Al-Anbar University

College of Sciences

Biology department

Subject name: Microbial Identification

Educational level: Master

Lecture title: Biochemical Tests

Subject teacher

Assistant prof. Dr. Jamal A. Rahman

Chapter 5

Biochemical Tests for the Identification of Bacteria

Introduction

Simple, differential, and structural stains (see Chapter 3), even if combined with cultivation and observation of colony characteristics (see Chapter 4), are not sufficient for the identification of bacterial isolates. Results of staining and cultivation must be combined with the results from biochemical tests. **Biochemical tests** evaluate the metabolic properties of an isolate, which are unique for each species.

A combination of biochemical tests can be used to determine the biochemical pattern for an isolate. This enables the identification of an isolate using an identification scheme (see Chapter 6).

Tips for Success

- *Use good aseptic technique* when transferring cultures to prevent contamination.
- Inoculate biochemical tests with *actively growing cultures* that are 24–48 hours old. This will ensure the presence of young, viable cells.
- *Check for growth* before determining the result of a test. When results are negative, the presence of growth indicates the absence of a metabolic step, and not just an absence of growth.

Lab Procedure

CARBOHYDRATE UTILIZATION

Purpose and Procedure Summary

Carbohydrate fermentation media, such as purple broth and phenol red broth, are used to determine whether an organism has the ability to ferment various carbohydrates. Carbohydrates typically tested include adonitol, arabinose, glucose, inositol, lactose, melibiose, rhamnose, sorbitol, and sucrose. These media are often used for distinguishing lactose-fermenting enterics, such as *Escherichia coli, Enterobacter aerogenes,* and *Klebsiella pneumoniae,* from non-lactose-fermenting enterics, such as *Proteus vulgaris, Shigella flexneri,* and *Salmonella typhimurium.*

Purple broth contains peptone, a single carbohydrate, plus the pH indicator bromcresol purple. This indicator is purple at an alkaline pH, but yellow at an acidic pH. **Phenol red broth** contains peptone, a single carbohydrate, plus the pH indicator phenol red. This indicator is red at an alkaline pH, but yellow at an acidic pH. A small, inverted glass tube,

called a **durham tube**, is placed at the bottom of each tube containing carbohydrate fermentation media. The tube collects gas, which is often a product of carbohydrate fermentation.

An isolate is inoculated into a purple broth tube or a phenol red broth tube with a sterile transfer loop. The tube is incubated at 35°C for 24–48 hours before examination. The broth is observed for color change and gas production.

Tips for Success

- When inoculating broth tubes, dip the loop into the medium and then *rub it against the tube wall* to ensure the transfer of inoculum.
- *Compare results with an uninoculated control tube* to determine whether changes have occurred in the medium.

Expected Results

Bacteria that ferment a carbohydrate produce acid, or acid and gas, as end-products (Figure 5.1). Acids lower the pH of the medium, causing the bromcresol purple or phenol red pH indicator to turn yellow, while gases (if produced) collect to form bubbles in the durham tube. If bacteria do not ferment a carbohydrate, the medium will remain purple (if purple broth was used) or red (if phenol red broth was used). In addition, no bubbles will be present in the durham tube. A range of results for purple broth with glucose are shown in Figure 5.2, while results for phenol red broth with lactose are shown in Figure 5.3

Positive test: carbohydrates (red, purple) ⟶ acid (pH decreases) or acid and gas **(yellow and bubbles in durham tube)**

Examples: ***Serratia marcescens*** (acid)
Escherichia coli (acid and gas)

Negative test: carbohydrates (red, purple) ⟶ carbohydrates (pH unchanged and no gas) **(red, purple and no bubbles in durham tube)**

Example: ***Alcaligenes faecalis*** (no acid or gas)

FIGURE 5.1 Possible reactions and results of carbohydrate utilization tests.

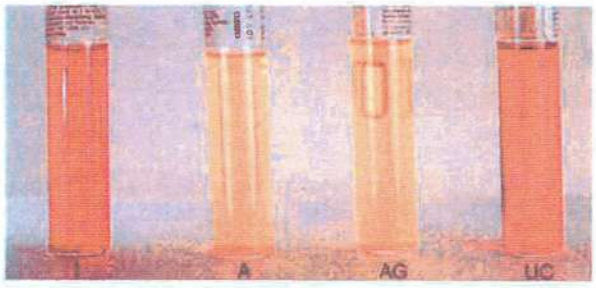

FIGURE 5.2 Carbohydrate utilization test results in purple glucose broth tubes. (Left to right): *Alcaligenes faecalis*, inert = no acid or gas (I); *Serratia marcescens*, acid (A); *Escherichia coli*, acid and gas (AG); uninoculated control (UC).

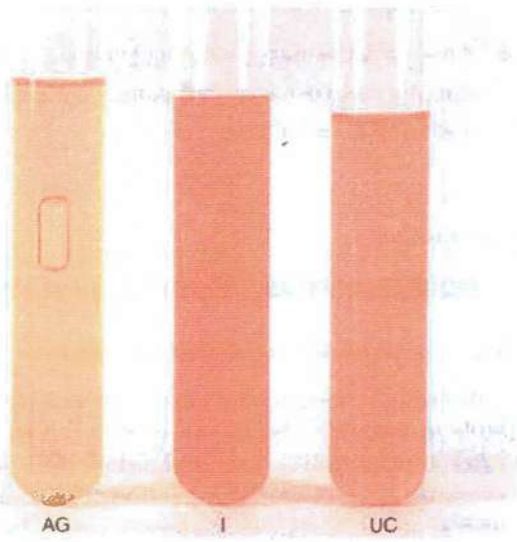

FIGURE 5.3 Carbohydrate utilization test results in phenol red lactose broth tubes. (Left to right): *Escherichia coli*, acid and gas (AG); *Alcaligenes faecalis*, inert (I); uninoculated control (UC).

Lab Procedure

CATALASE TEST

Purpose and Procedure Summary

Bacterial cells produce hydrogen peroxide during aerobic respiration. If hydrogen peroxide accumulates in the cell, it becomes toxic. For this reason, most aerobic and facultatively anaerobic bacteria possess an enzyme called catalase, which breaks down hydrogen peroxide. However, some bacteria, such as species of *Streptococcus* and *Enterococcus*, lack this enzyme. These bacteria are easily distinguished from catalase-positive bacteria, such as species of *Staphylococcus* and *Micrococcus*.

The catalase test is performed by adding 3% hydrogen peroxide to an 18–24 hour culture on an agar slant or glass slide. The culture is observed for the immediate appearance of bubbles.

Tips for Success

- *Do not use media containing blood*, because red blood cells contain catalase.
- *Use a fresh bottle of hydrogen peroxide*, because hydrogen peroxide is unstable. To ensure its chemical reactivity, test the bottle's contents on a known catalase-positive organism.

Expected Results

The catalase enzyme breaks down hydrogen peroxide into water and oxygen (Figure 5.4). The oxygen causes bubbles to form within seconds, indicating a positive test. The absence of bubbles is considered a negative test. Agar slant results are shown in Figure 5.5, while slide test results are shown in Figure 5.6.

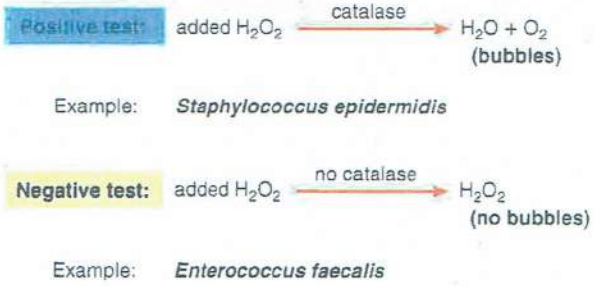

FIGURE 5.4 Catalase test: Reactions and results of positive and negative tests.

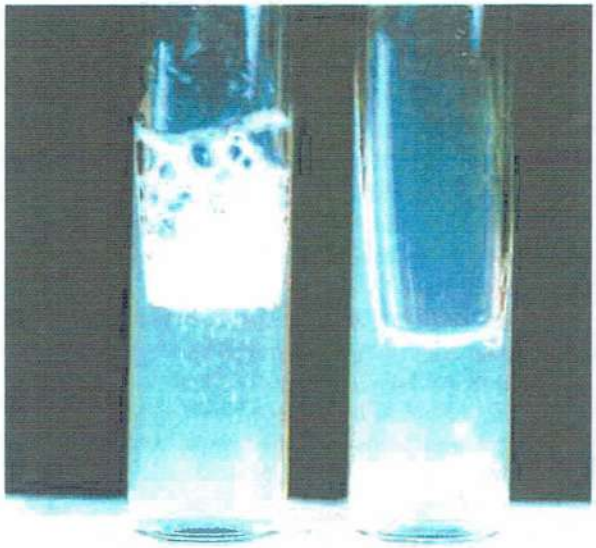

FIGURE 5.5 Results of catalase tests on nutrient agar slants. Each slant received 1 ml of hydrogen peroxide after incubation. Bubbles indicate the presence of the enzyme catalase. *Staphylococcus epidermidis* (left) is catalase positive, while *Enterococcus faecalis* (right) is catalase negative.

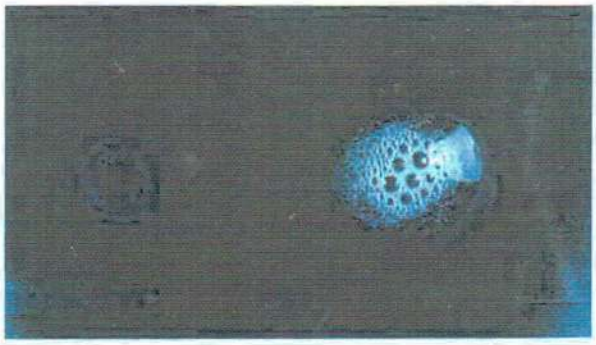

FIGURE 5.6 Results of catalase tests on a glass slide. Each culture suspension received several drops of hydrogen peroxide. *Enterococcus faecalis* (at left) is catalase negative, while *Staphylococcus epidermidis* (at right) is catalase positive.

CITRATE UTILIZATION

Purpose and Procedure Summary

Citrate utilization is the "C" portion of the four IMViC tests, which are used to characterize enteric bacteria. Citrate is an organic molecule that can be utilized by bacteria that produce the enzyme citrase. Citrase is produced by some bacteria, such as *Enterobacter aerogenes* and *Salmonella typhimurium*, but not by others, such as *Escherichia coli* and *Shigella flexneri*.

Simmon's citrate agar plates or slants are used to determine whether an organism produces citrase. This medium contains citrate as the only carbon source and the pH indicator bromthymol blue, which is green at the initial pH of 6.9 and blue at a pH of 7.6.

An isolate is inoculated onto a citrate agar plate or slant with a sterile transfer loop. The plate or slant is incubated at 35°C for 24–48 hours before examination. The medium is observed for growth and color change.

Tips for Success

- *Compare results to an uninoculated control* to aid in the detection of color changes.
- *Look for growth as well as color change,* because only citrase-positive bacteria will grow on a medium that contains citrate as the only carbon source.

Expected Results

Bacteria with the enzyme citrase metabolize citrate to produce alkaline end-products that raise the pH of the medium to 7.6, causing the bromthymol blue to turn blue (Figure 5.7). The presence of growth and a blue color represents a positive test for citrate utilization. The absence of growth and a green color represents a negative test for citrate utilization. These results are shown in Figure 5.8.

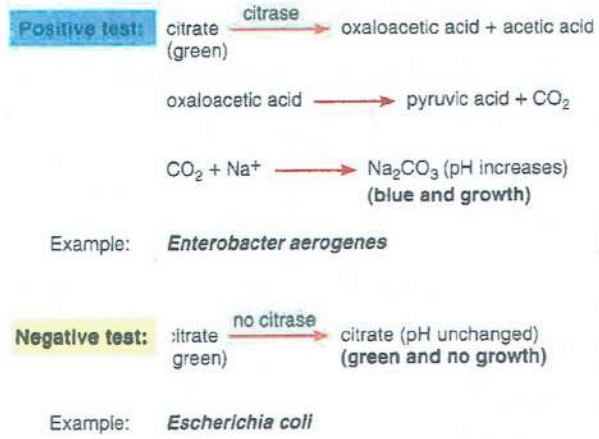

FIGURE 5.7 Citrate utilization: Reactions and results of positive and negative tests.

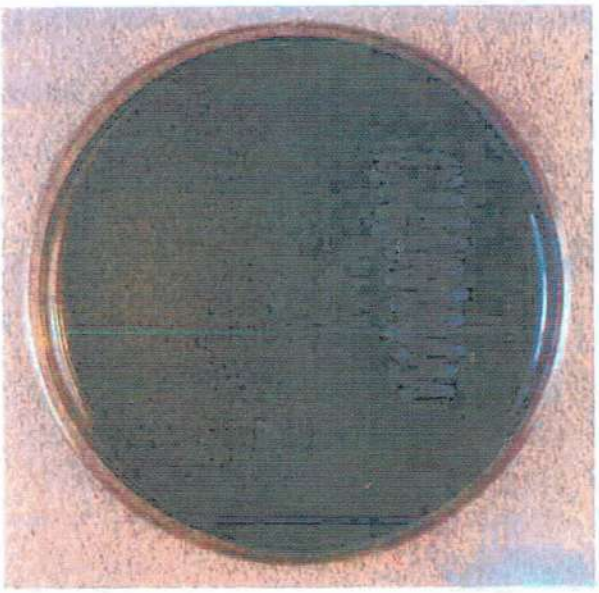

FIGURE 5.8 Simmon's citrate agar plate inoculated with citrate-negative *Escherichia coli* (at left) and citrate-positive *Enterobacter aerogenes* (at right). Citrate-positive organisms display growth and a blue color.

Lab Procedure

DECARBOXYLASE TESTS

Purpose and Procedure Summary

Decarboxylases are bacterial enzymes that remove the carboxyl (—COOH) group from amino acids. This process, called **decarboxylation**, is a step in the metabolism of amino acids. A specific decarboxylase enzyme exists for each amino acid, but only three are used to distinguish enteric bacteria: lysine decarboxylase, ornithine decarboxylase, and arginine decarboxylase. Lysine decarboxylase, for example, is produced by *Enterobacter aerogenes*, but not by *Proteus vulgaris*.

Decarboxylase medium is supplemented with one of the three amino acids lysine, ornithine, or arginine to detect decarboxylation. This medium also contains peptone and beef extract to support bacterial growth. The presence of a coenzyme called pyridoxal enhances decarboxylase activity. Glucose provides a fermentable carbohydrate. The pH indicator, bromcresol purple, turns purple at a pH above 6.8 and yellow at a pH below 5.2.

An isolate is inoculated into a tube with a sterile transfer loop. The tube is covered with 2–3 ml of sterile mineral oil to exclude oxygen. The lack of oxygen promotes the production of acids from glucose fermentation, thus creating the acidic environment necessary for the formation of decarboxylases. The tube is incubated at 35°C for 24–48 hours before examination. The medium is observed for color change.

Tips for Success

- *Compare results to an uninoculated control tube* to determine whether changes have occurred in the medium.
- *Any trace of purple is considered a positive test;* color may vary from light purple to dark purple.

Expected Results

Acids from glucose fermentation lower the pH of the medium, causing the bromcresol purple to turn yellow. The acidic environment promotes the formation of decarboxylases in those bacteria that produce these enzymes. The resultant decarboxylation results in alkaline end-products that raise the pH of the medium, causing the bromcresol purple to turn from yellow to purple (Figure 5.9). A purple color represents a positive test. The medium remains acidic, and yellow, in tubes inoculated with bacteria that lack decarboxylases. A yellow color represents a negative test. These colors, shown in Figure 5.10, are the same regardless of the amino acid tested.

Positive test: glucose → acids (pH decreases) (yellow)

amino acid (lysine) —decarboxylase→ amine + CO_2 (pH increases) **(purple)**

Example: ***Enterobacter aerogenes***

Negative test: glucose → acids (pH decreases) (yellow)

amino acid (lysine) —no decarboxylase→ amino acid (pH remains acidic) **(yellow)**

Example: ***Proteus vulgaris***

FIGURE 5.9 Reactions and results for decarboxylase tests.

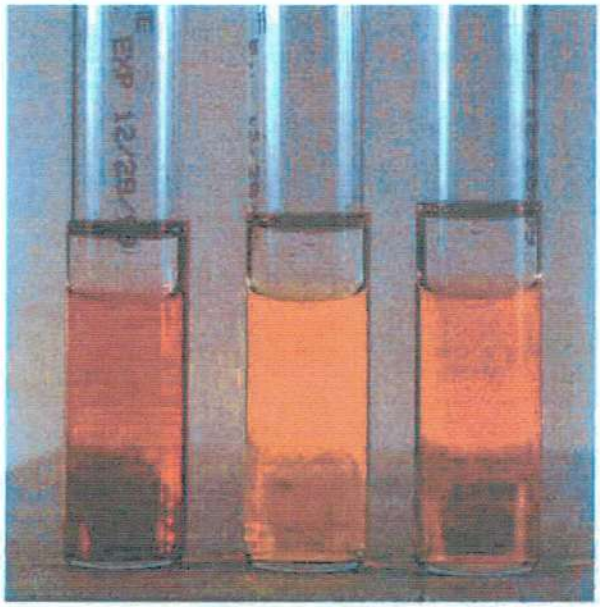

FIGURE 5.10 Results in lysine decarboxylase tubes. (Left to right): *Enterobacter aerogenes*, lysine decarboxylase positive; *Proteus vulgaris*, lysine decarboxylase negative; uninoculated control. The same colors seen here are produced when other amino acids are tested in this medium.

DENITRIFICATION

Purpose and Procedure Summary

The reduction of nitrate to nitrogen gas is called **denitrification**, a process that occurs in bacteria that produce the enzyme **nitrate reductase**. This enzyme allows bacteria to use nitrate as an electron acceptor in the absence of oxygen, resulting in the production of nitrogen gas. Nitrate reductase is produced by certain bacteria, such as *Pseudomonas aeruginosa,* but not by others, such as *Alcaligenes faecalis.*

Nitrate broth tubes containing a durham tube are used to detect an organism's ability to carry out denitrification. Nitrate broth contains beef extract and peptone to support bacterial growth, and KNO_3 as the source of nitrate.

An isolate is inoculated into a tube with a sterile transfer loop. The tube is incubated at 35°C for 24–48 hours before examination. The medium is observed for gas production.

Tips for Success

- Bubbles indicate the presence of nitrogen gas for *nonfermenters only;* fermenters may also produce gas from carbohydrate fermentation.
- Even a small amount of gas represents a positive result for nonfermenters.

Expected Results

Bacteria with nitrate reductase convert nitrate to nitrogen gas. This end-product causes bubbles to form in the durham tube (Figure 5.11). Therefore, the presence of bubbles for nonfermenters is considered a positive test for denitrification. The absence of bubbles indicates a negative test. These results are shown in Figure 5.12.

FIGURE 5.12 Results in nitrate broth tubes. (Left to right): uninoculated control; *Alcaligenes faecalis,* denitrification negative; *Escherichia coli,* denitrification negative; *Pseudomonas aeruginosa,* denitrification positive. The positive tube has bubbles in the durham tube and at the top of the medium.

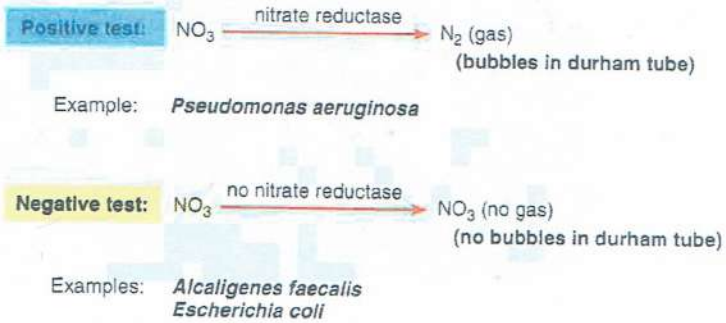

FIGURE 5.11 Positive and negative test results for denitrification.

Lab Procedure

GELATIN UTILIZATION

Purpose and Procedure Summary

Gelatin is a protein that is digested by bacterial extracellular enzymes called **gelatinases**. The end-products of this reaction are amino acids that are transported into the cell for utilization. Some bacteria, such as *Pseudomonas aeruginosa*, produce gelatinases, while others, such as *Alcaligenes faecalis*, do not.

Nutrient gelatin tubes are used to determine whether an organism produces gelatinases. Nutrient gelatin contains beef extract and peptone to support growth, and enough gelatin (120g/l) to cause the medium to gel.

An isolate is inoculated into a nutrient gelatin tube with a sterile transfer needle. The tube is incubated at 35°C for 24–48 hours. The medium is chilled thoroughly in a refrigerator before examination. Chilling is essential because gelatin is liquid at temperatures above 20°C. After chilling, the medium is observed for gelling by carefully tilting the tube to the side.

Tips for Success

- *Compare results to an uninoculated control tube.*
- *Do not shake the tube* when transferring it to the refrigerator; gelatin digestion may have occurred only at the surface.
- *Incubate tubes for up to 7 days* for slow gelatin-utilizers.

Expected Results

If gelatinases are produced, the medium will not gel when chilled because gelatin has been broken down into individual amino acids (Figure 5.13). A liquid medium after chilling represents a positive test for gelatin utilization. A gelled medium after chilling represents a negative test. These results are shown in Figure 5.14.

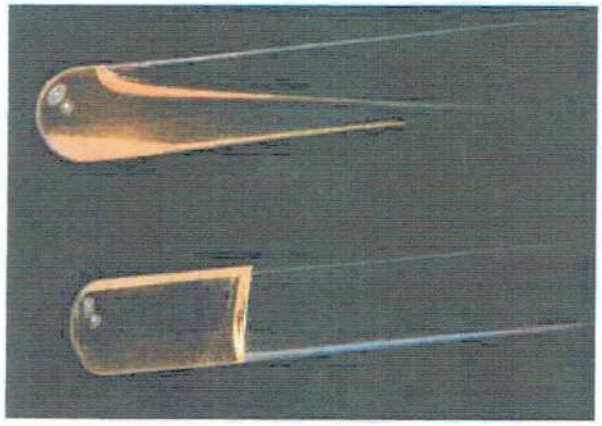

FIGURE 5.14 Nutrient gelatin tubes inoculated with *Pseudomonas aeruginosa* at the top (gelatin positive) and with *Alcaligenes faecalis* at the bottom (gelatin negative). Nutrient gelatin tubes must be thoroughly chilled before examination.

Positive test: gelatin —(gelatinase)→ polypeptides (chains of amino acids)

polypeptides —(gelatinase)→ single amino acids
(liquid medium after chilling)

Example: ***Pseudomonas aeruginosa***

Negative test: gelatin —(no gelatinase)→ gelatin
(gelled medium after chilling)

Example: ***Alcaligenes faecalis***

FIGURE 5.13 Gelatin utilization reactions and test results.

HYDROGEN SULFIDE (H_2S) PRODUCTION

Purpose and Procedure Summary

Bacteria use the enzyme **cysteine desulfurase** to hydrolyze the amino acid cysteine, forming hydrogen sulfide as an end-product. Hydrogen sulfide is also an end-product of the bacterial reduction of **thiosulfate**. Hydrogen sulfide is produced by *Proteus vulgaris* and *Salmonella typhimurium*, but not by *Escherichia coli* and *Shigella flexneri*.

SIM medium, also used to detect motility and indole production, is used to demonstrate hydrogen sulfide production. SIM medium contains peptone and beef extract to support growth. Peptone contains the amino acid cysteine. Sodium thiosulfate is also included in the medium. Ferrous sulfate is the source of iron used to detect hydrogen sulfide production from either cysteine or sodium thiosulfate. Agar is added to make the medium semisolid. **Triple sugar iron agar** and **Kligler iron agar**, described later in this chapter, are also used to demonstrate hydrogen sulfide production.

An isolate is inoculated into a tube with a sterile transfer needle. The tube is incubated at 35°C for 24–48 hours before examination. The medium is observed for blackening.

Tips for Success

- *Read hydrogen sulfide results first* if determining indole production in the same tube.
- *Any blackening of the medium* is considered a positive test.

Expected Results

If cysteine is broken down by cysteine desulfurase (Figure 5.15), or if sodium thiosulfate is reduced, then hydrogen sulfide is produced. Hydrogen sulfide combines with ferrous sulfate to form iron sulfide (FeS), which blackens the medium. Blackening of the medium is considered a positive test. No blackening of the medium is considered a negative test. These results are shown in Figure 5.16.

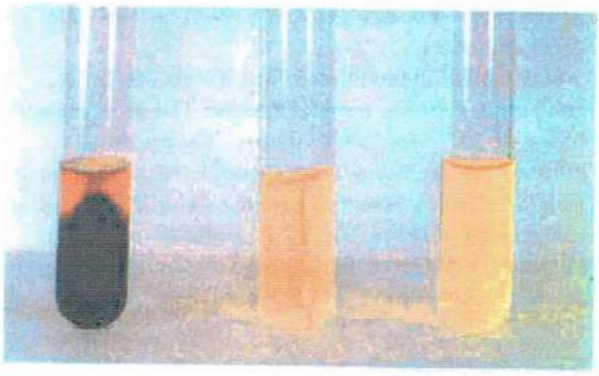

FIGURE 5.16 Results for H_2S production in SIM medium tubes. (Left to right): *Proteus vulgaris*, H_2S positive; *Escherichia coli*, H_2S negative; uninoculated control.

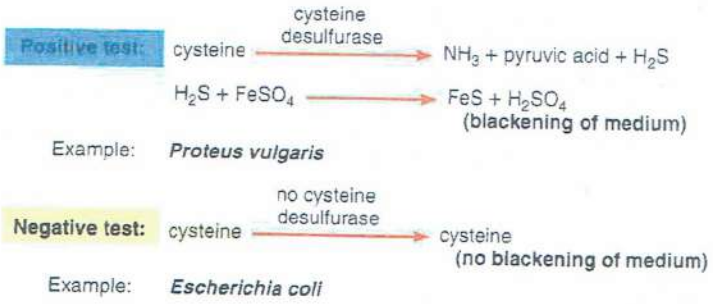

FIGURE 5.15 Positive and negative test results for hydrogen sulfide (H_2S) production.

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.**