Acetylene Reduction Assay (ARA): Measuring Nitrogenase Activity

Adapted from Hawkes Lab

Introduction:

The discovery that the nitrogenase enzyme responsible for N_2-fixation also reduced C_2H_2 (acetylene) to C_2H_4 (ethylene) (Dilworth, 1966) provided a useful assay for the quantification of the N_2-fixation process. For quantitative determinations of N_2-fixation, $_{15}N_2$ techniques should be used, however, the acetylene reduction assay is still used widely because it provides a highly sensitive and inexpensive way to quantify relative nitrogenase enzyme activity in N_2 fixing samples.

Because the presence of acetylene blocks the conversion of N_2O to N_2, we are able to simultaneously measure denitrification ($NO_3^- \rightarrow N_2O \rightarrow N_2$) by measuring N_2O.

Materials:

Calcium Carbide (CaC_2) (1 gram CaC_2= 130 mL of C_2H_2 gas; or ~0.3g per sample)
Nanopure DI water
24 inch section of tygon tubing with needle fitting
1000 mL volumetric flask with armhole and appropriate sized rubber stopper #8
Tedlar gas collection bag
5cm PVC coring cylinders with yellow caps
500 mL canning jar with lid bottom drilled hole
#4 one hole rubber stopper fitted with glass tubing
Septa Cyl Half-hole 1/4 in. 100pk (Fisher catalog #AT6526)
1000 µL pipette
60 mL gas-tight syringes with 2-way stopcocks
22 gauge syringe needles
Labco exetainer sample tubes (pre-evacuated)

Making acetylene gas:

Combine appropriate number of rocks of calcium carbide and ½ cup water in a flask ($CaC_2 + H_2O \rightarrow C_2H_4$). Quickly cover flask opening with a rubber stopper let flask vent for several seconds before inserting syringe needle into the collection bag. Make sure that needle does not puncture gas bag. Allow bag to fill with acetylene. Remove needle from bag and flask when finished. *Do not over inflate bags!*

Place flask in hood or allow flask to vent out the window until reaction is complete.

Sample "Wet-up":

Add appropriate volume (V_{water}) of nanopure DI water to 5cm sample core using a pipette. Take care to evenly disperse water over entire surface of the sample core without disturbing the surface. Place soil core into incubation chamber for 4 hours. Chambers settings should be as follows: all lights on (88), humidity 10% (humidity control is broken and will read 99% during the incubation period), and temperature set.

3. Add acetylene to create a 10% acetylene atmosphere:

Place soil cores into incubation jars and stopper the jar. For a 500 ml Mason jar that has a full 5cm core volume, remove 38 ml of air from the jar with a gas-tight syringe. Then inject 38 ml acetylene into the jar and let the acetylene equilibrate with the atmosphere by venting the jar with a needle so the jar is not over pressurized.

4. Take time zero (t_0) readings:
Pump the air in the jar gently with a syringe to mix the acetylene in the jar and remove 24mL from the sample jars with a gas-tight syringe and inject into a pre-evacuated labco exetainer. Exetainers should be over pressurized. Record time that sample was taken.

5. **Incubate and Take time zero (t_i) readings:**
Return sample jars to incubator at the same settings as "wet-up" incubation for the time period of interest (usually 3 hours). Again remove 24mL from jars and inject into Exetainer. Record the time. (This is the t_f sample.)

Samples can be stored in exetainers for quite a long time. However, it is recommended that you run the samples as soon as possible. Injecting ethylene standard into vial will also help to identify whether or not vials are leaking over time.

Calculations

1. Calculate $\Delta t = t_f - t_0$
2. Assuming a particle density of 2.6 g mL^{-1}, calculate the volume of solids: $V_{solid} = W_{OD} / 2.6$
3. Calculate the headspace volume: $V_{headspace} = V_{total} - V_{water} - V_{solid}$
4. Calculate the ethylene concentrations for the t_0 and t_f measurements from the calibration: $\mu mol\ C_2H_4\ mL^{-1} = a + bx$ (x is the peak area from the gas chromatograph, a and b are derived from the calibration curve).
5. Calculate the Δmol of ethylene in the jar at t_0 and t_f: $E_{total} = (\Delta mol\ C_2H_4\ mL^{-1}) \text{ x} V_{headspace}$ (For simplicity, we are ignoring any C_2H_4 dissolved in pore water)
6. Calculate the rate of acetylene reduction to ethylene: $Rate = [(E_{total})t_f - (E_{total})t_0)] / (\Delta t \text{ x} W_{OD})$

References

Dilworth, M.J. 1966. Acetylene reduction by nitrogen fixing preparations from *Clostridium pasteurianum*. Biochem. Biophys, Acta 127:285-294.

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