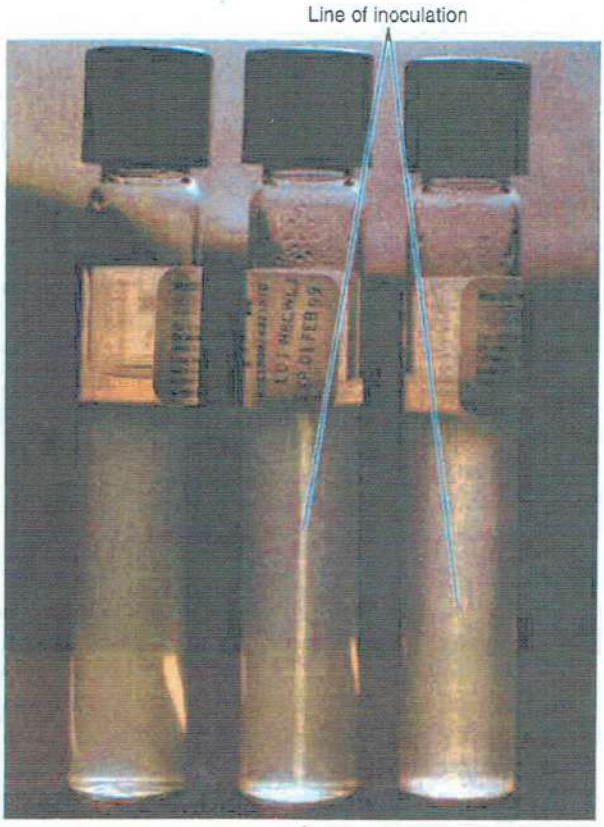

FIGURE 5.25 Results in motility test medium tubes. (Left to right): uninoculated control; *Staphylococcus epidermidis,* nonmotile; *Citrobacter freundii,* motile. Motile organisms grow out from the line of inoculation.

Positive test: motility test medium → **growth spreads away from line of inoculation**
(motile)

Example: ***Citrobacter freundii***

Negative test: motility test medium → **growth occurs only along line of inoculation**

Example: ***Staphylococcus epidermidis***

FIGURE 5.24 A positive and negative test for motility.

Lab Procedure

OXIDASE TEST

Purpose and Procedure Summary

The electron transport chain is a sequence of reactions that represent the final stage of bacterial cell respiration. The final reaction in this sequence is catalyzed by the enzyme cytochrome oxidase. In this final step, cytochrome oxidase oxidizes the electron transport molecule, cytochrome *c*, while reducing oxygen to form water. Bacteria that contain cytochrome oxidase, such as species of *Pseudomonas*, are oxidase positive, while those that lack this enzyme, such as *Escherichia coli* and other enterics, are oxidase negative.

The oxidase test requires the use of a reduced chemical reagent. This reagent does not interact directly with cytochrome oxidase, but instead interacts with the enzyme's product, oxidized cytochrome *c*. Cytochrome *c* changes the reduced reagent to an oxidized form.

The oxidase test can be performed either by adding oxidase reagent to bacterial growth on an agar plate or by transferring growth to a DrySlide™ that already contains the oxidase reagent. After the growth is combined with oxidase reagent, color change is observed for up to 60 seconds.

Tips for Success

- *Test the reactivity of the oxidase reagent* by using it on a known oxidase-positive organism; oxidase reagent is unstable.
- *Disregard color changes to the surrounding medium* if using the plate test; this is typical and not related to the test.
- *Disregard color changes after 60 seconds.*

Expected Results

Cytochrome oxidase oxidizes cytochrome *c*, which in turn oxidizes the oxidase reagent. The oxidized oxidase reagent changes from pink, to light purple, to dark purple, usually within 10–30 seconds after it is added to the plate or growth is added to a DrySlide™ (Figure 5.26). A dark purple color represents a positive test. If the oxidase reagent remains colorless, the test is negative. These test results for the plate test and DrySlide™ test are shown in Figures 5.27 and 5.28, respectively.

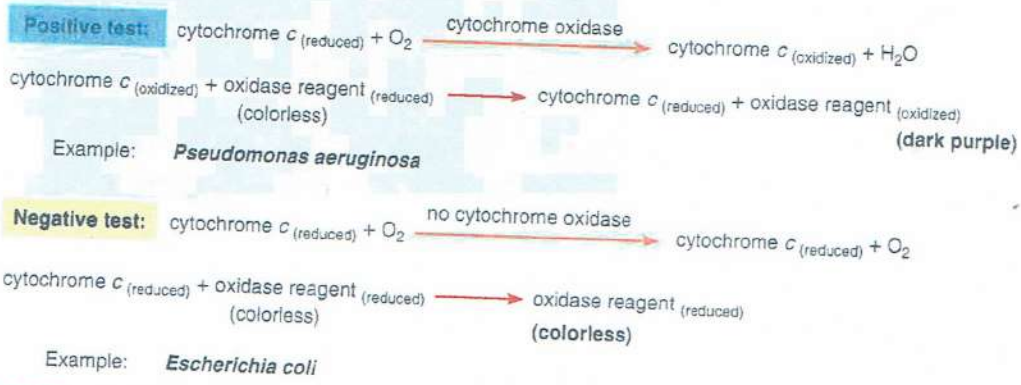

FIGURE 5.26 Oxidase test: Reactions and results of positive and negative tests.

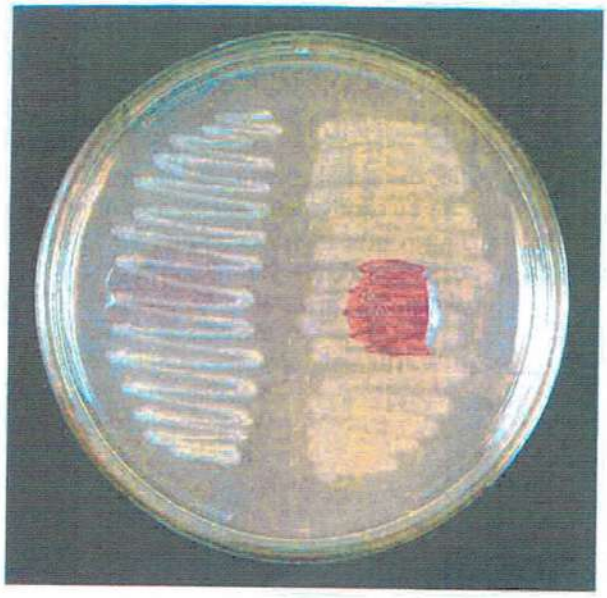

FIGURE 5.27 Results of an oxidase test on a nutrient agar plate inoculated with *Escherichia coli* (at left) and *Pseudomonas aeruginosa* (at right). Several drops of oxidase reagent were added to the growth of each organism. A purple color indicates that *Pseudomonas aeruginosa* is oxidase positive. *Escherichia coli* is oxidase negative.

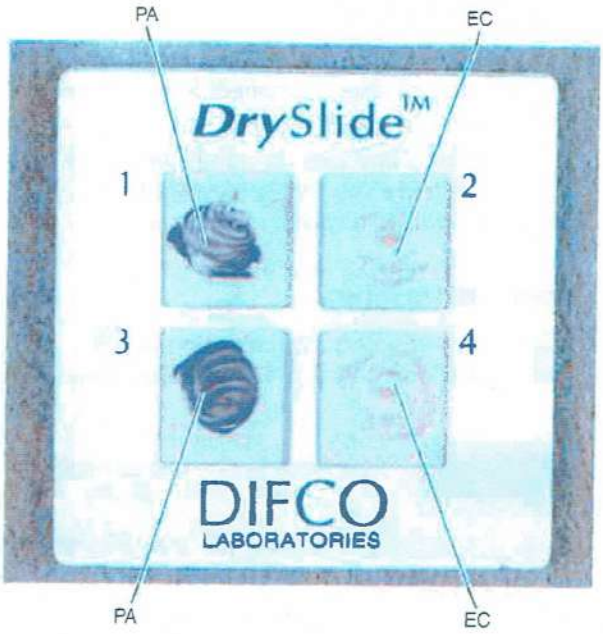

FIGURE 5.28 Results of a DrySlide™ oxidase test, with *Pseudomonas aeruginosa* (oxidase positive) on the left and *Escherichia coli* (oxidase negative) on the right.

Lab Procedure

OXIDATION-FERMENTATION (O-F) TEST

Purpose and Procedure Summary

Carbohydrate conversion to acidic products can occur either aerobically by oxidation or anaerobically by fermentation. Bacteria that oxidize carbohydrates are called **aerobes**, while bacteria that ferment carbohydrates are called **facultative anaerobes.** The oxidation-fermentation (O-F) test is used to determine whether bacteria are aerobes or facultative anaerobes. The O-F test distinguishes Gram-negative rods that are aerobes, such as *Pseudomonas aeruginosa,* from those that are facultative anaerobes, such as *Escherichia coli.* The O-F test also distinguishes Gram-positive cocci that are aerobes, such as species of *Micrococcus,* from those that are facultative anaerobes, such as species of *Staphylococcus.*

Oxidation-fermentation (O-F) medium is used for this test. This medium contains a low concentration of peptone, but enough to support growth. A low concentration of peptone is essential to limit the formation of alkaline products that would neutralize the effect of acidic products. A carbohydrate, such as glucose, lactose, maltose, sucrose, mannitol, or xylose, is added at a high concentration. This high concentration promotes carbohydrate utilization, with the resulting formation of acidic products. The pH indicator, bromthymol blue, is green at the initial pH of 7.1 and yellow at a pH of 6.0. Agar is added at a low concentration to make the medium semisolid. Although **phenol red broth** can also be used for the O-F test, it is not recommended because it has a high concentration of peptone, which may result in the formation of sufficient alkaline products to neutralize the effect of acidic products.

An isolate is inoculated into two tubes of O-F medium with a sterile transfer needle. The medium in one tube is then covered with 2–3 ml of sterile mineral oil to create an anaerobic environment. The medium in the other tube is left open to the air to provide an aerobic environment. The two tubes are incubated at 35°C for 48 hours before examination for color change.

Tips for Success

- *Compare results to an uninoculated control tube* to determine whether changes have occurred in the medium.
- If a yellow color appears *on the surface in the open tube only,* this finding should be interpreted as oxidation.

Expected Results

A green color in both tubes indicates that the organism is unable to utilize the carbohydrate tested (Figure 5.29). Facultative anaerobes convert glucose to acidic products in both tubes, changing the initial green to yellow. A yellow color in both tubes indicates a facultatively anaerobic organism. Aerobes convert glucose to acidic products in the open tube only, changing the initial green color to yellow. A green color in the tube containing oil and a yellow color in the open tube indicates an aerobe. These results are shown in Figure 5.30.

No reaction: (inert)

glucose (green) → glucose **(with oil)** **(green)**

glucose (green) → glucose **(open tube without oil)** **(green)**

Example: ***Alcaligenes faecalis***

Oxidation-Fermentation: (facultative anaerobe)

glucose (green) → acids, pH decreases **(with oil)** **(yellow)**

glucose (green) → acids, pH decreases **(open tube without oil)** **(yellow)**

Example: ***Escherichia coli***

Oxidation: (aerobe)

glucose (green) → glucose **(with oil)** **(green)**

glucose (green) → acids, pH decreases **(open tube without oil)** **(yellow)**

Example: ***Pseudomonas aeruginosa***

FIGURE 5.29 Possible reactions and results of oxidation-fermentation (O-F) tests.

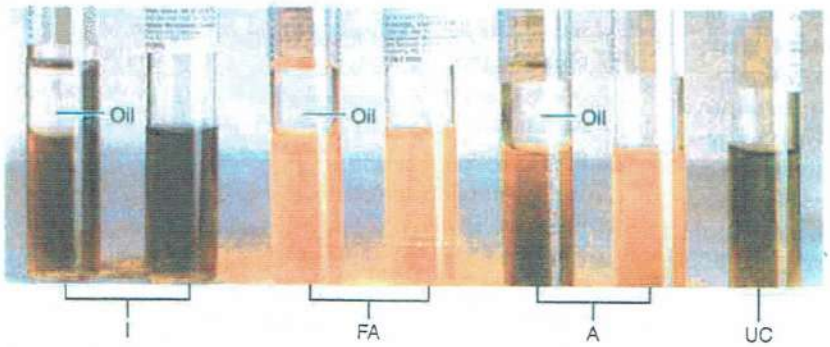

FIGURE 5.30 Results in O-F glucose tubes. (Left to right): *Alcaligenes faecalis* with and without oil, inert (I); *Escherichia coli* with and without oil, facultative anaerobe (FA); *Pseudomonas aeruginosa* with and without oil, aerobe (A); uninoculated control (UC).

Lab Procedure

PHENYLALANINE DEAMINASE TEST

Purpose and Procedure Summary

The amino acid phenylalanine can be broken down by the enzyme **phenylalanine deaminase** to form phenylpyruvic acid and ammonia. Phenylalanine deaminase is produced by some enterics, such as *Proteus vulgaris,* but not by others, such as *Escherichia coli* and *Enterobacter aerogenes.*

Phenylalanine agar is used to detect the deamination of phenylalanine. This agar contains yeast extract to support growth, DL-phenylalanine, and agar.

An isolate is inoculated onto a slant with a sterile transfer loop. The slant is incubated at 35°C for 18–24 hours. After incubation, four or five drops of 10% ferric chloride ($FeCl_3$) are added and rolled over the surface of the slant. The slant is then observed for color change.

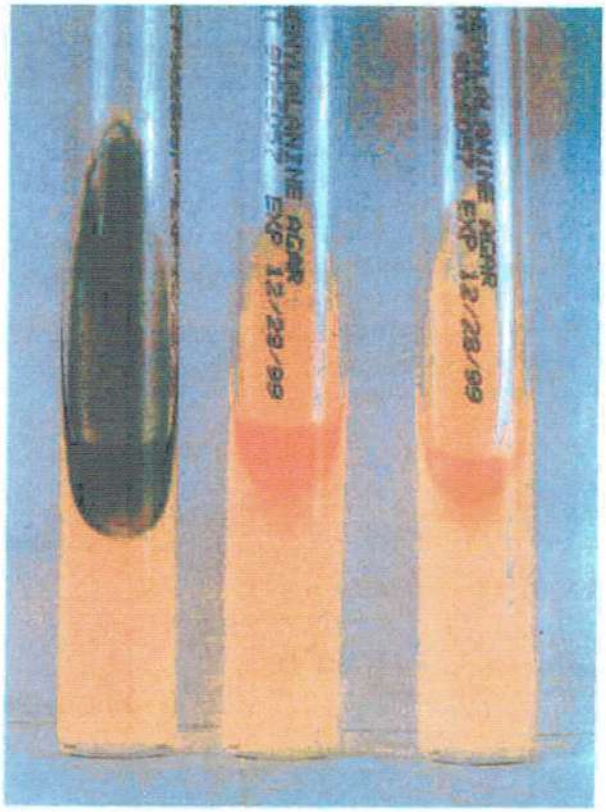

FIGURE 5.32 Results in phenylalanine agar tubes. (Left to right): *Proteus vulgaris,* phenylalanine deaminase positive; *Escherichia coli,* phenylalanine deaminase negative; uninoculated control. A positive test turns green after the addition of ferric chloride.

Tips for Success

- *Test the reactivity of the ferric chloride on a known positive.*
- *Read this test within 1–5 minutes,* because the green color disappears.

Expected Results

Ferric chloride reacts with phenylpyruvic acid to form a green color (Figure 5.31). A green color represents a positive test. No green color represents a negative test. Positive and negative test results are shown in Figure 5.32.

Positive test: phenylalanine $\xrightarrow{\text{phenylalanine deaminase}}$ phenylpyruvic acid + NH_3

phenylpyruvic acid + added $FeCl_3$ = **green color**

Example: ***Proteus vulgaris***

Negative test: phenylalanine $\xrightarrow{\text{no phenylalanine deaminase}}$ phenylalanine

phenylalanine + added $FeCl_3$ = **no green color**

Example: ***Escherichia coli***

FIGURE 5.31 Positive and negative test results for phenylalanine deaminase.

SKIM MILK UTILIZATION

Purpose and Procedure Summary

Casein is the primary protein in skim milk. Only bacteria that produce the extracellular enzyme caseinase, such as *Bacillus cereus*, can break down this protein, which gives milk its white color. The breakdown products are single amino acids that are transported into the cell and used in metabolism.

Skim milk agar is used to test for the presence of caseinase. This medium contains peptone to support growth, 10% skim milk, and agar.

An isolate is inoculated onto a plate with a sterile transfer loop. The plate is incubated at 35°C for 24–48 hours before examination.

Tips for Success

- *Incubate plates an extra 1–2 days* to eliminate the possibility of a slow caseinase-positive organism.

Expected Results

Skim milk agar appears white due to the presence of casein. The zone around growth of caseinase-positive organisms will be clear because of casein breakdown (Figure 5.33). The zone around growth of caseinase-negative organisms will remain white. These test results are shown in Figure 5.34.

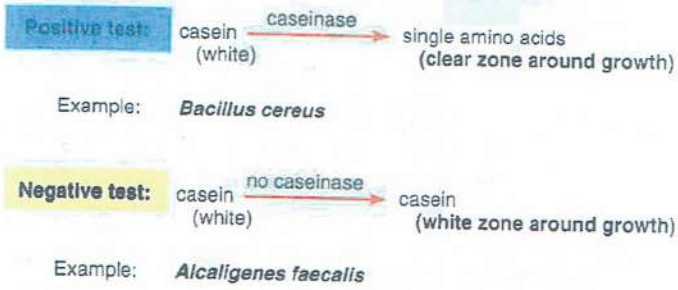

FIGURE 5.33 Skim milk utilization test results.

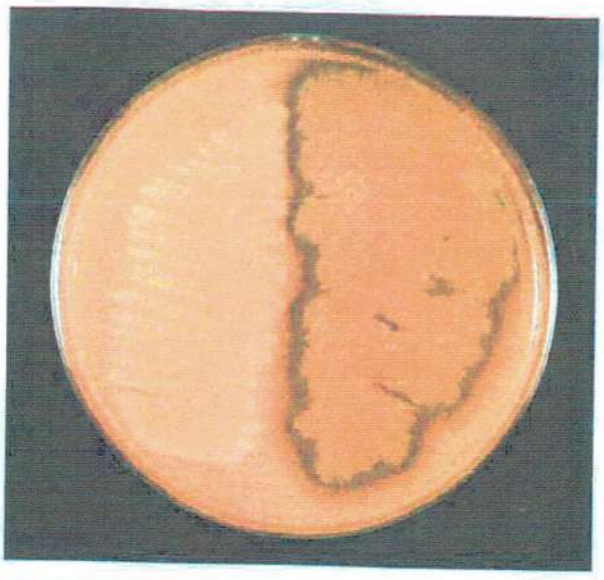

FIGURE 5.34 Results on skim milk agar plate inoculated with *Alcaligenes faecalis* on the left (caseinase negative) and *Bacillus cereus* on the right (caseinase positive).

Lab Procedure

STARCH HYDROLYSIS TEST

Purpose and Procedure Summary

Starch is a polysaccharide composed of repeating alpha-D-glucose subunits. Bacteria that produce the extracellular enzyme **amylase**, such as *Bacillus cereus*, break down starch into single subunits of alpha-D-glucose. These are transported into the cell, where they are broken down in cell respiration.

Starch agar is used to test for the breakdown of starch by amylase. This medium contains beef extract and peptone to support growth, soluble starch, and agar.

An isolate is inoculated onto a plate with a sterile transfer loop. The plate is incubated at 35°C for 48 hours. After incubation, the plate is flooded with Gram's iodine, which reacts with starch to produce a purple-blue color.

Tips for Success

- *Test the reactivity of the Gram's iodine* by testing it on a known amylase-positive organism.
- *Examine for color immediately after addition of Gram's iodine*, because the color may fade.

Expected Results

Gram's iodine reacts with starch to produce a purple-blue color throughout the agar medium. A clear zone around bacterial growth indicates starch hydrolysis (Figure 5.35). A purple-blue color to the edge of bacterial growth indicates no starch hydrolysis. These results are shown in Figure 5.36.

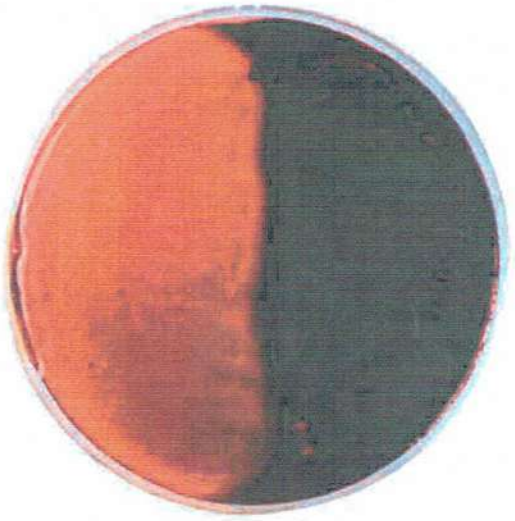

FIGURE 5.36 Results on starch agar plate inoculated with *Bacillus cereus* (at left) and *Escherichia coli* (at right). Starch hydrolysis by *Bacillus cereus* is indicated by a clear zone around growth after the addition of Gram's iodine. The reddish color seen on the left is due to the added Gram's iodine.

Positive test: starch —amylase→ glucose subunits

glucose subunits + added Gram's iodine = **clear zone around growth**

Example: ***Bacillus cereus***

Negative test: starch —no amylase→ starch

starch + added Gram's iodine = **purple-blue zone around growth**

Example: ***Escherichia coli***

FIGURE 5.35 Starch hydrolysis test results.

TRIPLE SUGAR IRON (TSI) AGAR AND KLIGLER IRON AGAR (KIA) TESTS

Purpose and Procedure Summary

Triple sugar iron (TSI) agar and Kligler iron agar (KIA) are used to determine both carbohydrate fermentation and hydrogen sulfide production in enteric bacteria. These media contain a variety of substrates that enteric bacteria can utilize, including several carbohydrates, proteins, and thiosulfate. Different enteric bacteria utilize these substrates differently, so they can be distinguished based on their pattern of utilization.

Both media contain beef extract, yeast extract, and peptone to support bacterial growth. The fermentable carbohydrates in TSI are glucose, lactose, and sucrose. KIA contains only glucose and lactose. Sodium thiosulfate is the source of sulfur for H_2S production in both media. Both TSI and KIA contain ferrous sulfate, which reacts with H_2S to form a black precipitate called ferrous sulfide. The pH indicator in both media is phenol red. Phenol red is red at the initial pH of 7.4 but turns yellow at an acidic pH and dark red at an alkaline pH. Agar is present in these two media as a solidifying agent.

An isolate is inoculated onto a TSI or KIA slant using a sterile transfer needle. First, the butt is stabbed. Then, the needle is withdrawn and the slant is streaked. The slant is incubated at 35°C for 18–24 hours before examination for color changes.

Tips for Success

- *Examine the tubes no earlier or later than 18–24 hours,* or incorrect patterns of carbohydrate fermentation may be obtained.
- Record the butt as acid production if the black color of ferrous sulfide masks the color in the butt.

Expected Results

Because the many reactions possible in TSI and KIA are similar, they are summarized together in Figure 5.37. These reactions are the following:

No carbohydrate fermentation or hydrogen sulfide production (Alk/Alk or Alk/NC): No acids are produced in the slant or butt to cause a drop in the pH of the medium. The slant and butt remain red or turn dark red due to the production of alkaline products from peptone.

Glucose fermentation only (Alk/A): Glucose fermentation results in the formation of acids that lower the pH of the butt. The butt turns yellow while the slant remains red or turns a dark red.

Glucose fermentation only with hydrogen sulfide production (Alk/A, H_2S): If glucose fermentation occurs with hydrogen sulfide production, the butt turns black and yellow while the slant remains red.

Lactose and/or sucrose and glucose fermentation (A/A): Lactose and/or sucrose and glucose fermentation results in the formation of acids that lower the pH of the slant and butt. The slant and butt turn yellow.

Lactose and/or sucrose and glucose fermentation with hydrogen sulfide production (A/A, H_2S): If lactose and/or sucrose and glucose fermentation occurs with hydrogen sulfide production, the butt turns black and yellow while the slant turns yellow.

The various reactions on TSI are shown in Figure 5.38, while the reactions on KIA are shown in Figure 5.39.

No carbohydrate fermentation or hydrogen sulfide production:

glucose, lactose, sucrose (red slant/red butt) → glucose, lactose, sucrose **(red slant/red butt)**

cysteine → cysteine **(no black color)**

Example: ***Alcaligenes faecalis***

Glucose fermentation only:

lactose, sucrose (red slant) → lactose, sucrose **(red slant)**

glucose (red butt) → acids, pH decreases **(yellow butt)**

cysteine → cysteine **(no black color)**

Example: ***Shigella flexneri***

Glucose fermentation only with hydrogen sulfide production:

lactose, sucrose (red slant) → lactose, sucrose **(red slant)**

glucose (red butt) → acids, pH decreases **(yellow butt)**

cysteine → H_2S + other products

$H_2S + FeSO_4$ → FeS **(black color)**

Example: ***Salmonella typhimurium***

Lactose and/or sucrose and glucose fermentation:

lactose and/or sucrose (red slant) → acids, pH decreases **(yellow slant)**

glucose (red butt) → acids, pH decreases **(yellow butt)**

cysteine → cysteine **(no black color)**

Example: ***Escherichia coli***

Lactose and/or sucrose and glucose fermentation with hydrogen sulfide production:

lactose and/or sucrose (red slant) → acids, pH decreases **(yellow slant)**

glucose (red butt) → acids, pH decreases **(yellow butt)**

cysteine → H_2S + other products

$H_2S + FeSO_4$ → FeS **(black color)**

Examples: ***Proteus vulgaris***
Citrobacter freundii

FIGURE 5.37 Possible reactions and results in triple sugar iron (TSI) agar and Kligler iron agar (KIA).

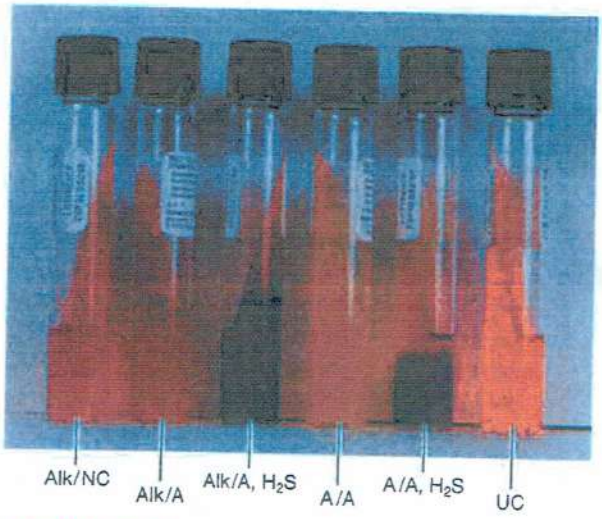

FIGURE 5.38 Results on triple sugar iron agar tubes. (Left to right): *Alcaligenes faecalis*, Alk/NC; *Shigella flexneri*, Alk/A; *Salmonella typhimurium*, Alk/A, H_2S; *Escherichia coli*, A/A; *Proteus vulgaris*, A/A, H_2S; uninoculated control (UC).

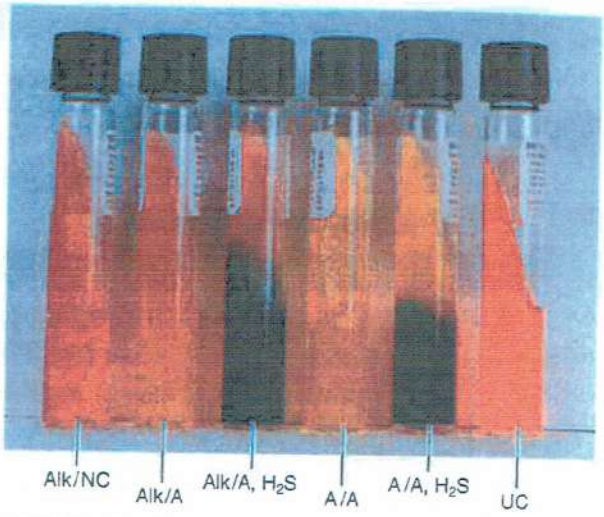

FIGURE 5.39 Results on Kligler iron agar tubes. (Left to right): *Alcaligenes faecalis*, Alk/NC; *Shigella flexneri*, Alk/A; *Salmonella typhimurium*, Alk/A, H_2S; *Escherichia coli*, A/A; *Citrobacter freundii*, A/A, H_2S; uninoculated control (UC).

Lab Procedure

UREA UTILIZATION

Purpose and Procedure Summary

Some bacteria produce **urease**, an enzyme capable of breaking down urea. The breakdown of urea within 24 hours is a trait used to distinguish species of *Proteus* from other enteric bacteria.

Urea broth contains yeast extract, urea, and the pH indicator phenol red. Phenol red is yellow-orange at the initial pH of 6.8 but changes to pinkish-red at a pH of 8.4.

An isolate is inoculated into a tube with a sterile transfer loop. The tube is incubated at 35°C for 24 hours before examination for color change.

Tips for Success

- *Examine the color of broth within 24 hours;* a pinkish-red color within 24 hours distinguishes species of *Proteus;* a pinkish-red color after 24 hours indicates the slow urease activity of other enterics.

Expected Results

The enzyme urease breaks down urea into alkaline end-products that raise the pH of the medium, causing the phenol red to turn a pinkish-red color (Figure 5.40). Therefore, a pinkish-red color represents a positive test for urea utilization. No color change represents a negative test. Test results are shown in Figure 5.41.

Positive test: urea (yellow-orange) —urease→ NH_3 (pH increases) + CO_2 **(pinkish-red)**

Example: ***Proteus vulgaris***

Negative test: urea (yellow-orange) —no urease→ urea **(yellow-orange)**

Example: ***Escherichia coli***

FIGURE 5.40 Urea utilization test results.

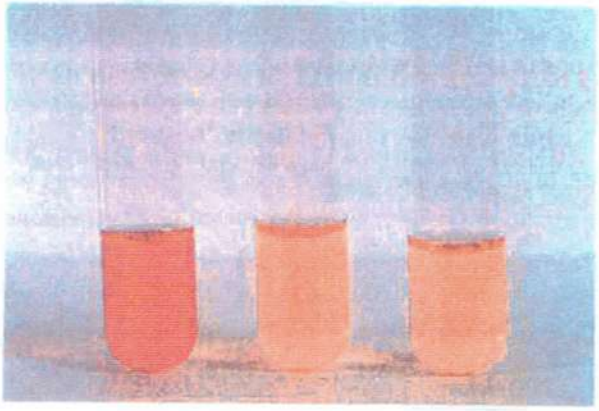

FIGURE 5.41 Results of urea broth tubes inoculated with *Proteus vulgaris* on the left (urease positive) and *Escherichia coli* in the center (urease negative). The tube on the right is uninoculated.

Lab Procedure

VOGES-PROSKAUER (VP) TEST

Purpose and Procedure Summary

The Voges-Proskauer (VP) test is the "Vi" portion of the four IMViC tests used to characterize enteric bacteria. The Voges-Proskauer test is used to identify enteric bacteria based on their pattern of glucose metabolism. All enterics initially produce pyruvic acid from glucose metabolism. The enterics that subsequently produce acidic products from pyruvic acid are detected with the methyl red test. The enterics that subsequently produce neutral end-products, such as acetoin (acetylmethylcarbinol), from pyruvic acid are detected with the Voges-Proskauer test. This test is used to distinguish acetoin-producing enterics, such as *Enterobacter aerogenes*, from enterics that do not produce acetoin, such as *Escherichia coli*.

Methyl Red-Voges Proskauer (MR-VP) medium, also used for the methyl red test, is used to detect acetoin production. MR-VP medium contains peptone to support growth and glucose as the fermentable carbohydrate.

An isolate is inoculated into a tube with a sterile transfer loop. The tube is incubated at 35°C for 48 hours. After incubation, 2.5 ml is transferred to another tube, and six drops of Barritt's Reagent A (containing alpha-naphthol) and two drops of Barritt's Reagent B (containing KOH) are added. The contents of the tube are gently mixed and then allowed to sit for 10–15 minutes to allow time for color development.

Tips for Success

- *Test the reactivity of Barritt's Reagents A and B on a known positive.*
- *Add Barritt's Reagents A and B in the order and amounts specified;* this increases the sensitivity of the test.

Expected Results

If acetoin is produced from glucose metabolism, it reacts with the alpha-naphthol and KOH to produce a red color in the medium (Figure 5.42). A red color represents a positive Voges-Proskauer test. If acetoin is not produced, the reagents themselves give the medium a copper color. A copper color represents a negative Voges-Proskauer test. These test results are shown in Figure 5.43.

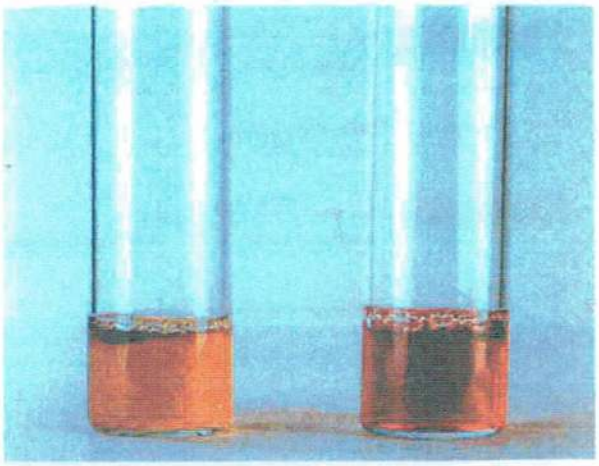

FIGURE 5.43 Results of the Voges-Proskauer test in MR-VP tubes. Tubes have received six drops of Barritt's Reagent A and two drops of Barritt's Reagent B. *Escherichia coli* (left) is VP negative, and *Enterobacter aerogenes* (right) is VP positive.

Positive test: glucose → pyruvic acid

pyruvic acid → acetoin

acetoin + added alpha-naphthol + added KOH = **red color**

Example: ***Enterobacter aerogenes***

Negative test: glucose → pyruvic acid

pyruvic acid → no acetoin

no acetoin + added alpha-naphthol + added KOH = **copper color**

Example: ***Escherichia coli***

FIGURE 5.42 Reactions and results for a positive and negative Voges-Proskauer (VP) test.

References:

(1) Michael J. Leboffe. Burton E. Pierce. (2016) A photographic Atlas for the Microbiology Laboratory. 5th Edition.

(2) FAIRBROTHER, R.W. (2013). **A Textbook of Bacteriology, Fourth Edition.**