3. Shake flask experiments

3a. Cell culture preparation

- Follow the steps detailed in section 2c. Prepare 24-48h culture to use it for any experiment. Harvest the cells once it reaches its early stationary phase (OD600~0.6-0.7) and re-suspend in 30-50 mL of Cu-free NMS and use as inoculum.
- Any experiment, make sure that the initial OD600 is measures as 0.05-0.1.

3b. Experiment set-up and monitoring.

- Prepare 30 or 50 mL final volume in 250 mL side arm flask for any experiment (Note: as a rule of thumb always have 1/4 of flask volume free head space). In which 27 or 45 mL will be the fresh NMS and 3 or 5 mL will be the inoculum (~10%).
- Always use triplicate flasks for each conditions and work close to flame to avoid any contamination.
- If working with Cu-free tests or with any other metals, after washing with soap solution, soak the flasks in acid bath overnight, rinse it with tap water followed by distilled water and sterilize it.
- Use fresh NMS every time. Also use the same NMS as Blank for any subsequent analysis from the same experiment (e.g. ICP-MS; Naphthalene assay, etc.).
- Ensure the required final Cu concentration and antibiotic requirements for the test strain and maintain throughout the experiment.
- If you plan to use Cu-preloaded Methanobactin (Cu-MB complex) instead of free Cu, prepare your Cu-MB incubations well before the start of your experiment (i.e., Cu-MB complex required 1 h incubation at 30^{o}C and 220 rpm)
- Incubate the flasks at 30^{o}C and 220 rpm and monitor the growth by OD600 measurement.
- Monitor the OD600 for every 6-8 h initial incubation period and once the OD600~0.35 or above, every 2-4h until it reaches its early stationary phase.
- Harvest the cells for different tests when the culture reaches its mid or late-exponential phase (or at early stationary phase).

3c. Harvesting and sample preparation for analysis

- Immediately after opening the experimental flask (do one flask at a time), set samples aside for following analysis;

    (i) RNA extraction: collect 10 mL of samples in a 50 mL falcon tube that contains stop solution. Set aside until you complete the naphthalene assay and sample preparation for metal extraction. However, finish first day extraction as quick as possible and cannot be stored for long (see section: ??).

(ii) Naphthalene assay: take 1.6 mL out and incubate the tubes at 30°C and 220 rpm for 1 h (see section: ???).

(iii) DNA extraction: collect 10 mL of samples, centrifuge at 6500 rpm for 10 min and store the biomass at -20°C.

(iv) Metal extraction: Collect 10-20 mL of samples in a 50 mL falcon tube. Centrifuge at 6500 rpm for 10 min and collect the supernatant in a new-labelled 15 mL flacon tube. Add equal amount of Cu-free NMS to wash the pellet (i.e. re-suspend the pellet and centrifuge again). Collect the wash in a different 15 mL labelled falcon tube. Add 1 mL of Cu-free NMS to the pellet and re-suspend and transfer it into 2 mL screw cap vial. Store all samples at -20°C until use (see section: ??).