

# Carbohydrate Fermentation Protocol

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## **Information**      **History**

In 1837 Cagniard-Latour, Schwann, and Kützing independently proposed that yeast, detected during alcoholic fermentation, was a microscopic plant and the responsible etiology for the conversion of sugars to ethyl alcohol and carbon dioxide (12). This theory was strongly opposed by leading contemporary chemists who subscribed to the popular belief that fermentation and putrefaction were both strictly chemical processes (12). The controversy over the matter continued for years, until Pasteur, a chemist by training, was able to convince the scientific community that “all fermentation processes are the results of microbial activity” (11, 12).

Pasteur’s work on fermentation was aided providentially by his involvement with “The distillers of Lille,” where alcohol was produced from beet sugar. This local industry had encountered difficulties with their production and being aware of Pasteur’s interest in the matter, solicited his assistance. Pasteur’s investigations showed that the alcoholic fermentation had been replaced, at least in part, by another kind of fermentative process, which resulted in the conversion of the sugar to lactic acid (12). Pasteur found that in the vats where lactic acid had been formed, a different or “new” yeast was present. Successive inoculation of these small rods and spheres on the same medium always resulted in the production of lactic acid and more rods and spheres. He concluded that these new microorganisms must specifically convert sugar to lactic acid as they grew (11, 12).

For the next 20 years (1856-1876), Pasteur continued to test many fermentative processes. He was able to show that fermentation was always accompanied by the growth of microorganisms and that each particular type of chemical fermentation, defined by its primary chemical end-product, was the result of a particular microorganism’s involvement. These “specific types of microorganisms” could be characterized by their size and shape and further distinguished by the specific environmental condition in which they thrived (12).

Pasteur was also the first to recognize that some microorganisms were able to obtain energy by breaking down organic compounds, and he was able to show that the amount of growth was directly related to the amount of energy that could be produced by the breakdown of an organic compound (12). He also noted that fermentation was less efficient than other biochemical pathways at producing energy (11, 12).

The term “fermentation” is often used to describe the breaking down or catabolism of a carbohydrate under anaerobic conditions. Therefore, bacteria capable of fermenting a carbohydrate are usually facultative anaerobes (6, 7, 9). It should also be noted that while the terms “carbohydrate” and “sugar” are often used interchangeably, the term sugar might not indicate the true chemical composition of certain substrates such as in the case of dulcitol and mannitol (5).

## Purpose

Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific carbohydrate. Fermentation patterns can be used to differentiate among bacterial groups or species (2, 6, 8, 9). For example, all members of the *Enterobacteriaceae* family are classified as glucose fermenters because they can metabolize glucose anaerobically (8, 9). Within this family however, maltose fermentation differentiates *Proteus vulgaris* (positive) from *Proteus mirabilis* (negative) (8, 9).

While fermentation tests can be performed on microorganisms other than bacteria, this protocol only addresses fermentation of carbohydrates by bacteria.

## Theory

During the fermentation process, an organic substrate serves as the final electron acceptor (8, 12). The end-product of carbohydrate fermentation is an acid or acid with gas production (6, 9). Various end-products of carbohydrate fermentation can be produced. The end-product depends on the organisms involved in the fermentation reaction, the substrate being fermented, the enzymes involved, and environmental factors such as pH and temperature (12). Common end-products of bacterial fermentation include lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide, and hydrogen (6, 8, 9).

Fermentation reactions are detected by the color change of a pH indicator when acid products are formed. This is accomplished by adding a single carbohydrate to a basal medium containing a pH indicator. Because bacteria can also utilize peptones in the medium resulting in alkaline by-products, the pH changes only when excess acid is produced as a result of carbohydrate fermentation (5).

Phenol red is commonly used as a pH indicator in carbohydrate fermentation tests because most of the end-products of carbohydrate utilization are organic acids (8). However, other pH indicators such as bromocresol/bromocresol purple, bromothymol/bromothymol blue, and Andrade's can be used (Table 1).

**Table 1** pH indicators for carbohydrate fermentation media

pH indicator	Uninoculated media		Acid (fermentation)		Alkaline (negative)	
	pH	Color	pH	Color	pH	Color
Andrade's	7.1-7.2	Light pink	5.0	Pink-red	12.0-14.0	Yellow, colorless
Bromocresol purple	7.4	Deep purple	5.2	Yellow	6.8	Purple
Bromothymol blue	7.0	Green	6.0	Yellow	7.6	Deep Prussian blue
Phenol red	7.4	Reddish-orange	6.8	Yellow	8.4	Pink-red

Fermentation tubes or Durham tubes are used to detect gas production (6, 8, 9). These small, slender test tubes (6 by 50 mm) are inserted upside down inside larger (13 by 100 mm) test tubes. After sterilization, Durham tubes become filled with the media. If gas is produced, it will be trapped inside the Durham tube and is evident by the presence of a visible air bubble.

Three characteristic reactions can be observed from the fermentation of a specific carbohydrate (8). Based on these reactions, bacteria are classified as:

- Fermenter with acid production only
- Fermenter with acid and gas production
- Nonfermenter
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## RECIPE

### Phenol red carbohydrate broth (3, 8, 13)

Trypticase or proteose peptone no. 3	10 g
Sodium chloride (NaCl)	5 g
Beef extract (optional)	1 g
Phenol red (7.2 ml of 0.25% phenol red solution)	0.018 g
Distilled and deionized water	1,000 ml
Carbohydrate	10 g

Prepare broth medium by mixing all ingredients in 1,000 ml of distilled and deionized water; heat gently to dissolve (8). Use a single carbohydrate for each batch of medium prepared. Fill 13- by 100-mm test tubes with 4 to 5 ml of phenol red carbohydrate broth (8). Insert an inverted fermentation tube or Durham tube to detect gas production. Sterilize media by autoclaving for 15 minutes at 116 to 118°C (8, 13). The sterilization process will also drive the broth into the inverted fermentation tube or Durham tube. When using arabinose, lactose, maltose, salicin, sucrose, trehalose, or xylose, autoclave at 121°C for only 3 minutes as these carbohydrates are subject to breakdown by autoclaving (8). Broth medium will be a light red color. The final pH should be  $7.4 \pm 0.2$ .

Alternatively, mix all ingredients except carbohydrate in 800 ml of distilled and deionized water to prepare the base broth. Heat while mixing. Fill 13- by 100-mm test tubes with 4.5 ml of base broth. Insert an inverted fermentation tube or Durham tube to detect gas production. Sterilize base broth by autoclaving for 15 minutes at 121°C (8, 13). Cool sterilized base broth in a 42 to 50°C water bath before adding carbohydrates (8). Prepare carbohydrate solution by dissolving 10 grams of desired carbohydrate in 200 ml of distilled and deionized water and sterilize by filtering the solution through a bacteria-retaining membrane filter with a 0.45- $\mu\text{m}$  pore size (8).

Aseptically add 0.5 ml of filtrate to each tube of sterilized and cooled broth. Shake gently to mix. Broth medium will be a light red color. The final pH should be  $7.4 \pm 0.2$ .

Cool all media before use. Store prepared media at 4 to 10°C (4). Refrigerated media has a shelf life of approximately 6 to 8 weeks (8). Run controls to check for possible breakdown of carbohydrates.

### Recipe notes

Generally, the carbohydrate concentration used for carbohydrate fermentation testing is 0.5% or 1.0%. The recipe provided will produce a 1.0% carbohydrate broth. It is preferred to use a 1.0% carbohydrate concentration to prevent the reaction from reverting due to the rapid depletion of the carbohydrate by some microorganisms (3).

A modified Andrade's pH indicator is recommended instead of the phenol red pH indicator when prolonged incubation is required (Streptococci and Enterococci) or when testing aerobic Gram-negative bacilli such as those in

the *Enterobacteriaceae* family (6, 8).

Add NaCl for a final concentration of 2% to 3% when preparing fermentation media for fermentation studies on halophilic *Vibrio* species (13).

For fermentation studies on *Neisseria* species enrich broth by adding 5% sterile rabbit serum (3) or use cystine-tryptic agar (a semisolid medium with only 0.25% agar) for the base broth (10, 14). Due to the semisolid nature of cystine-tryptic agar medium, the entire medium may not turn yellow. Therefore, the appearance of any yellow color is indicative of a positive reaction. Please note that the color change may only occur at the top of the medium. If no color change is evident at 24 hours, recheck results at 48 hours and then again at 72 hours.

## PROTOCOL

### A. Inoculation of media

Aseptically inoculate each test tube with the test microorganism using an inoculating needle or loop. Make sure to avoid the Durham tube, if present. Swirl the tube gently to mix contents. Avoid contact of liquid with tube cap (8).

Alternatively, inoculate each test tube with 1 to 2 drops of an 18- to 24-hour broth culture of the desired organism (6, 8). Examples of broth media that could be used include brain heart infusion, nutrient agar, and tryptic soy agar.

### B. Incubation

Incubate tubes at 35 to 37°C for 18 to 24 hours (6, 8). Longer incubation periods may be required to confirm a negative result (6, 8).

### C. Interpretation of results

#### 1. Fermentation results

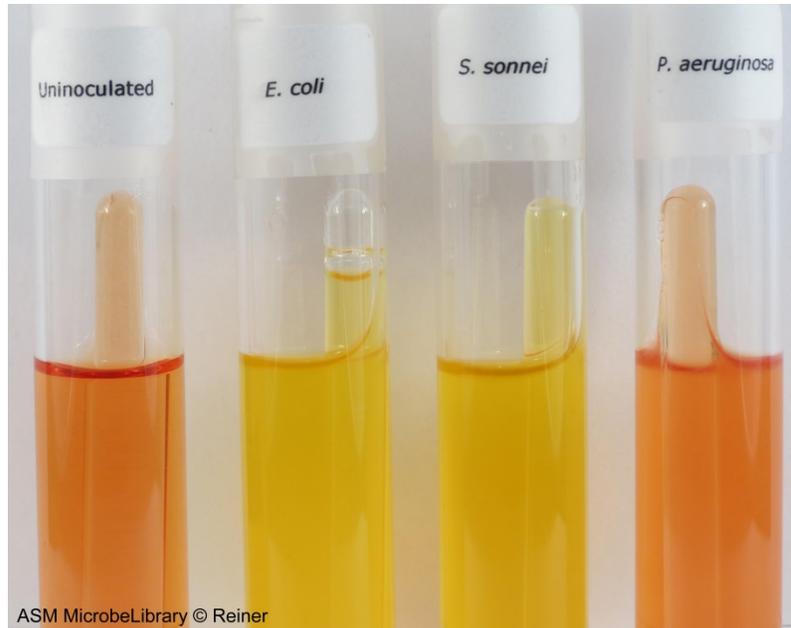
When using phenol red as the pH indicator, a yellow color indicates that enough acid products have been produced by fermentation of the sugar to lower the pH to 6.8 or less. A delayed fermentation reaction may produce an orange color. In such cases, it is best to reincubate the tube (8). Refer to Table 1 for other commonly used pH indicators and their corresponding colors.

#### 2. Gas production results

Bubbles trapped within the Durham tube indicate the production of gas. Even a single bubble is significant and denotes evidence of gas production (6, 8). No bubbles within the Durham tube indicate a non-gas-producing or anaerogenic organism (8).

#### 3. Negative results

A reddish or pink color indicates a negative reaction (8). In negative tubes, the presence of turbidity serves as control for growth. A reddish or pink color in a clear tube could indicate a false negative.



**FIG 1** Peptone media with phenol red indicator. From left to right: uninoculated tube; *Escherichia coli*, a glucose fermenter with gas production (visible air bubble in the inverted Durham tube); *Shigella sonnei*, a glucose fermenter without gas production (no visible air bubble in the inverted Durham tube); *Pseudomonas aeruginosa*, nonfermenter.



**FIG 2** Peptone media with bromocresol purple indicator. From left to right: uninoculated tube; *E. coli*, glucose fermenter with gas production (visible air bubble in the inverted Durham tube); *S. sonnei*, glucose fermenter without gas production (no visible air bubble in the inverted Durham tube); *P. aeruginosa*, nonfermenter.

### 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

- Always use strict aseptic techniques. If possible, perform test under a biosafety hood.
- Any yellow in the main tube and/or the Durham tube is significant and indicates fermentation.
- Not all fermentation reactions produce gas.
- Check the Durham tube before inoculating to ensure that there is no bubble inside the tube. To avoid false positives for gas production, do not use tubes with preexisting bubbles. If an uninoculated tube has a bubble in the Durham tube, proceed as follows: (i) Kim cap, discard tube; (ii) screw cap, to remove bubble, make sure cap is tight, invert tube, dislodge bubble, quickly return tube to upright position. After inoculation, loosen cap prior to incubation to prevent pressure build-up.
- Avoid excessive agitation of tubes during the entire inoculation and incubation process. Do not incubate tubes in a shaking incubator. Unnecessary agitation may inadvertently introduce air bubbles in the Durham tube and cause a false positive reaction for gas production. If air is introduced in the Durham tube by excessive agitation, discard the tube appropriately and repeat with a fresh tube and sterile media.
- For the teaching lab, a single set of controls for (i) uninoculated and incubated, (ii) positive for fermentation with gas, (iii) positive for fermentation without gas, and (iv) negative for fermentation will demonstrate to students the possible variations in color change. Controls incubated at varying lengths of time (e.g., 48 or 72 hours) are also helpful when class meeting times impose longer than 24-hour incubation periods.
- Be careful not to confuse organism pigmentation (e.g., *Serratia marcescens*) with a color change in the medium. Be aware that some organisms such as *Pseudomonas aeruginosa* may secrete pigments into the medium.
- Because the color of the media in the tubes looks identical regardless of the carbohydrate used, the tubes must be clearly identified and labeled. A color coding system, by color of cap or colored tape depending on the carbohydrate used, is recommended.

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