

Analysis of Formate Dehydrogenase

References:

Properties of formate dehydrogenase in *Methanobacterium formicicum*, Neil L. Schauer and James G. Ferry, *Journal of Bacteriology*, (1982), Vol. 150 No. 1, p.2

Metabolism of Formate in *Methanobacterium formicicum*, Neil L. Schauer and James G. Ferry, *Journal Bacteriology*, (1982), Vol. 142 No. 3 p.800

Materials needed:

A. Gases

- N₂ gas
- 90% N₂, 5% CO₂, 5% H₂ gas

Glassware and equipment

- 24-ml culture tubes
- butyl stoppers
- sealers/crimpers
- serum bottles
- anaerobic chamber

B. Glassware and equipment

- Quartz cuvettes (Biochrom 2.5ml Spectrophotometer Cuvettes)
- Spectrophotometer (Biochrom WPA S800 visible spectrophotometer range 300-700nm)
- High speed centrifuge (Eppendorf Centrifuge 5415D)
- 500 mL beaker

C. Chemicals

- 2-mercaptoethanol
- Methyl viologen
- 0.1M NaOH
- 0.1M KH₂PO₄
- 10 μmol of sodium formate

D. Preparation of reagents

Potassium phosphate buffer (pH 7.5)

1. 0.1M NaOH

Add 2 grams NaOH to a 500mL beaker. Fill to full volume with distilled water (The molecular mass of NaOH = 39.99711 g/mol (about 40 g/mol).

2. 0.1M KH₂PO₄

Add 6.8 grams KH₂PO₄ to a 500mL beaker. Fill to full volume with distilled water. (Molar mass of KH₂PO₄ = 136.085541 g/mol)

Preparation of potassium phosphate buffer (pH 7.5):

Add 82.2 mL of 0.1M NaOH to 100 mL 0.1M KH_2PO_4 to a 500 mL beaker.

E. Preparation of growth media

1.) Phosphate-buffered medium

Composition per 985 ml of distilled water:

CaCl ₂	1 g
NH ₄ Cl	1 g
MgCl ₂ .6H ₂ O	0.2 g
Wolfe's mineral solution	10 ml (see recipe below)
Wolfe's vitamin solution	5 ml (see recipe below)
Yeast extract	0.5 g
Reazurin	1 ml

Media preparation:

Add components except for the carbon source (i.e. glucose) and bring the volume to 985 ml. Adjust the pH to 7.2-7.4 with NaOH. Gently heat and boil. Cool medium and distribute anaerobically in test tubes in 10 ml volumes using 100% N₂ until the reazurin indicator. Stopper the tubes anaerobically. Autoclave for 15 min at 15 psi pressure, 121°C. Aseptically add 0.2 ml of NaS.9H₂O solution and 0.2 ml of phosphate buffer solution to each tube. Mix thoroughly. **Note:** These two solutions and other additions to the medium should be added in syringes. All solutions added after medium sterilization must be autoclaved or filter sterilized separately in sealed serum vials.

Wolfe's vitamin solution

Composition per liter:

Pyrodoxine-HCL	10 mg
Thiamine-HCL	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium pantothenate	5 mg
<i>p</i> -Aminobenzoic acid	5 mg
Thioctic acid	5 mg
Biotin	2 mg
Folic acid	2 mg
Cyanocobalamin	100g

2) Preparation of Wolfe's vitamin solution

Add above components to distilled/deionized water and bring the volume to 1L. Mix thoroughly and filter sterilize.

Wolfe's mineral solution

Composition per 1 liter:

MgSO ₄ .7H ₂ O	3 g
Nitroloacetic acid	1.5 g
NaCl	1 g

MnSO ₄ .H ₂ O	0.5 g
FeSO ₄ .7H ₂ O	0.1 g
CoCl ₂ .6H ₂ O	0.1 g
CaCl ₂	0.1 g
ZnSO ₄ .7H ₂ O	0.1 g
CuSO ₄ .5H ₂ O	0.01 g
AlK(SO ₄) ₂ .2H ₂ O	0.01 g
H ₃ BO ₃	0.01 g
Na ₂ MoO ₄ .2H ₂ O	0.01 g

3) Preparation of Wolfe's mineral solution

Add nitroloacetic acid to 500 ml distilled/deionized water. Dissolve by adjusting pH to 6.0 with KOH. Add remaining components. Add distilled/deionized water to 1.0 L.

4) Reducing agent:

Composition per 20 ml (2.5%)

NaS.9H₂O 0.5 g

Preparation of reducing agent

Add sodium sulfide NaS.9H₂O to distilled/deionized water and bring volume to 20 ml. Mix thoroughly. Gas with 100% N₂ for 20 min and cap with a rubber stopper. Autoclave for 15 min at 15 psi pressure, 121°C. Use fresh prepared solution. Cool medium to room temperature.

Note: Reducing agent must be prepared fresh each time there is an inoculation, the reducing agent will remain fresh for a week (it will start to lose its effectiveness if not used within that time period).

Phosphate buffer solution

Composition per 100 ml:

KH₂PO₄ 15%

Na₂HPO₄ 29%

5) Preparation of buffer solution

Gas with 100% N₂ for 20 min and cap with a rubber stopper. Autoclave for 15 min at 15 psi pressure, 121°C.

Glucose solution (400 mM)

When glucose is use as a source add 20 mM per tube containing 10 ml of Phosphate-buffered medium. Make sure that a stock solution (400 mM) is prepared. Gas glucose solution with 100% N₂ for 20 min. Filter sterilized in serum bottles. Capped with rubber stopper. Add 0.5 ml of filter sterilized glucose in per 10 ml of phosphate-buffered medium (final amount should be 20 mM).

Sodium formate (25% w/v stock solution)

Prepare 100 ml of 25% (3.68 M) by dissolving 25g of sodium formate in 100 ml distilled water in a serum bottle. Gas the reagent with N₂ for 30 minutes, seal with stopper and crimp. Autoclave for 15 min and store in dark cool place.

Sodium selenite (1 mM stock solution)

The final concentration of sodium selenite (NaSe) in the medium should be 1 μ M. To prepare the stock solution, dissolve 0.174g of sodium selenite in 990ml of water and bring the volume up to 1L. Prepare a working solution by diluting 10ml of the 1 mM stock and dilute to 100 ml with distilled water. The concentration of the working solution should be 0.1 mM. Gas the solution with N₂ for 30 minutes and seal with stopper and crimp. Autoclave for 15 min and store in dark cool place.

Buffers for adjusting pH

- 0.1N KOH for adjusting pH
- NaOH for adjusting pH

Lysis Buffer

Tris pH 8.0	100mM
NaCl	150m
Lysozyme	1mg/ml
antiprotease DNAse I	1mg/ml
MgCl ₂	2mM

Prepare 25ml of Lysis buffer for our 20 isolates. (1.25ml lysis buffer *20)

Tris pH 8.0	0.0025ml
NaCl	0.21915g
Lysozyme	25mg/ml
antiprotease DNAse I	25mg/ml
MgCl ₂	0.0101665g

Note: The lysis buffer must be stored at 4°C.

Standard Reaction Mixture with Methyl Viologen (in micromoles) per sample

Composition per 0.75 ml:

- | | |
|---------------------------------------|--------------|
| • 2-mercaptoethanol | 15 μ mol |
| • Potassium phosphate buffer (pH 7.5) | 45 μ mol |
| • Methyl viologen (MV) | 15 μ mol |

Note: Amount of solution that should be prepared is 0.75ml x # of isolates to be assayed.

For 60ml of Standard Reaction Mixture:

add the following to 20ml of distilled water in a serum bottle

- 84.1 μ mL of 2-mercaptoethanol
- 3.6mL of 1M Phosphate Buffer (pH7.5)
- 308.5mg of Methyl viologen

Preparation of Anaerobic Chamber

1.) Place the following items in anaerobic chamber:

- 70% ethanol
- Spectrophotometer

- thermometer
- 3ml syringe
- 20G x 1" needle
- prepared lysis buffer
- standard reaction mixture
- crimp remover
- centrifuge rack
- indicator strips should be used to ensure that the conditions within the chamber are anaerobic (BBL dry anaerobic indicator strips)
- Sodium formate
- 250 beaker
- Waste bottle (methyl viologen waste)

2.) Ensure that the chamber shell is flushed with the base and no leaks are present.

3.) Ensure that the condenser is properly connected to the unit.

NOTE – The condenser socket is extremely sensitive and can be disconnected easily.

4.) Ensure that the inside and outside doors are properly sealed.

5.) Check O-rings and ensure that the sleeves are not leaking or torn.

6.) Connect the 90% N₂, 5% CO₂, 5% H₂ gas tank to the anaerobic chamber.

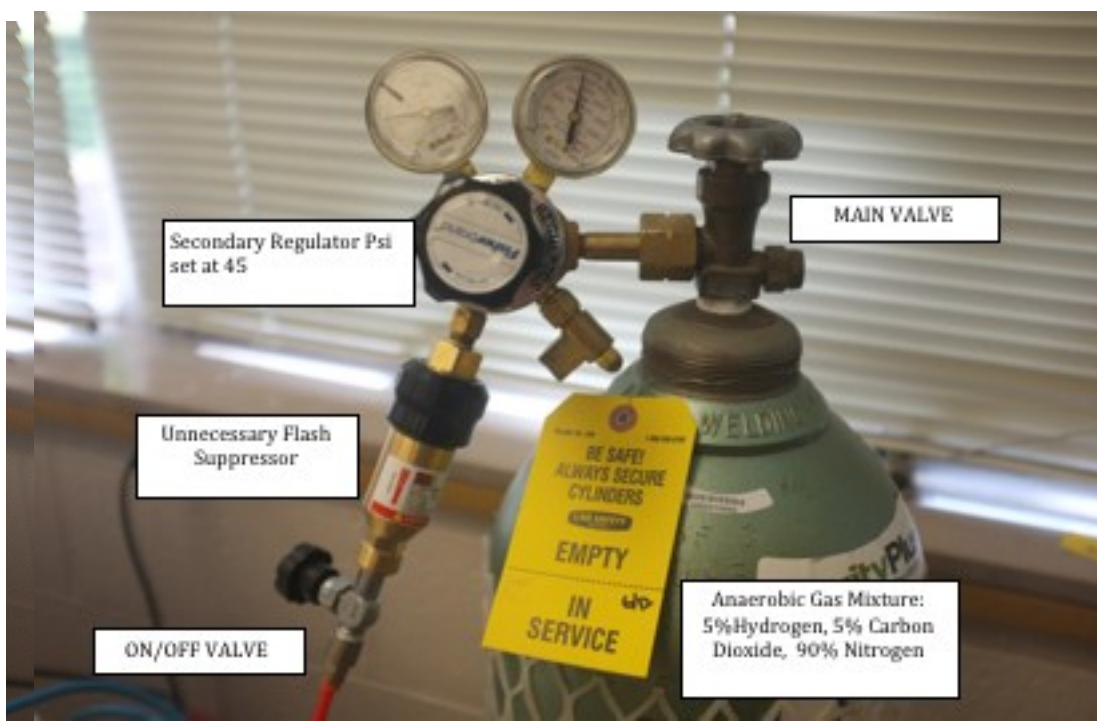
7.) Open the main valve entirely.

8.) Set the secondary regulator at 40 psi. The secondary regulator is a component of regulator that controls the pressure of gas being expelled from the tank which ranges from 0-200psi. The middle protruding lever is the secondary regulator.

9.) Plug in and turn on the anaerobic chamber. Both the gas and flow fault lights should light up.

10.) There should be a buzzing sound to signal that the fault alarm is on. (Toggle the buzzer switch until the check alarm is heard.)

11.) Turn the ON/OFF valve to allow gas to flow through and the alarm should stop.



- 12.) Run the commission cycle by pressing and holding the commission buzzer until 2 distinct beeps are heard (Gas should be sucked in approximately every 20 seconds for 20 minutes, if the chamber continues sucking in gas after this time period, then there may be a leak in the system and the operation should be aborted.)
- 13.) Allow the chamber to stabilize for 24 hours.

Protocol

A. Preparation of cells

Background information: Mesophilic bacteria are characterized as being able to grow at moderate temperatures (between a 20-45°C) and are labeled “M” on the culture tube and thermophilic bacteria are characterized as being able to survive at higher temperatures (between 45-80°C) and are labeled “T” on the culture tube.

1. Prior to adding media for inoculation, label anaerobic culture tubes by copying down the name provided on the frozen culture stock. **Note:** prepare two extra culture tubes and label one M-blank and the other T-blank. These uninoculated tubes will serve as blanks when measuring absorbance values.
2. Set the spectrophotometer to 660_{nm} and allow it to warm up for 30 minutes.
3. Obtain 1 ml of cells from frozen stock cultures and grow in 4 ml phosphate medium containing 8 ml phosphate-buffered medium with 0.1ml phosphate buffer, 0.2ml NaS.9H₂O, 0.1ml NaSe), and 0.5 ml glucose (400 mM) as carbon source. The cells should be incubated at 37°C (mesophiles), 55°C (thermophiles) for 20-24 hours.
4. After inoculation, place the M- blank in the spectrophotometer and adjust the absorbance is set to 0. Record the absorbance of the mesophilic isolates. Place the T-blank in the spectrophotometer and adjust the absorbance is set to 0. Record the absorbance of the thermophilic isolates. Incubate the culture tubes at 37°C (for mesophiles) and 55°C (for thermophiles).
5. Monitor and record absorbance hourly for the first 8 hours and again around 15-20 hours. Plot the growth curve and determine the growth rate. Note: Do not store the growth cultures in the refrigerator; store them room temperature in a cool dry place away from light.
6. Repeat steps 3 to 5 by inoculating a second batch of fresh culture medium. Incubate the culture tubes at 37°C (for mesophiles) and 55°C (for thermophiles). Monitor and record absorbance hourly for the first 8 hours and again around 15-20 hours. Plot the growth curve and determine the growth rate. The absorbance value of the cells should be at least 0.2 absorbance at 660 nm.
7. Repeat the process a third time by inoculating a third batch of fresh phosphate-buffered medium with 1 ml of cells from the second batch. Monitor and record absorbance hourly for the first 8 hours and again around 15-20 hours. Plot the growth curve and determine the growth rate. After the third batch of growth cycle, the cells should be successfully revived. The absorbance value of the cells should be at least 0.2 absorbance at 660_{nm}.
8. Transfer 1 ml of cells from Step 7 in a fresh phosphate-buffered medium 4 ml phosphate medium (with 0.1 ml Phosphate buffer, 0.1 ml Na₂S, 0.1ml NaSe and 0.5 % (w/v) sodium formate as the carbon source). Incubate cells at 55°C (thermophiles) and 37°C (mesophiles) for 20-24 hours. Record the absorbance hourly for the first 8 hours and around 15-20 hours at 660_{nm}. The target absorbance should be 0.2 at 660_{nm}.

9. After cells have grown in initial culture medium, place the remaining 5ml of media (containing concentrated cells at the bottom of the tube) into serum bottles filled with 45ml of fresh phosphate-buffered medium (40ml phosphate-buffered medium, 1ml phosphate buffer, 1ml Na₂S, 0.5ml NaSe and 2.5ml sodium formate [final volume should be 50ml]).
10. Incubate cells in serum bottles at 55°C (for thermophiles) and 37°C (for mesophiles). Measure the initial absorbance at 660 nm by drawing 1 ml of the culture. Grow for 20-24 hours and check for signs of growth periodically (increase in turbidity of the solution). Cells should be incubated until absorbance of 0.2 at 660 nm is reached. (Withdraw 1ml of the culture from bottle and place in cuvette and measure with spectrophotometer).
11. Pipet 10ml of culture medium from the serum bottle and place into 10 ml Falcon tube centrifuge at 4000 g for 5 minutes.
12. Repeat 4 more times (4 x 10ml centrifugation step) by withdrawing 10ml of solution from the serum vial, injecting it into the Falcon tube containing the pellet, and centrifuging for 5 minutes at 4000g to pelletize the rest of the cells from the solution. Repeat for the rest of the isolates. The target goal is 1.50 mg of cells.
13. Add 1.25 ml of lysis buffer (50mM Tris-HCl (pH8.0), 2mM Na-dithionite, 0.1mg of lysozyme per ml, 0.05mg of DNase I per ml, and 0.2mM phenylmethylsulfonyl fluoride) into the culture tube containing the pellet and resuspend the cells by pipetting the cell suspension up and down.

Lysis Buffer

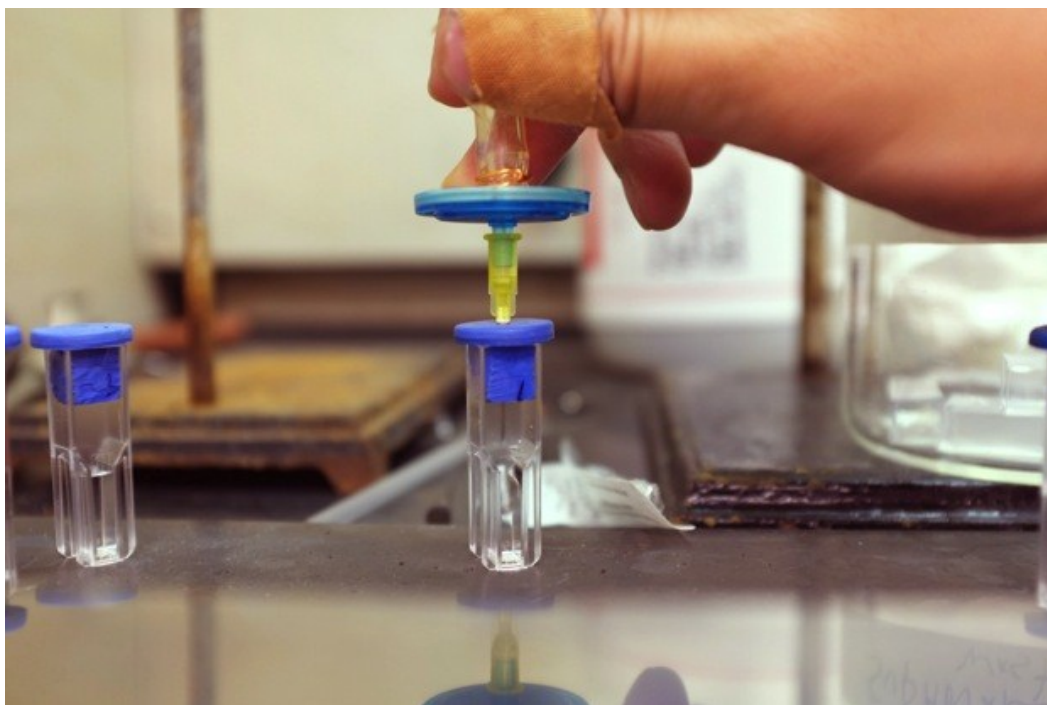
Tris pH 8.0	100mM
NaCl	150m
Lysozyme	1mg/ml
antiprotease DNase I	1mg/ml
MgCl ₂	2mM

Prepare 25ml of Lysis buffer for our 20 isolates. (1.25ml lysis buffer *20)

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NaCl	0.21915g
Lysozyme	25mg/ml
antiprotease DNase I	25mg/ml
MgCl ₂	0.0101665g

Note: The lysis buffer must be stored at 4°C.

14. Transfer the cell suspension into a sterile 1.5ml eppendorf tube using the 3ml syringe with a 20G x 1" syringe.
15. Incubate cells at 55°C for 10 minutes in a dry heating block (Labnet Int. Accublock Digital Dry bath). Vortex the cells every 2 minutes.
16. Add 0.1mg of glass beads (Bio 101 systems) and place on a vortexer (Daigger vortex genie 2 -22220A) with a microcentrifuge attachment and vortex for 10 minutes at maximum speed. Note: repeat for the rest of the isolates.
17. Place centrifuge tubes on a rack and prepare for formate dehydrogenase assay.



Example of modified butyl stopper for air tight cuvette.

Sodium Formate Enzyme assay:

Background information about the assay:

Methyl viologen is reduced photochemically in the presence of dehydrogenase activity at 603 nm. The disappearance of methyl viologen is a sign of this enzymatic activity and can be measured using the spectrophotometer. It is blue in color and turns colorless when reduced.

A. Sodium Formate Assay Preparation of Standard Reaction Mixture

1. Standard Reaction Mixture with Methyl Viologen (in micromoles) per sample

Composition per 0.75 ml:

- | | |
|---------------------------------------|--------------------|
| • 2-mercaptoethanol | 15 μmol |
| • Potassium phosphate buffer (pH 7.5) | 45 μmol |
| • Methyl viologen (MV) | 15 μmol |

Note: Amount of solution that should be prepared is 0.75ml x # of isolates to be assayed.

For 60ml of Standard Reaction Mixture:

add the following to 20ml of distilled water in a serum bottle

- 84.1 μmL of 2-mercaptoethanol
- 3.6mL of 1M Phosphate Buffer (pH7.5)
- 308.5mg of Methyl viologen

Dissolve the reagents to 60 ml distilled or MilliQ water and gas with N_2 for 30 min. Seal with rubber stopper and cap and autoclave for 15 minutes.

2. Sodium formate

0.1 mL of 0.1M sodium formate (10 μmol) needs to be added to the standard reaction mixture to initiate the reaction. The formate ion is the substrate for the formate dehydrogenase enzyme.

Note: The total volume of the reaction mixture altogether (including sodium formate and the cells) should be 1.1 ml. The addition of 10 μmol sodium formate and 0.25 ml crude cell extract should add 0.35 ml to the 0.75 ml reaction mixture.

B. Enzyme assay protocol

1. Set spectrophotometer to 603_{nm} and allow it to warm up for 30 minutes.
2. Pipette 0.75 ml of standard reaction mixture into four separate 1 ml cuvettes with modified butyl stoppers (labeled 1, 2, 3, and B).
3. Add 0.25ml of the crude cell extract into each of the four cuvettes with a 1" 20G needle and syringe.
4. Blank the spectrophotometer using the mixture in cuvette B.
5. Add 0.1ml of 0.1 M sodium formate stock solution into cuvette 1, 2, 3 and measure and record absorbance after a one minute interval for 5 minutes.
6. Repeat steps 2-5 for the rest of the isolates.
7. For enzymatic assays, a unit was the amount of enzyme that reduced 1 μmol of acceptor per min. By this it means that the presence of sodium format dehydrogenase activity is directly proportional to the methyl viologen absorbance activity. That is, the amount of enzyme that the bacterium has determines the rate in which there is a color change (from the methyl viologen), which will cause an increase in absorbance. An $\text{OD}_{603\text{nm}}$ of 1.0 corresponds to $11.3 \text{ mM}^{-1} \text{ cm}^{-1}$.