2. Culture preparation, Harvest and maintenance

2a. Methanotroph culture stock maintenance (Freeze stock at -80°C)

- 15% Glycerol stock can be prepared for long-term storage. Glycerol is inhibitory to few methanotrophs. A protocol that is successful for most strains is to freeze in 1%, TT and 5% DMSO or only 10% DMSO (0.9 ml of a culture with OD600 0.5-1 plus 100 µl DMSO, stored at -80C).
  (For more details please read this article "Survival or Revival: Long-Term Preservation Induces a Reversible Viable but Non-Culturalable State in Methane-Oxidizing Bacteria" by Hofeman et al., 2012 https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0034196)
- It is advisable to check the cultures for freezer stocks carefully for purity and discard any that are suspect.
- Before use, ensure that the culture is same as written on the lid by PCR amplification or sequencing of suspect short region (if required) using specific primer sets.

2b. Methanotroph culture stock maintenance (NMS-Agar plates)

- For routine use and culture maintenance, streak and transfer the cultures on NMS-agar plates once in every 25-30 days (10µM-final Cu concentration and if required appropriate antibiotics).
- Incubate under pure-CH_4 conditions and at 30°C (optimum for most methanotrophs) at gas tight chamber. Some impurities inhibit the growth of methanotrophs (e.g. natural gas cannot be used as methane source).
- On plates, it is common for contaminants to grow under methanotroph colonies, and only after the methanotroph cells have lysed (often after weeks of incubation) is it possible to see thin films of contaminants growing out from the colonies.
- It is recommended that cultures routinely be checked under the microscope for low levels of contamination (at the few % level), and routinely streaked onto a minimal medium with methanol and also onto a rich medium, such as nutrient broth, to test for the presence of contaminants.
- LB is not recommended as a test medium, since the higher salt may inhibit heterotrophic contaminants.

2c. Methanotroph culture preparation from stock/NMS-agar for experiment (NMS-liquid)

- As a standard procedure, the culture from freeze stocks (~1 mL) will be added in 30 mL of NMS medium and sealed with rubber septa and purged with CH_4. The final OD600 ~ 0.07-0.08 on time 0.
- If it's from NMS-agar, scoop a loop full of culture and inoculate in 30 mL NMS-liquid.
- For purging, use gas manifold. Start the compressor and open the valves at gas cylinder. Connect the gas manifold tube with the flask outlet tube, turn the red-valve anti-clockwise and create negative pressure of ~15psi. Then turn the same valve on clockwise to feed the CH_4 until the gas pressure reaches 0 psi (Note: make sure that blue vale at the gas manifold is on for CH_4).
- Measure OD600 at point 0 and every 24 h until the culture reaches its OD600~0.6-0.7.

- Once culture is up, streak on NMS-agar plates and check for any contamination using Nutrient Agar plates. (Note: LB-agar usually not allow many heterotrophs to grow).
- Also, do PCR using specific primers to confirm the strain before use for any experiment. Follow colony PCR steps (see section:??) or liquid DNA extraction step followed by PCR (see section:??)
- For any subsequent experiments using this stock culture, plan the volume of culture required well before and inoculate in appropriate volume 24-48 h before starting any experiment (to harvest the cells at early-mid stationary phase).
- Add $CuCl_2$ to get 1μM –final Cu concentrations, if required. Some methanotrophs may not require Cu. (Note: can grow those using Cu-free NMS).
- If needed add specific antibiotic to grow the mutant strains (Check the table 1 in Appendix).
- To start with the OD600~0.05-0.1 should be ideal for any experiment. Measure OD600 intermittently and record the growth of culture.
- Harvest the cells when OD600~0.65-0.7 (at early stationary phase) that may reach between 24 and 48h incubations at 30°C and 220 rpm.
- Cell harvesting: Centrifuge the cultures @6500 rpm, 10 min; use 50 mL sterile falcon tubes and wash the pellets with fresh Cu-free NMS. Re-suspend the pellet and use it as inoculum for any experiment.
- To maintain the sterility, always work close to the flame.