

Oxidative-Fermentative Test Protocol

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Information

History

The oxidative-fermentative (OF) test was developed by Hugh and Leifson and outlined in their 1953 paper (4). 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” (4). Production of acid from the metabolism of carbohydrates in aerobic and anaerobic metabolism was at this time defined as fermentation. Hugh and Leifson were the first to refer to the production of acid from carbohydrates under aerobic conditions only, as oxidative (4). 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.

Hugh and Leifson developed OF media to differentiate between these two populations of bacteria (4). The original OF medium contained glucose as the carbohydrate source. The medium was made by increasing the amount of glucose above that found in medium used to detect fermentation and by decreasing the amount of peptone. This medium therefore enhanced the acid production even during oxidative metabolism and decreased the amount of alkaline product produced by the metabolism of peptone. The medium allowed researchers to, for the first time, easily distinguish between gram-negative bacteria that metabolize glucose oxidatively or fermentatively. These characteristics then became important in the identification and taxonomy of gram-negative bacteria (4).

Prior to the work of Hugh and Liefson, taxonomists “paid little attention to the type of carbohydrate breakdown” (3). Cowan and Steele in their 1965 *Manual of the Identification of Medical Bacteria* state that, “this test is one of the most important in the identification of aerobic bacteria. Most genera are composed of bacteria that either oxidize or ferment glucose: when a genus contains some species that attack glucose by oxidation and others by fermentation, there would seem to be reason to reconsider the taxonomy of the genus” (3).

Purpose

The oxidative-fermentative test is used 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.

Theory

The oxidative-fermentative test determines if certain gram-negative rods metabolize glucose by fermentation or aerobic respiration (oxidatively) (6, 8). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromthymol blue indicator in OF media from green to yellow in the presence or absence of oxygen (6,8).

Certain nonfermenting gram-negative bacteria metabolize glucose using aerobic respiration and therefore only produce a small amount of weak acids during the Krebs cycle and Entner Doudoroff (glycolysis) (8). The increased concentration of glucose in the medium enhances the production of these weak acids to a level that can be detected by bromthymol blue indicator. To further enhance detection of these weak acids, this medium contains a reduced concentration of peptones. This reduces the production of amines from the metabolism of amino acids, therefore reducing the neutralizing effect of these products (8). Dipotassium phosphate buffer is added to further promote acid detection (6, 8). Bacteria giving this reaction in OF media are oxidative.

RECIPE

Hugh and Leifson's OF basal medium (6, 8)

Peptone (tryptone)	2.0 g
Sodium chloride	5.0 g
Glucose (or other carbohydrate) ^a	10.0 g
Bromthymol blue	0.03 g
Agar	3.0 g
Dipotassium phosphate	0.30 g

Bring to 1 liter with distilled water. The pH should be adjusted to 7.1 prior to autoclaving (7).

After the medium is autoclaved at 121°C for 15 minutes, a filter sterilized solution of 10% solution of carbohydrate (6, 8) is aseptically added to the medium to a final concentration of 1%. The sterile medium containing the carbohydrate is aliquoted aseptically into sterile test tubes and cooled unslanted as stabs (5). Some procedures call for the addition of 10 g/liter of carbohydrate to the medium prior to sterilization. The medium is then dissolved by heating to a boil on a hot plate or by steaming for 20 minutes prior to aliquoting into test tubes. The tubed medium is then steamed for 20 minutes in place of autoclaving to prevent breaking down of the carbohydrate (7).

OF basal medium is commercially available in a premixed form from biological supply companies. The carbohydrate source is not included and must be added as stated above.

PROTOCOL

I. Oxidative-fermentative test using OF media with glucose

A. Inoculation of media

Two tubes of oxidative-fermentative medium are inoculated by stabbing "half way to the bottom" (8) or ¼-inch from the bottom (6) with the test organism. Overlay one of the two tubes with 1 cm of mineral oil (8) (Fig. 1, 2, 3). This overlay prevents the diffusion of oxygen into the medium and creates an anaerobic condition in the tube.

B. Incubation conditions

Incubation at 35°C for 48 hours is recommended for most gram-negative rods (5, 6). Slow growing bacteria may take 3 to 4 days before results can be observed (6).

C. Interpretation of results

1. 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, bromthymol blue, from green to yellow. The semisolid consistency of the medium also allows for detection of motility. Note hazy growth away from the stab line (Fig. 1) (8).

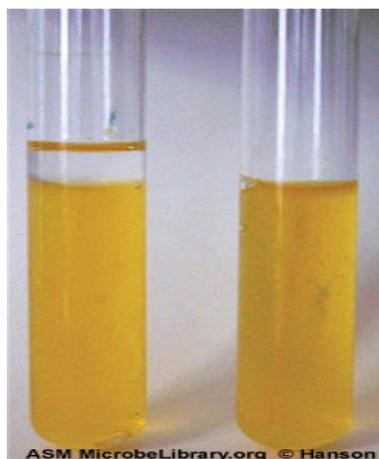
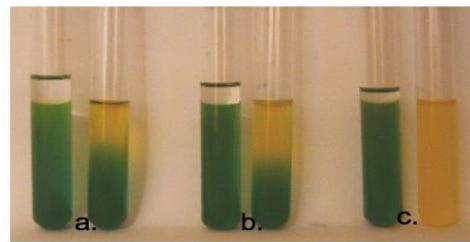


FIG. 1. Oxidative-fermentative test inoculated with *Escherichia coli*. Acid production in both the open and oil-covered tubes indicates a fermentative result. Hazy growth throughout is positive for motility.

See Atlas images for other fermentative results

2. 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, bromthymol blue, from green to yellow (6, 8). After a 24-hour incubation a change in pH is observed at the surface of the open tube where growth in the presence of oxygen is observed (2) (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.



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FIG. 2. Oxidative-fermentative test inoculated with *Pseudomonas aeruginosa*. Acid production in the open tube and not the oil-covered tube indicates an oxidative result. (a) *P. aeruginosa* incubated for 24 hours. Note pH change in the top of the open tube only. (b) *P. aeruginosa* incubated for 48 hours. Note the diffusion of the acid down the tube. (c) *P. aeruginosa* incubated for 5 days. Note the diffusion of the acid throughout the tube. The result is best if read within 24 to 48 hours.

See Atlas images of oxidative results

3. Negative results

Nonsacchrolytic bacteria give a negative OF result. 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 bromthymol blue from green to blue (6, 8) 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 (1, 5). Other bacteria give a negative result indicated by no growth or color change in the medium (5, 6, 8) (Fig. 3).

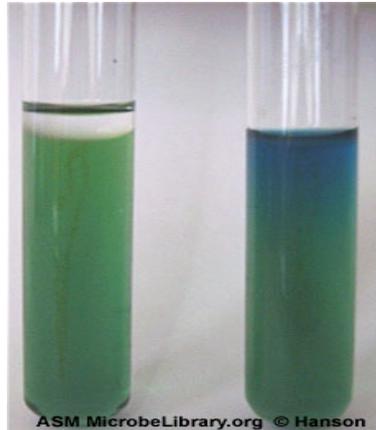


FIG. 3. Oxidative-fermentative test inoculated with *Alcaligenes faecalis*. No color change in the oil-covered tube and color change to alkaline in the open tube indicates a negative result. *A. faecalis* cannot use glucose fermentatively or oxidatively. The blue at the top of the open tube is due to amine production resulting from the metabolism of protein in the media.

See Atlas images for other negative results

II. The oxidative-fermentative test using carbohydrates other than glucose

Nonfermenting gram-negative rods that have been shown to give an oxidative result in an OF glucose test can be further tested for their ability to metabolize other carbohydrates oxidatively. The glucose is replaced by maltose, lactose, mannitol, or sucrose in the medium and only one tube per carbohydrate is inoculated. A heavy inoculum should be used, as many of these nonfermenters are slow growing (6). As the result being detected is based on aerobic respiration no mineral oil or agar layer is used. A positive result is indicated by an acid production and a change in pH in the top of the tube after 24 hours. Some slow growing nonfermenters may take several days to produce enough acid to be detected by the bromthymol blue (6, 8).

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

COMMENTS AND TIPS

1. Results of the OF test should be recorded as fermentative (F), oxidative (O), or negative (-). There is no positive (+) result designation. Negative indicates no fermentative or oxidative metabolism of the carbohydrate in the media.

2. If screw cap tubes are used, do not close them too tightly.
3. Use uninoculated controls with and without oil that are incubated and not incubated as OF media may change color during exposure to incubation temperatures.

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