Denitrifier Method

*Pseudomonas aureofaciens* edition

*My personal working protocol, not for official use*

*Paul Magyar, 14 October 2020*

Four Days Before Harvest: Inoculation of Growth Medium

Prepare a sterile working environment with 70% ethanol, wear gloves, and otherwise use sterile technique. Take one (for 40 vials) or two (for eighty vials) 400 mL bottles of growth medium, previously prepared according to the recipe in the Appendix. For each bottle, defrost one *Pseudomonas aureofaciens* seed culture from the box in the -80°C freezer. Use a disposable needle and syringe to inject 1 mL of seed culture into each medium bottle. Wrap the medium bottle in foil to exclude light and place in a 30°C incubator, shaking at 70 rpm.

Harvest Day

1. Checking for nitrite

Again, prepare a sterile workspace. Withdraw 1 mL from each bottle of growth medium, using a syringe and sterile technique. Into this medium, add (1) 5 drops of SAN and then (2) 5 drops of NED. While adding the NED, watch carefully for the appearance of a purple color, which indicates the presence of nitrite. If nitrite is present, the medium is not ready for use. You can try again tomorrow.

1. Centrifugation

From this point on, it is still important to work cleanly, but sterile technique is not required. Glassware should be rinsed 5x with Milli-Q water before use. Open each bottle of medium. Transfer the contents into new 50 mL Falcon tubes (8 per bottle of medium). Centrifuge at 4750 rpm for 10 minutes (room temperature). Then, discard the supernatant from each tube, while leaving the pellet of bacteria. Note the color of the bacterial pellet (it should be at least light pink).

1. Resuspension

Take a fresh bottle of nitrate-free resuspension medium (see Appendix for recipe). Transfer 15.6 mL of this medium to each tube containing a bacterial pellet. Agitate the tube thoroughly to suspend the whole pellet and combine all resuspended bacteria in a clean beaker. Add one drop of Antifoam per vial prepared (40 for one bottle, 80 for two). Stir well with a glass stir

1. Transfer to vials and purging

Load a standard vial rack with 20 mL vials that have been muffled at 450°C for 6 hours[[1]](#footnote-1) (40 for one bottle, 80 for two) Into each vial, pipet 3 mL of resuspended bacteria. Cap each vial with a new rubber septum and crimp with an aluminum crimpcap. Place a new green needle into each vial as a vent needle and then put each vial upside-down onto the brown needle on helium vent rack. Turn on the flow of helium so that all vials are gently bubbling, and check the pressure on the He cylinder. Purge for ≥5 hours.

1. Removal of vials from He purge rack and sample loading

At the end of the purge time, remove vials from the purge rack, taking care to withdraw the green vent needle first. Return vials to the sample rack and number each vial. Load samples and standards according to your runsheet (see attached Runsheet Template), taking care to include 1-2 blanks, and always measuring IAEA-N3, UBN-1, and Deep Pacific for long-term comparability. Additional standards can be chosen based on the expected composition of the samples. Working solutions of standards (20 µM) are maintained frozen at -20°C; defrosting can be accelerated by careful immersion of the tube in warm water.

For samples, inject with appropriately-size glass syringes (Hamilton). Sizes available: 25 µL, 100 µL, 250 µL, 500 µL, 1 mL, 5 mL). For standards, it is important to avoid any contamination of the working stock with either another sample or standard or with bacterial culture. One approach for protecting it is to use a fresh disposable plastic syringes for each standard, and a fresh disposable needle (blue) for each injection. Another approach is to subsample the amount required for a given sequence into a separate working vessel.

One samples and standards are injected, let the bacteria work overnight.

Day 2: Bacteria killing

The day after a harvest, kill bacteria by injecting 100 µL of 10M NaOH into each vial. Use a Luer-Lok disposable syringe with a blue needle. Take care to maintain pressure on the plunger of the needle, so as to prevent leakage of any gas. I prefer to keep the vial upside-down and inject the NaOH through the liquid. Shake each vial well. Samples are now ready for measurement.

*Appendix*

Look up the recipes for growth and resuspension media in the binder, stored in the downstairs lab.

1. We use new vials for each harvest, but if vials are reused, they should be washed overnight in a 10% HCl bath and then rinsed 5x in Milli-Q water (en-masse, in their box) before muffling. [↑](#footnote-ref-1)