

Cyanobacteria - Tips and Tricks

IISER-Pune-India, iGEM 2021

Synechococcus elongatus is an up-and-coming phototrophic chassis, which differs significantly from *E. coli* in terms of the protocols for its upkeep. We were fortunate to have Ph.D. mentors who have worked on this chassis, and have benefited from the support provided by them and other researchers working in the area.

There is a body of knowledge that is passed down orally from mentor to student in the cyanobacterial research community that is often undocumented in the literature and cannot be accessed by anyone wishing to start off working with these niche model organisms. For information on reviving cyanobacterial strains, practical knowledge on gradual passaging of the cultures, ways to handle aggregation of cells, and a quick guide to setting up a phototrophic culture without a photo-incubator or a CO₂ pump - our written guide, *Cyanobacteria - Tips and Tricks* is an invaluable resource.

I - Revival:

- *S. elongatus* cannot be revived onto a plate from a cryopreservation stock (glycerol or DMSO stock). Revival of a stock can be done in liquid media (BG-11), but takes time. Since it takes a few days to revive, **do not rely** on stocks as a regular backup; instead go for **plate-to-plate** transfer (see under § Plates).
- During revival from a stock, start with no (or low) concentration of any antibiotic markers, and add full antibiotics after passaging into higher volumes of medium.
- Overly dilute populations tend to crash; avoid this by starting with a 2 mL culture in a test tube and growing it overnight. Transfer this to a 50 mL flask and make up the culture to 10 mL in volume. Once it reaches a limegreen colour (#32CD32), add fresh medium to make the culture up to 50 mL culture in a 150 or 250 mL flask. Further, make this culture up to 100 mL in a 250 mL flask once it returns to limegreen.
- Cultures being revived from a stock may form a ring-shaped aggregate along the perimeter of a cross-section of the flask, above the surface of the culture. (This is where the culture splashes while shaking in the incubator). **This is indicative of stress, but does not mean the culture is doomed.** Aggregated cultures can be in a state of highly resilient stasis, and have been known to survive autoclaving. Wash such flasks well with

detergent, followed by ethanol and then UV-sterilise them before autoclaving. Aggregation can also lead to inaccurate estimates of population density, as it can affect the turbidity and therefore the OD measurements. Aggregates along the bottom of a culture can be re-suspended by shaking the flask manually; **do not vortex** the flask, as the cells will die.

- A culture **should not turn yellowish**. This means it is dying.
- Stock revivals are not favoured under high light conditions (unlike growth of a revived culture). If your incubator light is set to a certain high light level (depending on the strain you're using), placing a thin, single layer of tissue paper around the flask can attenuate the light and help revive your culture.



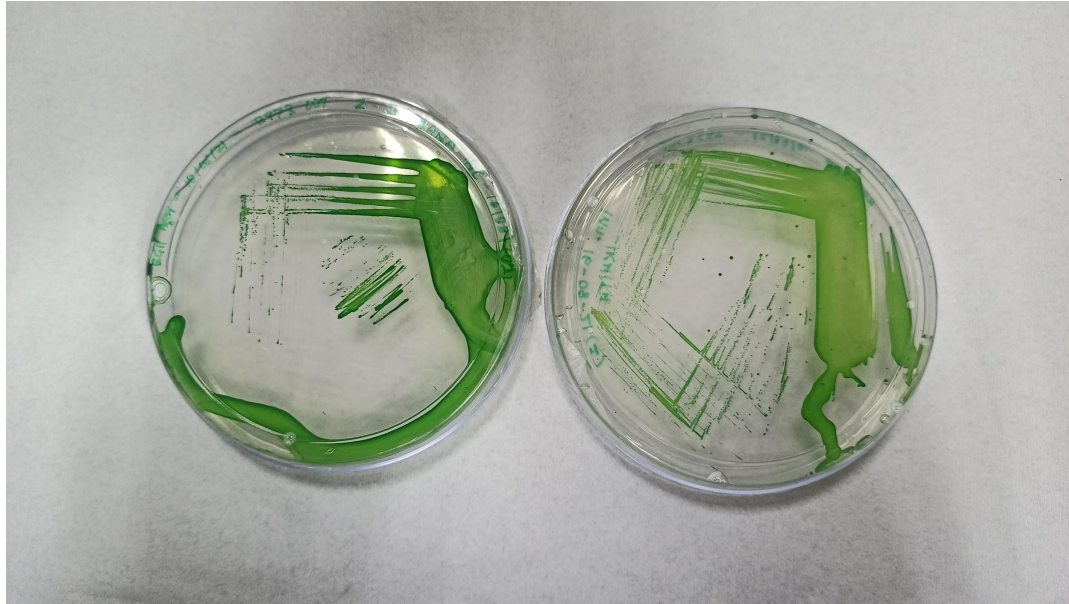
From the left: Limegreen #32CD32 coloured cultures; aggregation along perimeter of flasks; aggregation at the bottom of the flask due to lack of mixing/shaking



From left to right: A vibrant, healthy culture; reviving culture; dying culture; uninoculated BG-11 medium

II - Plates

- A BG-11 agar plate should be **thick**; about 30 mL of the solid medium should be used per plate. This relatively high value (as compared with *E. coli*) is due to the longer doubling time of the species. The plates need to be kept in the incubator for longer, and therefore run a higher risk of drying out.
- As always, store the plate with the BG-11 Agar medium on the upper lid, so that the moisture in the plate does not condense and drip onto it.
- Streaking a plate: Pellet down 1-2mL of a culture (see § Centrifugation and Pelleting) Gently resuspend your cell pellet in about 20 microliters of BG11 by mixing with a pipette and drop it onto the plate, near the circumference. Discard your pipette tip. Use a new tip to spread this liquid medium across a patch on the plate. Discard this tip and use a new tip to streak 5 parallel lines from the patch (closer to the perimeter). Discard this tip. Use a new tip to streak 5 new parallel lines, each originating from a different “parent line” perpendicular to the previous set. Continue this procedure into the third or fourth “generation”.



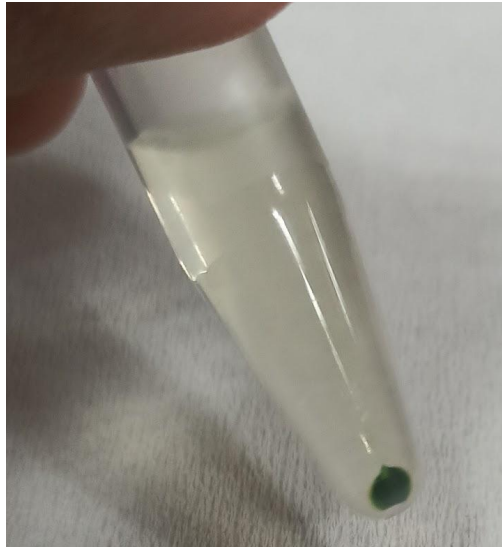
Cyanobacteria Plate Streaking Pattern

- Parafilm this plate (to prevent the solid medium from drying out; there is no concern for aeration as the CO₂ in the plate would be sufficient) and leave it in the incubator until you observe single colonies. (Usually a couple of days). Place the plate in a 4°C fridge for storage. Plates can be used to inoculate healthy liquid cultures for upto a month after they were placed in 4°C, after which a new plate must be streaked from a liquid culture. Set a reminder for 22 days from the date of refrigeration to begin inoculating new cultures to prepare a new plate.
- To inoculate a liquid culture from a plate, pick a single colony with a pipette tip and mix it into 10 mL of medium (in a 50 mL flask) or 20 mL of medium (in a 100 mL flask).

III - Centrifugation and Pelleting:

- Once a culture has reached an OD₇₃₀ between 0.6 and 0.8, centrifuge an aliquot of the culture at 4000-5000 RPM for 4-7 minutes to pellet it. Temperature can be set to any point in the interval [15°C, 40°C] - this might depend on the specific strain you are using, we pelleted our *S. elongatus* UTEX 2973 cultures at 25°C. If you are using a larger amount of culture (of the order of tens of milliliters instead of 1.5-2 mL) you might try increasing this time to 10-15 minutes.

- Discard the supernatant, and resuspend the pellet in the little remaining medium in the tube, or in a few microlitres of freshly added BG11. Use this pellet to make stocks or streak plates. This pellet need not be preserved/transferred in ice.



Cyanobacteria cell pellet

- How do we zero in on an appropriate volume to pellet down? Well, the optical density times the volume of culture is a proxy for the total cell number. This product should be >5 for a stock. If you instead plan on inoculating a second culture (see § OD measurements), back-calculate the required volume, from the desired initial OD of the second culture, and add about 5 mL excess in case you lose some cells.

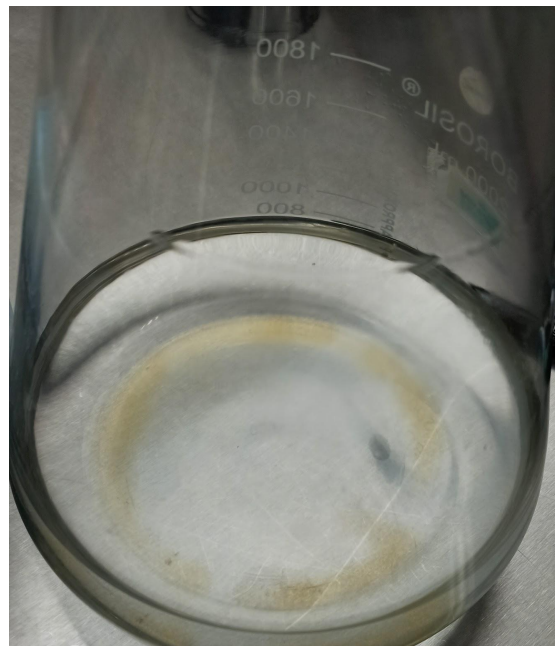
Example 1. Suppose you have a 100 mL culture at OD 0.8. You take 80 mL to make stocks from, and keep the remaining 20mL to passage into a higher volume (this lets you keep a live liquid culture). Now, the 80 mL culture you've chosen has an OD-volume product of 64. We can pellet this down and mix this with 13 mL of your stock prep solution (which depends on your protocol). Now, one mL of each can be preserved as a cryopreservation stock. Each 1mL stock corresponds, roughly, to an OD-volume product of 5. Alternatively, we can aim for a higher product of 8 or 10 per stock, at the cost of having fewer 1mL stocks.

Example 2. Suppose you wish to inoculate a new culture of 100 mL at an initial OD_{730} in the range 0.1-0.2 to begin a growth curve, and your initial liquid culture is of volume 100 mL at an OD of 0.6. A liquid-to-liquid passage tends to lead to cells dying and a dip in the OD shortly after the passage. Pelleting sends the cells into stasis and helps us calculate the initial OD of the new culture more accurately, but delays the onset of growth. Now, the intended OD-volume product of the new culture is 20, which means we strictly need $20/0.6 = 33.33$ mL. Adding in an extra 5 mL to be on the safe side, we can pellet down around 38 mL of the initial culture, and inoculate it into a 100 mL of medium.

- Sometimes, we might need to pellet down cultures of really low OD to assay the supernatant for the production of a desired metabolite over time. At low OD's (<0.4), we have successfully used 8000 RPM for over 10 minutes to pellet down a culture. Be careful to not disturb the cell pellet and to not let it disaggregate into the supernatant. These pellets aren't very stable so carry them and rest them in a stand in a perfectly upright orientation before decanting the supernatant.

IV - Medium Preparation

- Take the medium in powdered form and mix it with doubly distilled water. Add a common stock of micronutrients made in ddH₂O. The total volume of this mix should be a hundredth of the final intended volume of medium. Mix it well.
- Dilute it a hundredfold in double distilled water. Again, mix it well. Homogenise the mixture, then bring it to the desired pH (depending on the strain) by adding HCl/NaOH as required. Stir using a magnetic mixer continuously, to homogenize the pH and to prevent precipitation of certain constituents.
- Autoclave this mixture. In a sterile space, such as a flow hood, check the autoclaved mixture for pH and homogeneity. A whitish-yellow precipitate might form after autoclaving, and can be easily re-solubilised by mixing. If the pH has changed, adjust it back to the intended value. If these adjustments cannot be done in a sterile way - for example the instrument or the NaOH and HCl solutions are not sterile - then it must be re-autoclaved.

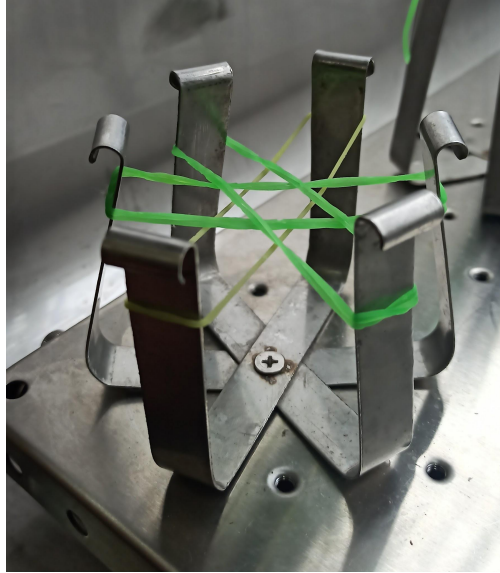


Precipitate formation in BG-11 medium

- To make BG-11 Agar plates, add Agar-Agar Type-1 equivalent to 1.5% (w/v) of liquid media and autoclave. Then before it solidifies (when the flask is bearable to touch), add the required antibiotics to the media and pour it onto plates. If it solidifies after autoclaving, re-heat and homogenize completely in an oven (but never heat after adding antibiotics - those will degrade). Pour 30mL of BG-11 agar per plate (This is to prevent the complete loss of agar due to evaporation which might happen during the long growth period of cyanobacteria).
- Add a buffer to maintain PH. TES buffer, or TES-Na, was what we used to maintain pH at 8.2 - check the requirements for your particular strain.

V - Incubating Cultures

- First things first - cyanobacteria are phototrophs. Do not use aluminium foil to cover the cotton plug on any culture in the incubator. You will restrict its access to light!
- If you do not have access to a photoincubator, you can make do by connecting white LED strips in series to form a panel of lights. This can be set at the top of an incubator **with an internal electrical outlet**. To vary the light intensity, you can use an ordinary fan regulator.
- When you install the light for the first time, and every time you dial up the intensity of the light, watch the incubator's temperature for an hour or two to ensure that its thermoregulatory apparatus is sufficient to counteract the heat produced by the light source. The temperature may fluctuate around the set point, but these fluctuations should die down to zero.
- As discussed under § Revival, *S. elongatus* cultures are continually passaged from lower to higher volumes. This means you may use smaller flasks than your institution or laboratory typically uses. For this reason, the shaking slots in your incubator may be too large to accommodate your flasks. **Do not use cotton, thermocol or tissue paper to fill the space between the flask and the boundary of the shaker slot.** This is common practice in labs for non-photoautotrophic microbial culture but will **block the light**. Instead, use **rubber bands to stabilise the flask** and hold it in place.



Rubber bands to fasten flask in place

- If you cannot maintain carbon dioxide gas at the concentrations you require (i.e, if your incubator cannot be connected to a gas outlet) then you can use *bicarbonate flashing*, a procedure in which filter sterilised sodium bicarbonate solution is added to the culture every day (or every two days if the culture begins to die of excess CO₂).
- How to calculate the amount of bicarbonate equivalent to a particular partial pressure of CO₂:

The relevant equilibrium is $CO_2(g) \rightleftharpoons CO_2(aq) \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$.

We have been able to find literature values for the equilibrium constants of the first reaction, and for the combined second and third reactions. These values, along with the corresponding enthalpies, are given below:

$K[T = 25^\circ\text{C}]$	ΔH^0
$K_1 = 0.03411 \text{ mol/L/atm}$	$\Delta H_1 = 19.799 \text{ kJ mol}^{-1}$
$K_2 = 4.457 \times 10^{-7}$	$\Delta H_2 = 9.154 \pm 0.063 \text{ kJ mol}^{-1}$

To this we may apply the van 't Hoff equation:

$$\ln \frac{K(T_2)}{K(T_1)} = \frac{\Delta H}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

And we can thus extract values for K_1 and K_2 at the incubator temperature. We can then use the product of these two as our K_{net} to calculate how much bicarbonate to add.

Example 3. Suppose we need to calculate the amount of bicarbonate to add to a culture at 37°C and pH=8.3 to be equivalent to 0.5% CO₂. We can treat this as being 5×10^{-3} atmospheres. We can also calculate the K -values at that temperature to be 0.0465 and 5.141×10^{-7} , giving us a value of $K_{net} = 2.389 \times 10^{-8} \text{ molL}^{-1} \text{ atm}^{-1}$. We can apply this equilibrium constant to formulate the equation:

$$10^{-8.3} \times [HCO_3^-] / (5 \times 10^{-3}) = 2.389 \times 10^{-8}$$

which, when simplified, yields:

$$[HCO_3^-] = 0.0238 \text{ molL}^{-1}.$$

- A simple way to add the sodium bicarbonate is to make a concentrated solution of it using an aliquot of **your growth medium as solvent**. Then, adding a few milliliters or few hundred microlitres of this stock to your culture will not alter the micronutrient concentrations significantly.
- Add bicarbonate as you see the culture growing duller/yellower. You may add either bicarbonate of the same concentration as your initial concentration, or start off slow by adding half the concentration - whatever helps restore a dark, vibrant green colour. Over time, you will converge to a better flashing frequency.
- If your culture has reached a dark green, you may leave it outside the incubator under a light in our laboratory (**make sure the cotton plug is tight**). The culture should be able to survive the night, and is unlikely to grow too much. This is very helpful during logistical emergencies - for example, if you have run out of incubator space, or don't have sterilised petri dishes to make agar plates, or autoclaved flasks to passage into.

VI - OD Measurements

- OD measurements for *S. elongatus* are typically taken at 730 or 750 nm, to avoid interference due to photosynthetic pigments.
- You typically start a growth curve in a range of 0.1 to 0.2 OD. You can do this by passing from a pre-existing liquid culture (if that culture is above 0.8 OD) or by inoculating a new liquid culture with a cell pellet (recommended if the OD of the previous

culture is between 0.6 and 0.8). The former would typically lead to some loss of cells and a dip in OD, while the latter would typically lead to a longer initial plateau in the sigmoid growth curve.

- We observed the *S. elongatus* UTEX 2973 cultures turning a much darker green before a sharp increase in growth rate.
- It is important **not to confuse** greenness with cell density. An unhealthy or dead culture can be yellow or colourless (“bleached”) but have a higher cell density than a very dark green and vibrant culture. The greenness is due to absorbance by chlorophyll at a particular set of wavelengths, whereas the OD at 730 nm is due to turbidity caused by the physical diffraction around the cells.
- Measurements of OD are to be made every 3 hours, but once you’ve estimated the growth curve, the plateau phases of the curve can be sampled once every six hours (for later growth assays).
- If the culture has reached a high OD, beyond the reliable range of your instrument, dilute the sample and mix well. Take the OD of the diluted sample and multiply by the dilution factor to estimate the initial OD.
- Unfortunately, as these measurements are not at 600 nm, we cannot use iGEM’s measurement kit to calibrate the results to our instrument. Instead, we suggest using either a flow cytometer or counting colony forming units on plates prepared using serially diluted cultures to estimate the cell count in a hyper-diluted cell culture. Multiplying by the dilution factor allows us to estimate the OD-to-cell density ratio, which should be constant for most of the range we need to measure. **We did not get to this in time before wiki freeze, and have no practical insights to share on the matter.**