# PW 02: BIOCHEMICAL TESTS

# O-F TEST and TSI TEST

## 1. OXIDATIVE-FERMENTATIVE Test (HUGH and LEIFSON Test)

### 1.1. Introduction

Carbohydrates are organic molecules that contain carbon, hydrogen and oxygen in the ratio $(CH_2O)_n$. Organisms use carbohydrate differently depending upon their enzyme complement. The pattern of fermentation is characteristics of certain species, genera or groups of organisms and for this reason this property has been extensively used as method for biochemical differentiation of microbes.

The Oxidative-Fermentative (OF) test was developed by Hugh and Leifson. Prior to this time microbiologists "had observed that some bacteria produced acid from carbohydrates only under aerobic conditions and others produced acid both under aerobic and anaerobic conditions". Hugh and Leifson were the first to refer to the production of acid from carbohydrates under aerobic conditions only, as oxidative. It was noted that the amount of acid produced by bacteria using carbohydrates under aerobic conditions was less than the amount of acid produced during fermentative metabolism. **OF medium** allowed researchers to, for the first time, easily distinguish between Gram-negative bacteria that metabolize glucose oxidatively or fermentatively.

### 1.2. Test objectives

To determine if Gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are nonsacchrolytic and therefore have no ability to use the carbohydrate in the media.

### 1.3. Test principal

Whether an organism is oxidative or fermentative can be determined by using Hugh and Leifson's medium, which contain tryptone and bromothymol blue (an indicator). A sugars, such as glucose is added to the medium which serves as the fermentable carbohydrate.

An organism is inoculated to two tubes of each OF medium. Once inoculated, one tube is overlaid with mineral oil (or melted paraffin) producing an anaerobic environment. The other tube is left open to the air. Growth of microorganisms in this medium is either by **utilizing the tryptone which results in an alkaline reaction (dark blue color)** or **by utilizing glucose, which results in the production of acid (turning bromothymol blue to yellow)**.

### 1.4. Procedure

- Obtain pure, isolated colonies from an 18-24 hours culture.
- For each test organism, inoculate tubes in duplicate. Inoculate by stabbing the agar to approximately 1/4 inch from the bottom.
- Apply sterile mineral oil (or sterile melted paraffin) to one of each duplicate tubes. Tighten the cap of the overlaid tube, and loosen the cap of the non-overlaid tube.
- Incubate both tubes aerobically at 37°C for 24-48 hours, this is recommended for most Gram-negative rods. Slow growing bacteria may take 3 to 4 days before results can be observed.
- Examine tubes daily for color change.

### 1.5. Expected results

#### 1.5.1. Positive results

A positive carbohydrate utilization test is indicated by the development of a yellow color in the medium.

**a. Fermentative results:** bacteria that can ferment glucose give a fermentative result as indicated by acid production in both the open (aerobic) and oil covered (anaerobic) tube. The acid produced (pH 6.0) changes the pH indicator, bromothymol blue, from green to yellow. The semisolid consistency of the medium also allows for detection of motility (Fig. 1).

**b. Oxidative results:** nonfermenting bacteria that metabolize glucose via oxidative metabolism give an oxidative result. This result is indicated by a small amount of acid production in the open tube. The acid produced (pH 6.0) changes the pH indicator, bromothymol blue, from green to yellow. After 24 hours of incubation, a change in pH is observed at the surface of the open tube where growth in the presence of oxygen is observed (Fig. 2a). With prolonged incubation (more than 48 hours), the reduced concentration of agar in the medium allows for the eventual diffusion of the weak acid throughout the whole tube (Fig. 2c). No color change or reaction occurs in the oil-covered tube.

#### 1.5.2. Negative results

Nonsacchrolytic bacteria give a negative OF result (Non-oxidizer/Non-fermenter). The negative result is indicated by no color change in the oil-covered tube and in some cases an increase in pH (pH 7.6) changing the bromothymol blue from green to blue in the top of the open tube. The increase in pH

is due to amine production by bacteria that break down the peptone (protein) in the medium. Other bacteria give a negative result indicated by no growth or color change in the medium (Fig. 3).

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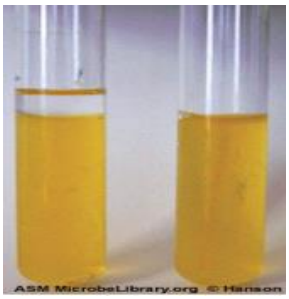
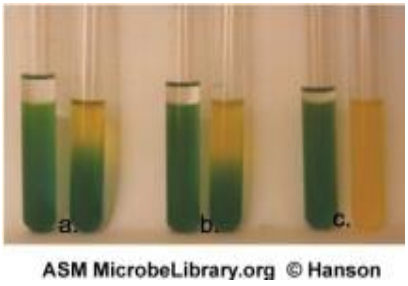
|  |   |  |
| **Fig. 01:** Fermentative results. | **Fig. 02:** Oxidative results. | **Fig. 03:** Negative result. |

### 1.6. Study questions

**1.** What type of bacteria would typically show an oxidative result in the Hugh and Leifson test?

**2.** How do you interpret the test results when the medium turns yellow in both the sealed and unsealed tubes?

**3.** What is the significance of the oil overlay in the Hugh and Leifson test?

**4.** Can the Hugh and Leifson test be used to identify anaerobic bacteria? Why or why not?

**5.** What are some examples of bacteria (2 examples) that would test positive for oxidative metabolism in the Hugh and Leifson test?

**6.** What are some limitations of the Hugh and Leifson test in identifying bacterial metabolic characteristics?

## 2. TRIPLE SUGAR IRON AGAR Test

### 2.1. Introduction

Most bacteria have the ability to ferment carbohydrates, particularly sugars. Among them, each bacterium can ferment only some of the sugars, while it cannot ferment the others. Thus, the sugars, which a bacterium can ferment, and the sugars, which it cannot is the characteristic of the bacteria and thus an important criterion for its identification. The Triple Sugar Iron (TSI) agar is a culture medium named for its ability to test a microorganism's ability to ferment sugars and to produce hydrogen sulfide and gas.

### 2.2. Test objectives

To determine the ability of an organism to ferment glucose, lactose, and sucrose, and their ability to produce hydrogen sulfide and gas. TSI is most frequently used in the identification of the *Enterobacteriaceae*, although it is useful for other Gram-negative bacteria

### 2.3. Test principal

TSI agar slants are used to differentiate between various Gram-negative bacilli. There are seven ingredients in a TSI slant: (1) 0.1% glucose (2) 1.0% sucrose (3) 1.0% lactose (4) peptones (5) phenol red – pH indicator (6) sodium thiosulfate and (7) ferrous sulfate. Gas production can be determined with this test by observing the butt of the tube for gas bubbles or cracks in the agar. If the microorganism produces hydrogen sulfide, it will utilize the sodium thiosulfate as a substrate, producing $H_2S$ which reacts with the ferrous sulfate (the $H_2S$ indicator) to cause a black precipitate. Carbohydrate fermentation is indicated by the production of gas and a change in the color of the pH indicator from orange red to yellow.

Due to the building of acid during fermentation, the pH falls. The acid base indicator Phenol red is incorporated for detecting carbohydrate fermentation that is indicated by the change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In 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.

To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an

alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension. After depletion of the limited dextrose, organisms able to do so will begin to utilize the lactose or sucrose. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely. If the tube is tightly closed, an acid reaction (caused solely by dextrose fermentation) will also involve the slant.

### 2.4. Procedure

- With a straight inoculation needle, touch the top of a well-isolated colony.
- Inoculate TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.
- Incubate with caps loosened at 37°C and examine after 18-24 hours for carbohydrate fermentation, gas production and hydrogen sulfide production.
- Do not incubate longer than 24 hours because the acid reaction in the slant of lactose and sucrose fermenters may revert to an alkaline reaction

### 2.5. Results

**1.** Observe your TSI slant for the following reactions:

**a.** Slant: acid (yellow) or alkaline (red, orange, or pink);

**b.** Butt: acid (yellow) or alkaline (red, orange, or pink);

**c.** Gas production: bubbles or cracks throughout the media;

**d.** Hydrogen sulfide production: indicated by black coloration in the tube.

**Note:** Hydrogen sulfide production is always acidic, even though black coloration masks yellow color of pH indicator.

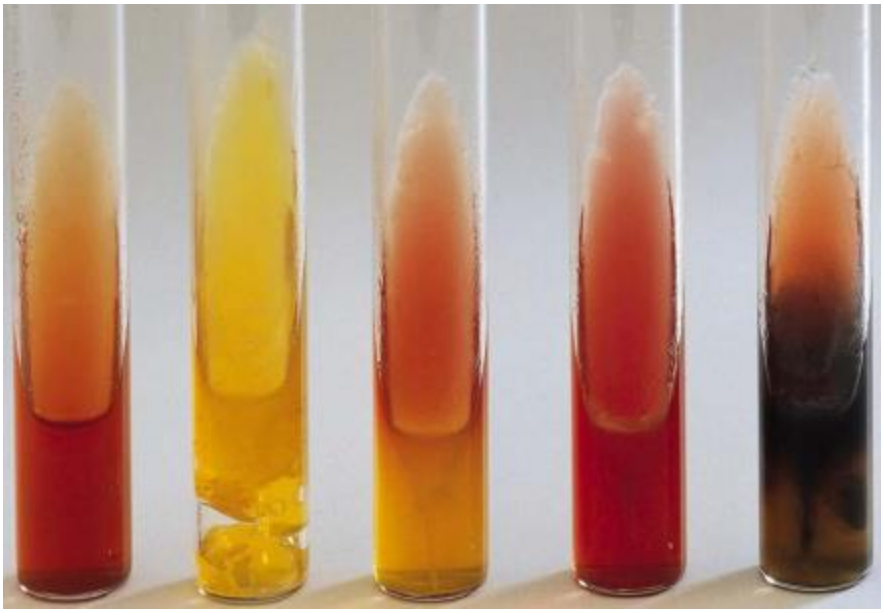
**2.** Interpretation:

**a.** Red (alk) slant and yellow (acid) butt: only glucose fermentation has occurred. Since glucose is present in a 0.1% concentration, the small amount of acid produced by glucose fermentation is rapidly oxidized on the slant, resulting in an alkaline reaction. In the butt, the acid reaction is maintained because of reduced oxygen tension and slower growth of the organisms.

**b.** Yellow (acid) slant and yellow (acid) butt: lactose and/or sucrose fermentation has occurred.

**c.** Red (alk) slant and red or orange-red (alk) butt: no carbohydrate fermentation has occurred. Instead, peptones are catabolized resulting in an alkaline pH due to the production of ammonia.

**3.** Record your results.



**2.6. Study questions**

**1.** What does a red slant and yellow butt in a TSI test result signify?

**2.** Why is the TSI test incubated for 18-24 hours, and what could happen if it is left for a longer period?

**3.** What are the typical biochemical reactions that can be observed using TSI, and how do they help identify different bacterial species?

**4.** Why is the inoculation technique critical in performing a correct TSI test?