Analysis of Carbon Monoxide Dehydrogenase

A. Reference:

Two Membrane-Associated NiFeS-Carbon Monoxide Dehydrogenases from the Anaerobic Carbon-Monoxide-Utilizing Eubacterium *Carboxydothermus hydrogenoformans*. Journal of Bacteriology (2001). 183: 5134-44.

Materials needed:

B. Gases

- N_2 gas
- 90% N_2, 5% CO_2, 5% H_2 gas
- CO gas (99% Purity)

C. Glassware and equipment

- -24-ml culture tubes
- -butyl stoppers
- -sealers/crimpers
- -serum bottles
- -anaerobic chamber
  -50ml Falcon Tubes
- Quartz Cuvettes
- Spectrophotometer (Biochrom WPA S800 visible spectrophotometer range 300-700nm)
- High speed centrifuge (MCE model GT-2)
- 500 mL beaker
- 3-5ml screw cap conical vials (Microscale Distillation of Fermented Products)

D. Chemicals

- 50 mM Tris-HCl (pH 8.0)
- Na-dithionite (buffer A)
- lysozyme
- DNase I
- Phenylmethylsulfonyl fluoride
- 0.6 M sucrose (comment: Could you please check the reference paper to make sure that sucrose is needed in this protocol?)
- Methyl viologen
- dithioerythritol (DTE)
- 50mM HEPES-NaOH [pH 8.0] (buffer B)

E. Preparation of growth media

1.) Phosphate-buffered medium

Composition per 985 ml of distilled water:

$CaCl_2$	1 g
NH_4Cl	1 g
$MgCl_2.6H_2O$	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% N_2 until the reazurin indicator. Stopper the tubes anaerobically. Autoclave for 15 min at 15 psi pressure, 121^{o}C. Aseptically add 0.2 ml of $NaS.9H_2O$ 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
p-Aminobenzoic acid	5 mg
Thioctic acid	5 mg
Biotin	2 mg
Folic acid	2 mg
Cyanocobalamin	100 ⌈g

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:

$MgSO_4.7H_2O$	3 g
Nitroloacetic acid	1.5 g
NaCl	1 g
$MnSO_4.H_2O$	0.5 g
$FeSO_4.7H_2O$	0.1 g
$CoCl_2.6H_2O$	0.1 g
$CaCl_2$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
$CuSO_4.5H_2O$	0.01 g
$AlK(SO_4)_2.2H_2O$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4.2H_2O$	0.01 g

2. 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.

Reducing agent:
Composition per 20 ml (2.5%)
$NaS.9H_2O$ 0.5 g

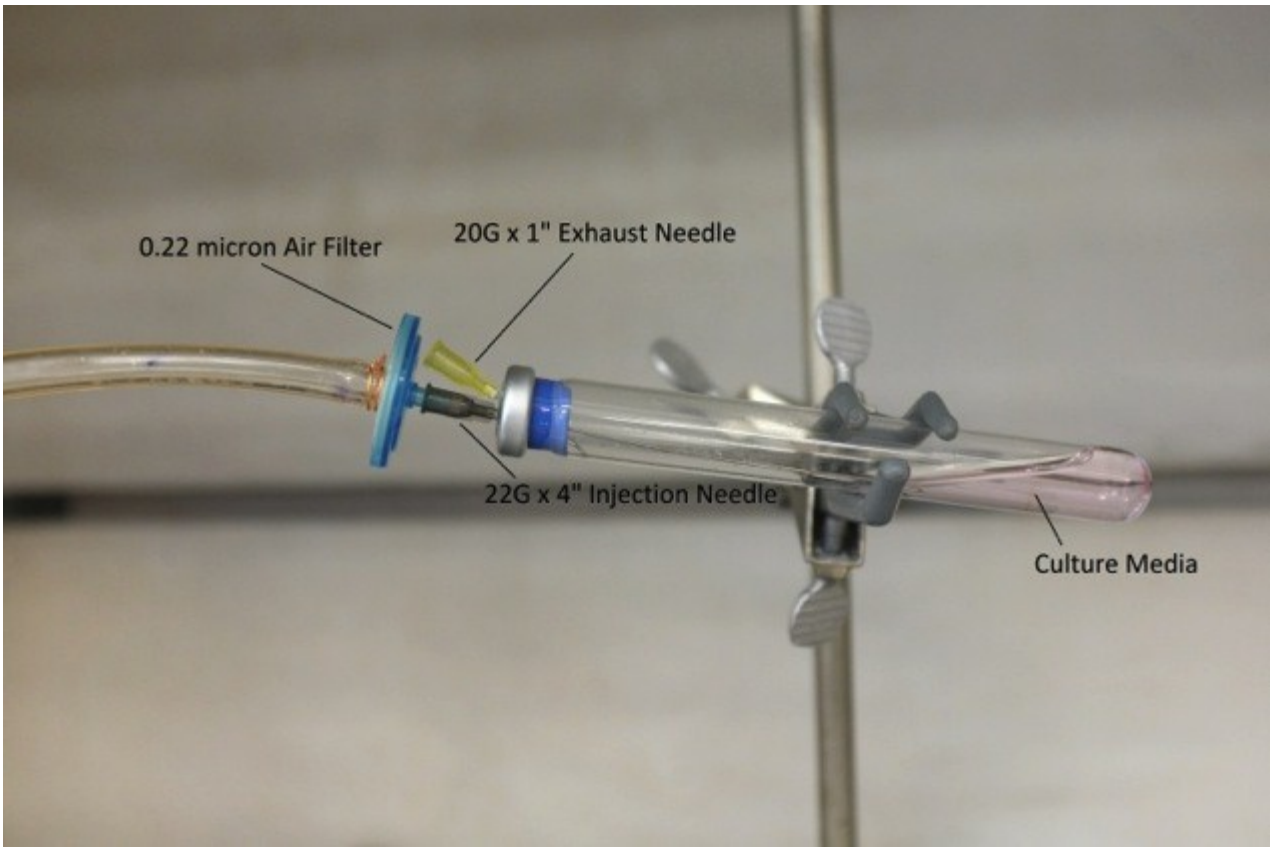
3. Preparation of reducing agent
Add $NaS.9H_2O$ to distilled/deionized water and bring volume to 20 ml. Mix thoroughly. Gas with 100% N_2 for 20 min and cap with a rubber stopper. Autoclave for 15 min at 15 psi pressure, 121°C. Use fresh prepared solution. Cool medium to room temperature.

4. Phosphate buffer solution
Composition per 100 ml:
KH_2PO4 15%
Na_2HPO4 29%

5. Preparation of buffer solution
Gas with 100% N_2 for 20 min and cap with a rubber stopper. Autoclave for 15 min at 15 psi pressure, 121°C.

F. Glucose solution (400 mM)
When glucose is used 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% N_2 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).

G. Carbon monoxide medium
When carbon monoxide is used as a carbon source, gas a tube containing 10ml of culture medium (containing 0.2ml Na_2S, 0.2ml phosphate buffer solution, 8.5ml phosphate-buffered medium) with CO gas for a duration of 2 minutes. The CO gas apparatus should have a line that runs from the CO gas tank to a 0.22 micron air filter. The air filter then passes the gas through a 22G x 4" injecting needle. The anaerobic culture tube should have an exhaust system consisting of a 20G x 1" needle in the rubber stopper cap. Bacteria can then be injected into the CO media at a volume of 1ml.



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

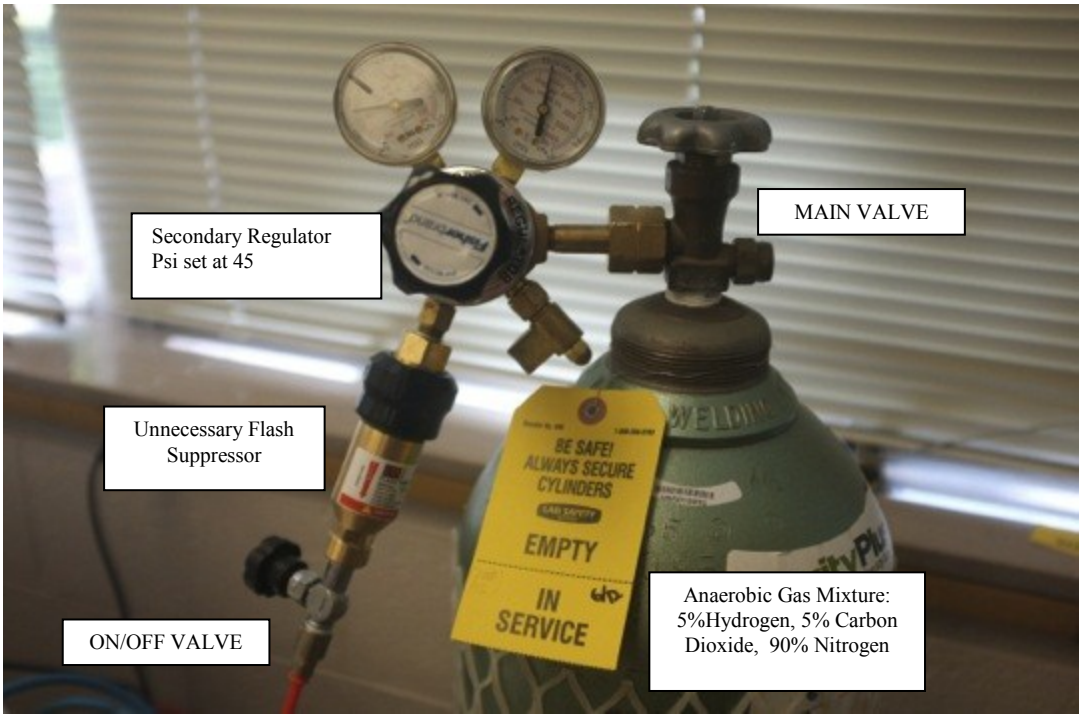
Note: lysis buffer must be stored at 4^oC.

Preparation of Anaerobic Chamber

1.) Place the following items in anaerobic chamber:

- sterile 1.5ml eppendorf tubes
- 70% ethanol
- Spectrophotometer
- vortexer with eppendorf attachment
- heating block
- thermometer
- 3ml syringe
- 20G x 1" needle
- prepared lysis buffer
- standard reaction mixture
- crimp remover
- test tube rack
- glass beads (Bio 101 systems)
- BBL dry anaerobic indicator strips (should be used to ensure that the conditions within the chamber are anaerobic)
- 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% N_2, 5% CO_2, 5% H_2 gas tank to the anaerobic chamber.
7.) Open the main valve entirely.
8.) Set the secondary regulator at 40 psi. The secondary regulator is the lever in the middle protruding out.
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 on the ON/OFF 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 20-45ºC) and are labeled "M" on the culture tube and thermophilic bacteria are characterized as being able to survive are higher temperatures (between 45-80ºC) 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 9 ml culture medium containing 8.6 ml phosphate-buffered medium with 0.2ml phosphate buffer, and 0.2ml $NaS.9H_2O$, and 0.5 ml glucose (400 mM) as carbon source. The cells should be incubated at 37ºC (mesophiles), 55ºC (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 37ºC (for mesophiles) and 55ºC (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 37ºC (for mesophiles) and 55ºC (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 OD valueof the cells should be at least 0.2 at 660 nm.
7. Repeat the process a third time by inoculating a third batch of fresh culture 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 culture medium (8 ml phosphate-buffered medium with 0.2ml phosphate buffer solution, and 0.2ml $Na_2S.9H_2O$ and 99% CO gas as the carbon source. Add 0.6 ml more of phosphate-buffered medium to ensure the volume is 9.9ml. Incubate cells at 55ºC (thermophiles) and 37ºC (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. Withdraw and discard 5 ml of the cells and inoculate the remaining 5 ml(containing concentrated cells a$_t$ the bot$_t$om of the tube) into serum bottles filled with 45ml of fresh medium (40 ml phosphate-buffered medium, 1ml phosphate buffer solution, and 1 ml $Na_2S.9H_2O$, Supplement with 3ml of phosphate-buffered medium to ensure volume is 50 ml.
10. Incubate cells in serum bottles at 55ºC (for thermophiles) and 37ºC (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 660nm is reached. (withdraw 1ml of the culture from bottle and place in cuvette and measure with spectrophotometer).
11. Pipet 10 ml of 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 x10ml 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, 0.05 mg 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	150mM

Lysozyme	1 mg/ml
antiprotease DNAse I	1 mg/ml
$MgCl_2$	2 mM

*Prepare 100 ml of 100mM Tris (pH 8.0) separately by dissolving 1.21 g of Tris in 80ml of distilled water, adjust pH to pH 8.0 using 1M NaOH and bring the volume to 100ml. Autoclave and store at room temperature.

To prepare 25ml of lysis buffer for 20 samples (1.25 ml of lysis buffer per sample), using 100mM Tris pH 8.0. (1.25 ml lysis buffer *20), dissolve the following components into 20 ml of 100mM Tris pH 8.0 prepared above:

NaCl	0.22g
Lysozyme	25mg/ml
antiprotease DNAse I	25mg/ml
$MgCl_2$	0.01g

Bring the solution up to 25 ml and filter sterilize. Do not autoclave.

Note: lysis buffer must be stored at 4^{o}C.

14. Transfer the cell suspension into a sterile 1.5ml eppendorf tube using the 3 ml syringe with a 20G x 1"syringe.
15. Incubate cells at 55ºC 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 CO dehydrogenase assay.

Carbon Monoxide Dehydrogenase Enzyme Assay:

Background information about the assay:
Methyl viologen is reduced photochemically in the presence of carbon monoxide dehydrogenase activity at 578_{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. Carbon Monoxide Dehydrogenase Assay Preparation of Standard Reaction Mixture

1. Standard Reaction Mixture with Methyl Viologen (in micromoles) per sample
Compostion per 0.75 ml:

Dithioerythritol (DTE)	2 mM
Methyl viologen (MV)	20 mM
Buffer B*	50mM HEPES-NaOH [pH 8.0]

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

*Prepare 100mL of 50 mM HEPES-NaOH (pH 8.0) separately by mixing 1.2 g of HEPES in 70 mL distilled water in a 250ml beaker. 2M NaOH hydroxide will need to be added drop wise into the solution in order to adjust the pH to 8.0, this will alow for the HEPES to dissolve completely. Bring the volume to 100ml and be sure to correct the pH of the solution to 8.0, filter sterilize and pour the solution into a clean serum bottle.

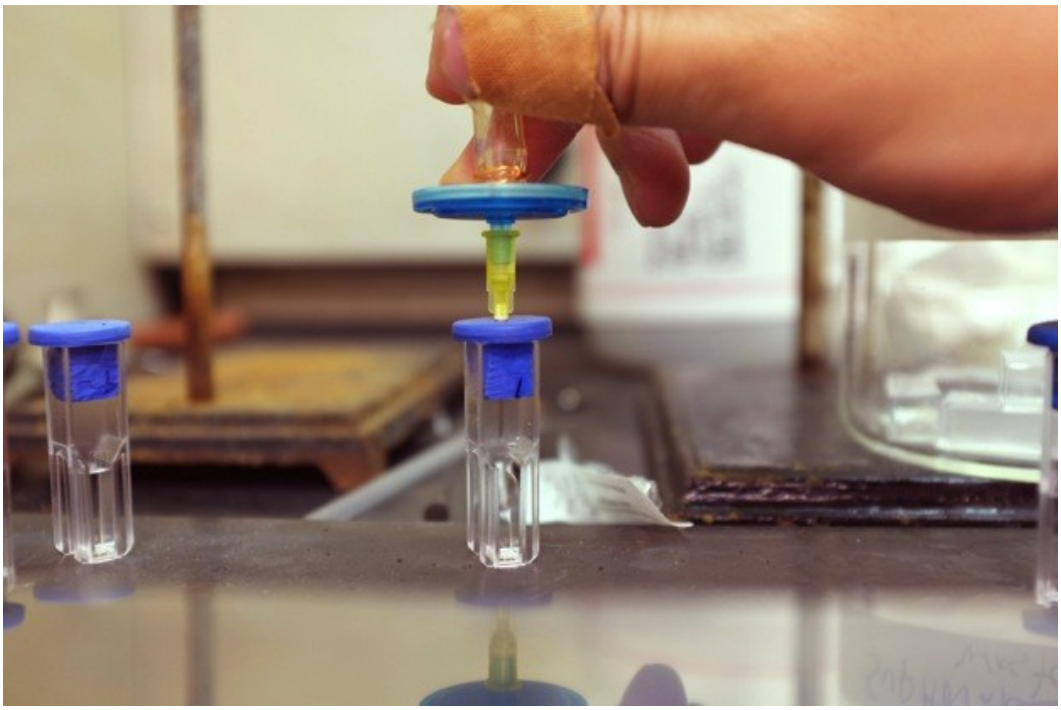
Slowly add NaOH pellets to adjust the pH to 8.0, and to completely dissolve the HEPES and bring the volume of the solution to 100 ml.

Alternatively, NaOH pellets can be dissolved in a separate beaker containing NaOH ml of distilled water and then add the dissolved NaOH in HEPES solution using a medicine dropper until the pH 8.0 is reached. Filter sterilize .

To prepare a 60-ml of standard reaction mixture, add the following to 30 ml of 50mM HEPES-NaOH solution in a sterile serum bottle:

Dithioerythritol (DTE): 0.02g
Methyl viologen (MV): 0.31g

Filter sterilize the reaction mixture and then gas with N_2.



Example of modified butyl stopper for air tight cuvette.

B. Enzyme assay protocol

1. Set spectrophotometer to 578_{nm} and allow it to warm up for 30 minutes.
2. Pipette 0.75 ml of standard reaction mixture into four cuvettes sealed with modified blue stoppers labeled 1 (replicate 1), 2 (replicate 2), 3 (replicate 3), and B (blank).
3. Gas the sealed cuvettes containing the reaction mixture with CO at 0.5-1psi for 2 minutes outside of the anaerobic chamber. **Note:** make sure the CO does not escape the cuvettes.
4. Place the gassed, sealed cuvettes back in the anaerobic chamber utilizing the proper vacuum and anaerobic gassing procedure.
5. Add 0.25ml of the crude cell extract (lyzed cells) into cuvettes labeled 1,2, and 3.
6. Blank the spectrophotometer (at 578_{nm}) using the mixture in cuvette B (without cell extract).
7. Take cuvette B (the blank) and place cuvettes 1 (replicate 1) into the spectrophotometere a begin to record the absorbance every minute for 5 minutes.
8. Repeat steps 2-7 for the rest of the isolates.

9. For enzymatic assays, 1 unit is the amount of enzyme that reduced 1 μmol of acceptor per min. This it means that the presence of carbon monoxide dehydrogenase activity is directly proportional to the reduction of methyl viologen. That is, that the amount of enzyme that the bacterium has determines the color change (from the methyl viologen) and subsequent increase in absorbance. At an OD_{578nm} 1.0 corresponds to $9.7mM^{-1}cm^{-1}$.