METHYL RED-VOGES PROSKAUER (MR-VP) TEST

Principle and Purpose

The enteric bacteria comprise those microbes that are often part of the normal flora of the intestinal tracts of animals. All enteric bacteria ferment glucose. Some species produce small amounts of organic acids via glucose fermentation. Usually, such species, typified by the genera *Enterobacter* and *Klebsiella*, accumulate 2,3-butanediol and acetoin as a significant glucose fermentation products. In contrast, other enteric species, like *Escherichia coli*, liberate relatively high concentrations of acidic substances including lactate, acetate, and formate to name a few. Often, these substances are produced in combination, which has led to defining such microbes as "mixed acid fermenters". Based upon the differences in fermentation products, these two groups of enterics can be differentiated by the Methyl Red-Voges Proskauer (MR-VP) test.

Methyl red is a pH indicator. In the presence of highly acidic conditions, as generated by mixed acid fermenters, the indicator appears read (Fig. 1). As the pH rises, i.e., becomes alkaline, methyl red turns yellow. Hence, the addition of methyl red to a culture of a mixed acid fermenter grown in MR-VP broth would make it appear red, that is, a positive MR reaction. In contrast, a non-mixed acid fermenter grown in MR-VP broth would cause the dye to appear yellow, a negative MR reaction. It is important to note that resulting color of a postive and negative MR test is opposite of tests that incorporate phenol red as a pH indicator (phenol red is yellow under acidic conditions and red under alkaline conditions).

Figure 1. Methyl Red Test Using MR-VP Media. The left image depicts the positive methyl read reaction of *Escherichia coli*. The right image shows the negative methyl red reaction of *Enterobacter cloacae*.

Non-mixed acid fermenters can readily detectable using the Voges-Proskauer (VP) test. In this test, alpha-napthol (termed Barritt's A reagent) and potassium hydroxide (Barritt's B reagent) are added to a culture of a grown in MR-VP broth. The potassium hydroxide causes acetoin to form diacetyl, which in the presence of Barritt's A reagent forms a red complex (Fig. 2). The latter is indicative of a positive VP reaction. The absence of a red color is considered a negative VP result. It is generally true that a MR positive enteric species is VP negative, and vice versa.

Figure 2. Voges-Proskauer Test Using MR-VP Media. The left image shows the positive Voges-Proskauer reaction of *Enterobacter cloacae*. The right image depicts the negative Voges-Proskauer reaction of *Escherichia coli*.

In this exercise, students will examine the fermentation patterns of selected bacterial species. Specifically, students will examine how these bacteria ferment glucose to generate either mixed acid products or 2,3-butanediol and acetoin. Students will employ a commercially available MR-VP broth (Hardy Diagnostics).

Learning Objectives

Upon completion of this exercise, a student should be able to:

- Understand the various underlying mechanism of methyl red and Voges-Proskauer tests;
- Properly conduct the methyl red and Voges-Proskauer tests; and
- Accurately interpret the results of these tests.

Materials Required

The following materials are necessary to successfully conduct this exercise:

Organisms - The following organisms should be provided as 24-48 hour-old TSA slant cultures:

- *Enterobacter cloacae* (ATCC 23355) [abbreviated as *Ent. cloacae*]
- *Escherichia coli* (ATCC 25922) [abbreviated as *E. coli*]
- *Klebsiella oxytoca* (ATCC 49131) [abbreviated as *K. oxytoca*]
- *Shigella flexneri (*ATCC 12022) [abbreviated as *S. flexneri*]

Media and Reagents

- Methyl Red-Voges Proskauer (MR-VP) broth [5 ml] (Cat. No. K37; Hardy Diagnostics; https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/MR-VPBroth.htm)
- Methyl Red Test Reagent [0.2% methyl red in 57% ethanol] (Cat. No. Z117; Hardy Diagnostics; https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/MR-VPBroth.htm)

Media and Reagents (cont.)

- Voges-Proskauer Reagent A [Barritt's Reagent A; 5% alpha-naphthol in absolute ethanol] (Cat. No. Z91; Hardy Diagnostics; https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/Voges-ProskauerTestRgnts.htm)
- Voges-Proskauer Reagent A [Barritt's Reagent B; 40% potassium hydroxide] (Cat. No. Z92; Hardy Diagnostics; https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/Voges-ProskauerTestRgnts.htm)

Materials

- Test tubes, 13 x 100 mm
- Sterile plastic bulbs pipettes

Procedures

Students shall review and use the BIOL 3702L Standard Practices regarding the labeling, incubation, and disposal of materials.

Initial Cultures (Day 1)

1) Obtain four (4) MR-VP broth tubes and allow them to warm to room temperature before use.
2) Label one of the tubes as '*E. coli*', a second as '*Ent. cloacae*', a third as '*S. flexneri*', and the remaining tube as '*K. oxytoca*'. Be sure to add other identifying information as appropriate.
3) Using a microbiological loop and aseptic technique, lightly inoculate the labeled tube of medium with cells from the TSA slant culture matched to the appropriate bacterium.
4) Incubate all the tubes at 37°C for 18-24 hours. Be sure that the screw-cap lid is loosened, but not to the degree at which it can fall off.

Voges-Proskauer (VP) Test (Day 2)

Note of Precaution: The reagents used in the VP test can cause irritation and/or burns to the skin. Use appropriate handling procedures with these materials. Avoid contact with the skin. If contact does occur, rinse thoroughly with copious volumes of water.

1) Remove the tubes from the incubator. Using a separate sterile, plastic bulb pipet for each MR-VP culture, aseptically transfer 1 ml to separate, clean, and appropriately labeled test tube. These tubes will be used in step 4 below.
2) Return the original cultures to 37°C for an additional 18-24 hours of incubation.
3) To each 1 ml aliquot prepared in step 5, add 15 drops of Voges-Proskauer Reagent A (Barritt's A) followed by 5 drops of Voges-Proskauer Reagent B (Barritt's B). Gently shake the tube to provide atmospheric oxygen.
4) Allow the tube to set at room temperature for 10-15 minutes undisturbed.
5) Observe the tube for the formation of a pink-red color.

Interpretation of Results: The formation of the pink-red color is taken as a positive result. The test may be read for up to, *but not longer than*, one hour following the addition of the Voges-Proskauer Reagents A and B.

Record your observations on the report sheet attached to this exercise.

Note: If the results appear negative (no red color produced), the test can be repeated using the re-incubated broth (step 3). There should be enough culture broth remaining after the Methyl Red Test (see below) to perform one repeated VP Test. The repeat test can be performed on the broth culture up to 5 days post inoculation.

Methyl Red (MR) Test (Day 3)

1) Remove the tubes (see step 3 above) from the incubator after 48 hours. Using a separate sterile, plastic bulb pipet for each MR-VP culture, aseptically transfer 2.5 ml to separate, clean, and appropriately labeled test tubes.
2) Discard each used bulb pipet in the appropriate waste bin.
3) To each tube, add five drops of Methyl Red Reagent.
4) Observe the tubes for the immediate development of a red color.

Interpretation of Results: A positive MR test is indicated by the development of a stable red color on the broth surface, whereas negative test result is depicted by a yellow color on the broth surface.

Record your observations on the report sheet attached to this exercise.

Student Name: _____

COMPLETE THE FOLLOWING TABLE BASED UPON YOUR OBSERVATIONS

Observations	Bacteria Tested			
	Escherichia coli	*Enterobacter cloacae*	*Shigella flexneri*	*Klebsiella oxytoca*
Color After Adding Methyl Red Reagent				
Methyl Red Positive/Negative?				
Color after Adding Voges-Proskauer Reagent				
Voges-Proskauer Positive/Negative?				

Discussion Question

Some enteric bacteria (e.g., *Hafnia alvei*) are variable in their VP reactions. When grown at 37°C, these microbes are VP negative. But when grown at lower temperatures (25°C to 30°C) these bacteria are VP positive. Speculate why this is so. (This answer may require some additional literature research.)