Yonsei Environmental Bio-Technology Laboratory

Enrichment and Cultivation of Reductively Dechlorinating Bacteria

- Wear gloves throughout. - All laboratory procedures should be performed in the absence of air (O_2). - Label vials should be done before putting them in the anoxic chamber. - Check the O_2 concentration is zero, before use the anoxic chamber. - Clean inside the chamber with alcohol, before and after use. - Flame sterilization is not possible inside the anoxic chamber. - Use tweezers to pick up a spatula.	Writer	Jaejin Lee
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<Sample collection, transportation, and storage>

- Apparatus –
(1) Sample containers
(2) Ice box
(3) Cooling materials
(4) Bucket
(5) Scoop
(6) Vinyl bags
(7) Boots (if needed)
(8) Permanent pen
(9) Green tapes

1. No standard protocols for sample collecting, handling, transportation, and storage exist; however, note that many reductively dechlorinating populations are strict anaerobes. Procedures should obviously limit exposure of the sample materials to air.

2. In case of estuarine or tidal flat sediments, it is recommended to use a core type sample container, like a PVC pipe. When it is not necessary to take samples in-depth, it is good enough to use an autoclaved jar. (It is recommended to flush those containers with N_2 gas in advance and to make head space as small as possible.)

3. During transportation, keep the samples on ice. In any event, microcosm setup should commence as soon as possible. In an anoxic chamber, transfer the samples into proper jars with wide-mouth and keep them in 4 ℃ until use.

<Preparing electron donors and electron acceptors>

(This part referred to "Frank E. Loeffler, Robert A. Sanford, and Kirsti M. Ritalahti. 2005. Enrichment, Cultivation, and Detection of Reductively Dechlorinating Bacteria. Methods in Enzymology. vol. 397; 77-111".)

1. Electron donor

All known MRDBs (Metabolic reductive dechlorination bacteria) use acetate, H_2, or both compounds as electron donors. For example, *Desulfuromonas* strain BB1 use acetate as carbon source and electron donor and *Dehalococcoides* sp. use acetate as carbon source and H_2 as electron donor. The addition of electron donors is differed by each case. Thus it is required to review related papers to decide proper electron donors and their final concentration.

Generally, H_2 (5-30% of the head space volume of a vessel) is added by syringe and acetate is added directly to the medium before dispensing it to individual culture vessels or is added to individual microcosms from a sterile, 1 M, anoxic aqueous stock solution. Acetate is added in two to fivefold excess of the theoretically needed amount to achieve complete reductive dechlorination.

An alternate strategy to provide the key electron donors is the addition of 2-10 mM lactate as a substrate. The fermentation of lactate typically yields acetate and H_2, the key electron donors for MRDBs. Lactate is also used directly as an electron donor by certain MRDBs, such as *Desulfitobacterium* and *Sulfurospirillum* species. Lactate can be added to the medium and autoclaved before dispensing to individual culture vessels or be added to individual microcosms.

2. Electron acceptors

The time when the chlorinated electron acceptor is added depends on the physical-chemical properties of the chloroorganic compounds of interest. Water-soluble and nonvolatile chlorinated compounds such as chlorobenzoates are added directly to the bulk medium or are added by syringe to sealed microcosms from anoxic, neutralized, sterile stock solutions.

Volatile compounds, such as chlorinated solvents, are added undiluted with a pipette immediately prior to sealing the microcosms or to sealed microcosms using gas-tight syringes. In the case of chlorinated solvents, inhibitory effects can be avoided by adding the chlorinated electron acceptor dissolved in an inert, hydrophobic carrier such as hexadecane.

Hydrophobic, nonvolatile, chlorinated compounds are dissolved in an organic solvent and added to sterile culture vessels.

Because many chloroorganic compounds are toxic, the initial strategy is to add the minimum amount of chlorinated electron acceptor that can be quantified readily with the available analytical instrumentation. It is important, however, to provide sufficient chlorinated substrate to support growth during the enrichment process, as the chloroorganic compounds serve as energy-yielding electron acceptors for the target organisms.

In the case of Tetrachloroethene (PCE) addition, it is possible to use PCE-medium mixture, which has formed DNAPL. It is also possible to use PCE stock dissolved in MeOH. However, keep in mind that the fermentation of MeOH can decrease pH, which can interrupt dechlorination process. In some cases, 1-4 μl of neat PCE (pure), which was stored at 4 ℃ in 1 ml stock, can be directly added using a 5 μl syringe. All depends on the purpose of microcosm study, so please refer to related papers before deciding electron acceptors and their final concentrations.

<Sample homogenization and transfer into vials>

- Apparatus –
(1) Samples in jars
(2) Proper medium (refer to medium preparation protocols; anoxic, autoclaved)
(3) Autoclaved beakers (1 liter) with autoclaved star bars (as much as needed)
(4) Stir plate
(5) Sterile spatula (a lot)
(6) Tweezers
(7) Sterile pipettes (as much as needed)
(8) Autopipette
(9) Autoclaved vials with aluminum foil on top (20 ml to 160 ml, as much as needed)
(10) Autoclaved Teflon septa or black butyl septa in beaker (in water, as much as needed)
(11) Aluminum caps (as much as needed)
(11) Alcohol bottle
(12) Permanent pen

(13) Waste bucket
(14) Labmat Pad
(15) Kim wipes

1. Make sure that proper medium is ready. Anoxic water collected at the sampling location, quarter- to full-strength saline (5mM potassium phosphate, with similar NaCl percentage with the sampling location, pH 7.2 to 7.6) or DCB-1 medium is good enough. Note that it should be anoxic and autoclaved.

2. Calculate the amount of required solids and liquid medium. The total amount of solids added ranges between 5 and 50 weight percent of the final liquid volume in each vessel.

3. Label the vials, before working in the chamber.

4. Check the O_2 concentration in the anoxic chamber is zero.

5. Put all apparatus in the chamber.

6. Clean inside the chamber with alcohol and Kim wipes. Also, clean the gloves with alcohol as frequent as required.

7. Pave the floor with some Labmat Pad. Set the stir plate and arrange things

8. Put a beaker with a stir bar on the stir plate.

9. Transfer required amount of liquid into the beaker using Autopipette and sterile pipettes.

10. Transfer required amount of solid samples into the beaker using a sterile spatula and make the slurry mixed constantly on the magnetic stirrer.

11. When the homogeneous slurry is ready, transfer the slurry into each vial. Medium is added to yield liquid to headspace ratios of 1-1.6:1.

12. Seal the vials with Teflon septa or butyl septa and aluminum caps.

13. Clean inside the chamber, wipe the floor and gloves with alcohol and take things out of the chamber.

14. Wash used beakers, spatula, stir bars, and so on. Replace everything back to the right places.

15. Proceed to electron donor and electron acceptor addition as soon as possible.

<Electron donors and electron acceptors addition>

- Apparatus –
(1) Vials containing medium and sample
(2) Stock solutions of electron donors and electron acceptors (should be done with final concentration calculations)
(3) Disposable syringes and needles (as much as needed)
(4) Alcohol for sterilizing top of bottles (in 50 ml falcon tube)
(5) (Applicator) Swab
(6) Torch or alcohol lamp

1. Wipe clean bench with alcohol before use. Place everything into the clean bench.

2. Before and after you inject, sterilize the top of bottles using alcohol, swap and flame.

3. Inject electron donors into each vial as much as needed.

4. Inject electron acceptors into each vial as much as needed. (Electron acceptors in gas phase, such as vinyl chloride, should be added in the chemical hood. DO NOT inject them in the clean bench.)

5. Used syringes and needles for chlorinated compounds should be separated from other wastes. Waste them in the engaged place.

6. Turn the vials upside down and store them, in dark, at 25℃ or at room temperature.

7. After a few days, take 0.1 ml of head space gas using gas-tight syringe and measure the peak area using Gas Chromatography. Those measurements are for Day 0.

8. Keep incubating microcosms and monitor the changes every two weeks or every month.