KOMBUCHA'S CELLULOSE QUANTIFICATION

Julio Coll, Diciembre 2021

PRELIMINARY MINI-EXPERIMENT IN 24 WELL PLATES

24p6 6XI21

CELLULOSE BIOFILMS BY KOMBUCHA: SUSTITUTION OF SUGAR BY *D.ACIDOPHILA* EXTRACTS

- KOMBUCHA INOCULUM:
- To reduce sugar in the inoculum, the Kombucha inoculum was from a > 6 month culture, when most sugar was converted into cellulose biofilm.

- To reduce cellulose in the inoculum, the kombucha biofilm was first cut with scissors, passed through a glass-cut Pasteur pipette >100 times and a ~1mm nylon filter to obtain a low-sugar cellulose-free kombucha inoculum

- *DUNALIELLA* INOCULUM:

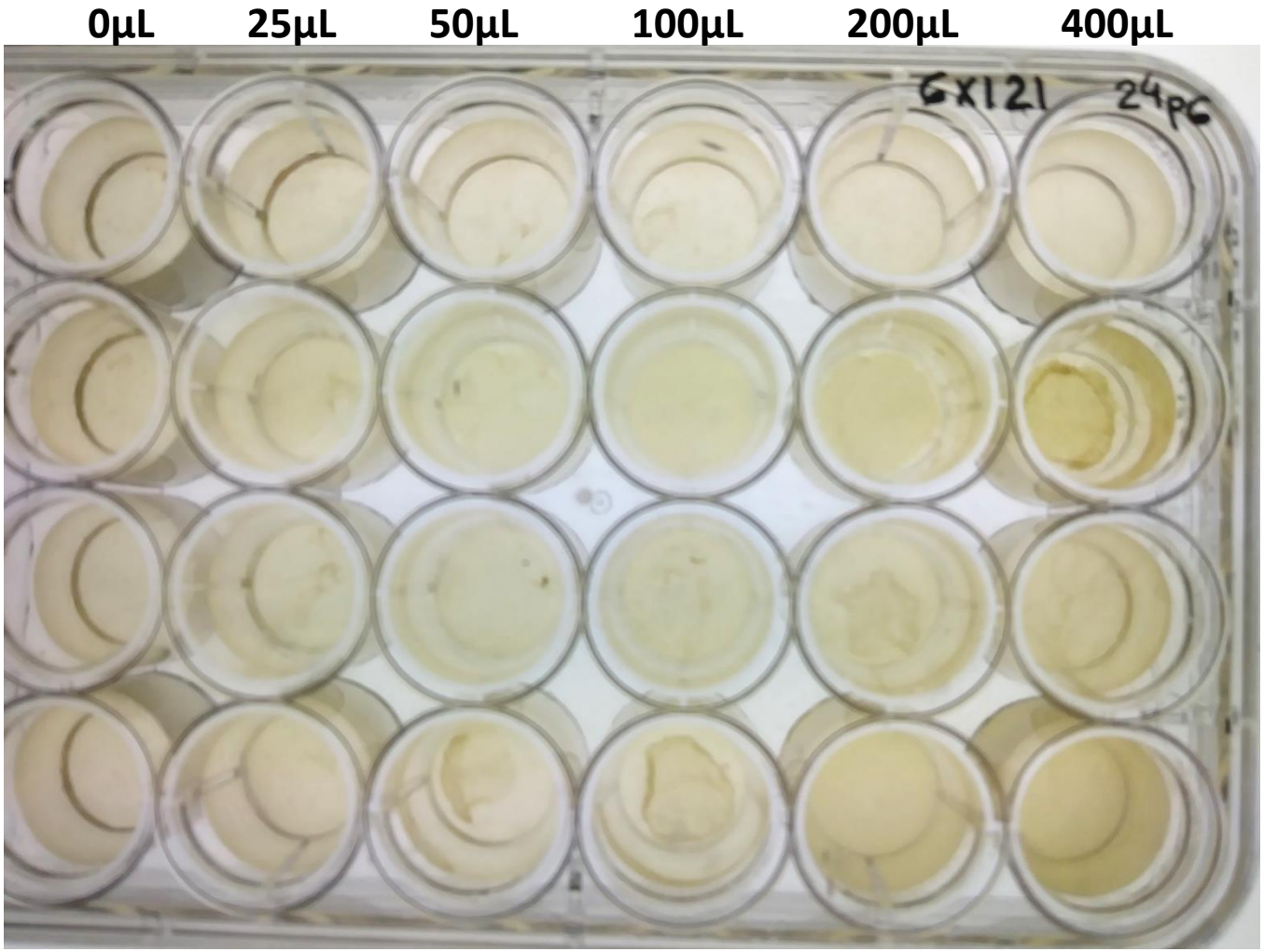
** A Dunaliella acidophila* culture of 50 mL was separated by centrifugation into pellet (cells, pp) & supernatant (ss):

- pp: The cell pp was resuspended in 8 mL of distilled water, frozen-defrozen several times to break the cells and decanted to eliminate large fragments. Te greenish liquid above (pp) was used for the mini-kombucha culture
- ss: One volume was mixed with 2 volumes of ethanol, the white granules (polysacharides) were dissolved in 8 mL of water and used for the mini-kombucha culture.

The experiment was performed in a 24-well plate, filled with 2 mL of mixtures per well.
The Kombucha inoculum (10%), acetic acid (0.1M), tea extract (0.2%) and water were added to 1600 µL total volume to each of the wells.
Rows, contained microliters of the additives: sugar (20%), pp, ss or ½ pp+ ½ ss as shown below in the blue table.
Columns, contained increasing amounts of additives (0, 25, 50, 100, 200, 400 µL additives & 400, 375, 350, 300, 200, 0 µL water)

+sugar	+0µL	+25µL	+50µL	+100µL	+200µL	+400µL
+pp	+0µL	+25µL	+50µL	+100µL	+200µL	+400µL
+ss	+0µL	+25µL	+50µL	+100µL	+200µL	+400µL
+½ pp	+0µL	+25µL	+50µL	+100µL	+200µL	+400µL
+½ ss						

Visible results after 12 days at 25-28ºC



PREPARATION OF REAGENTS FOR CONGO-RED STAINING OF CELLULOSE

- **2.5 mg/mL of water of Congo-Red to stain cellulose (add mertiolate to preserve sterility)**
- **0.5 M NaCl of diluent in water required by Congo-red binding**
- **0.1 M NaOH to dissolve the biofilm yeast / bacteria scoby**

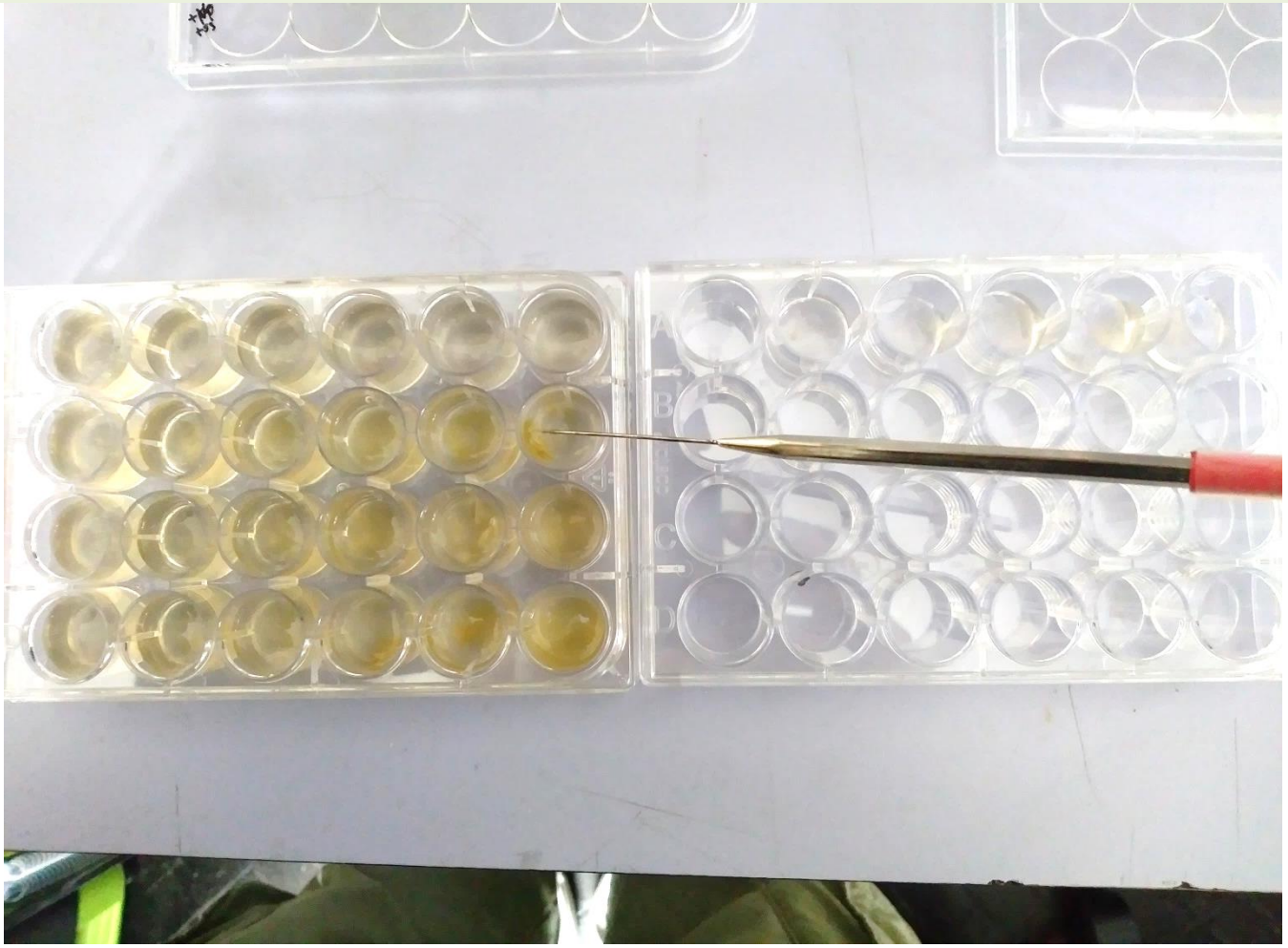
STAINING PROTOCOL (24 well-plates):

1. **+200µL Diluent**
2. **+100 µL Congo-Red**
3. **Manual gentle horizontal agitation**
4. **Wait > 4h**

Note. Other combinations are possible depending on the saturation of the Congo-red binding which depends also of the amounts of cellulose

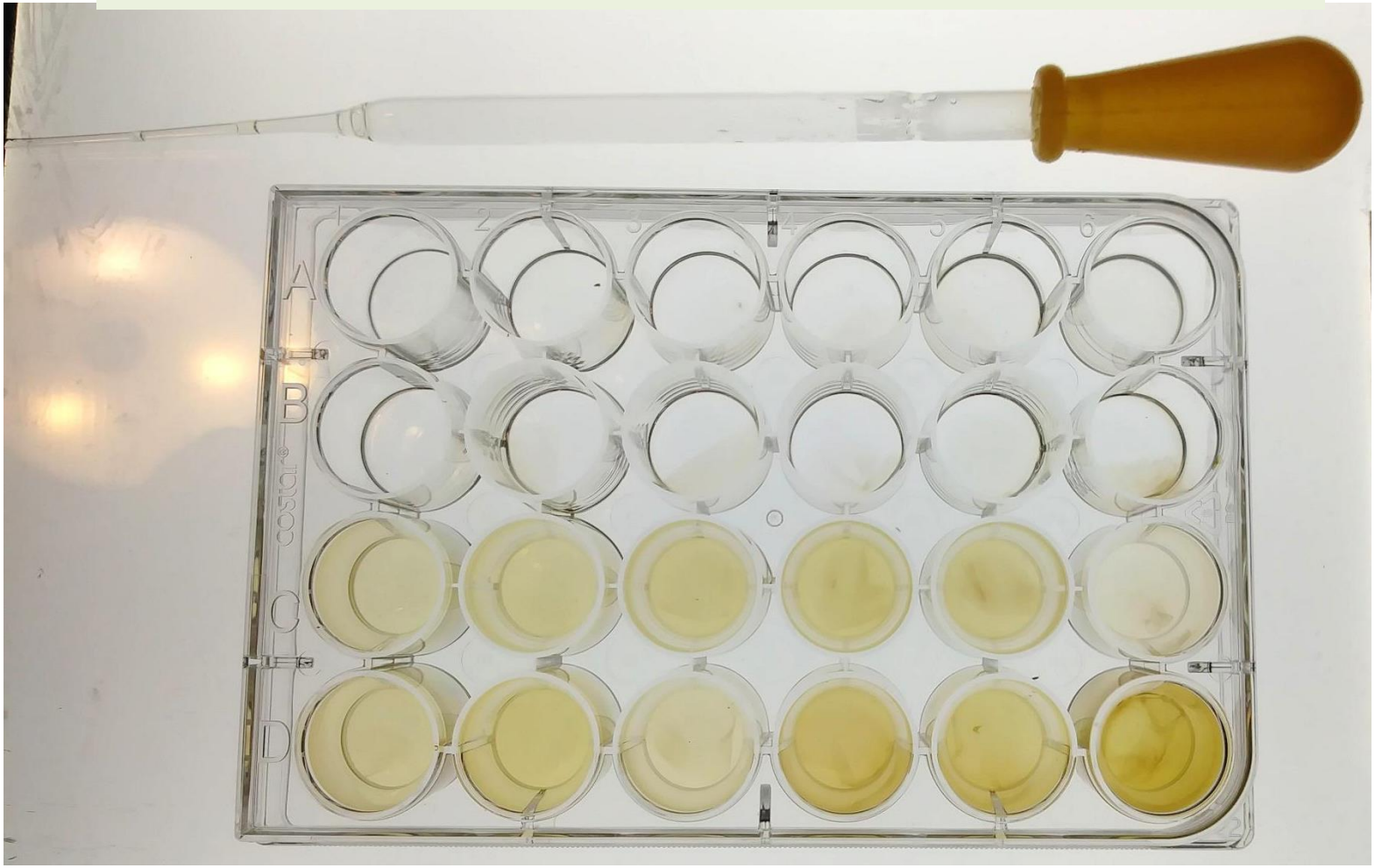
TRANSFER (HARVEST) BIOFILMS FROM THE CULTURES TO AN EMPTY PLATE

"Fishing" the floating or bottom biofilms with a needle

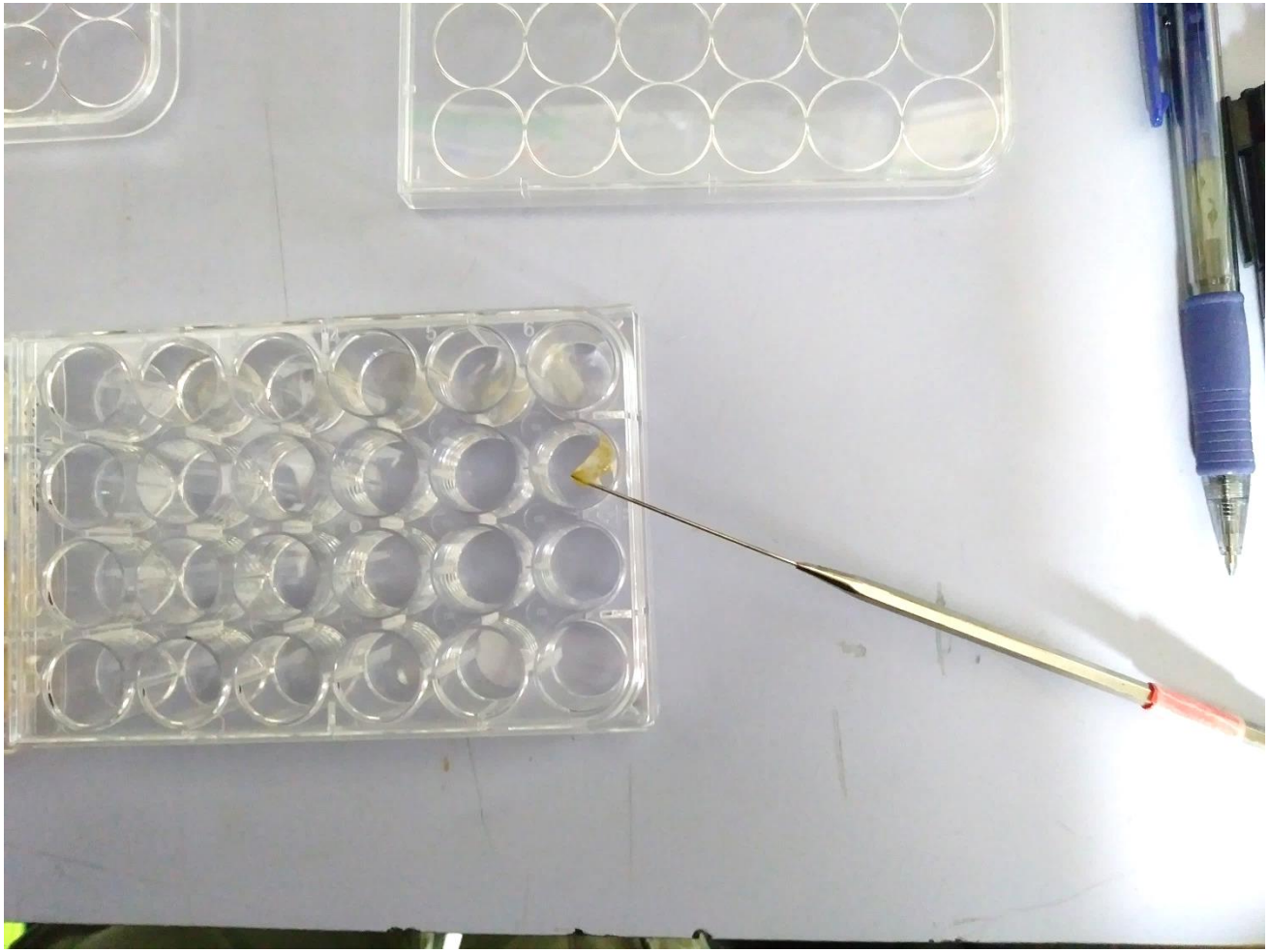


WASHING THE BIOFILMS WITH DILUENT

Carefully eliminate excess of culture media preserving the biofilms.
The AB rows are already washed, the CD are not yet washed



ASPECT OF THE WASHED BIOFILMS

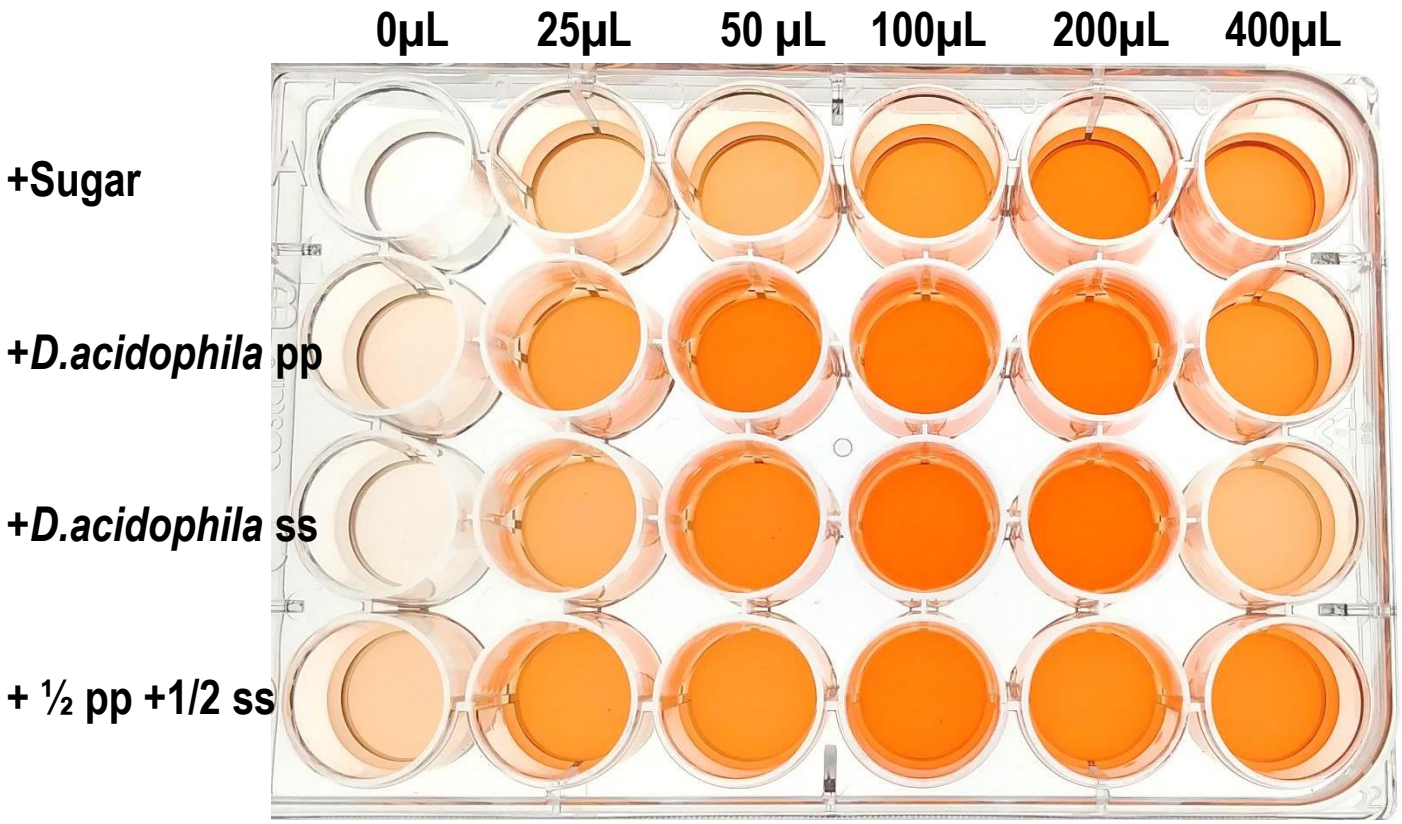


ASPECT OF THE HARVESTED BIOFILMS AFTER BEEN INCUBATED WITH 1mL OF NaOH 0.1N OVERNIGHT TO ELIMINATE THE SCOBY

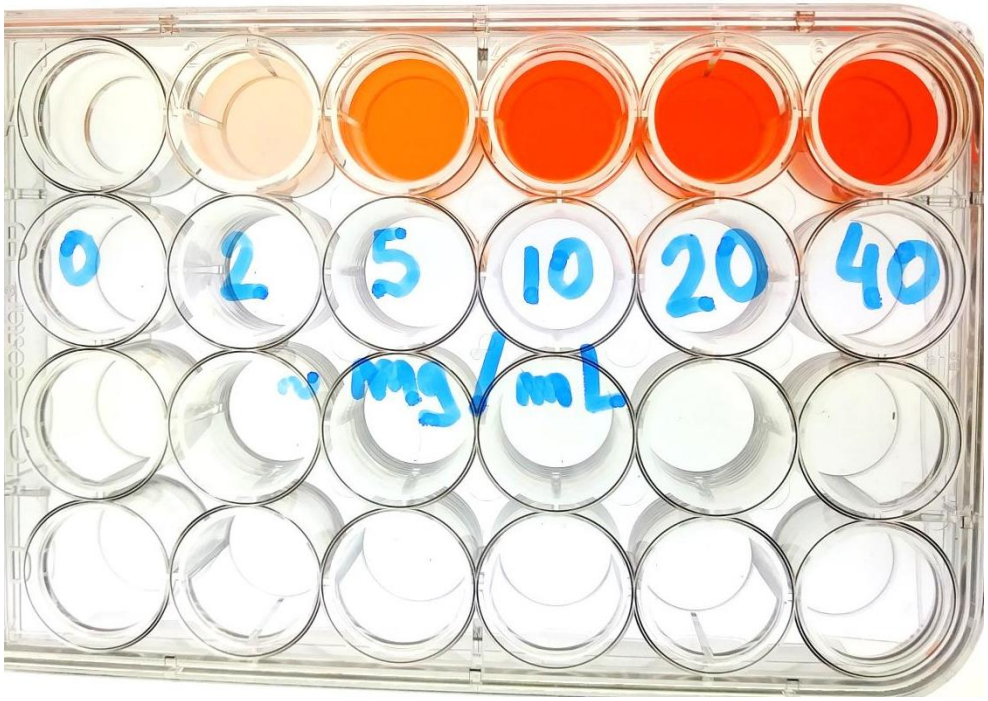
The excess of NaOH was eliminated with a Pasteur pipette

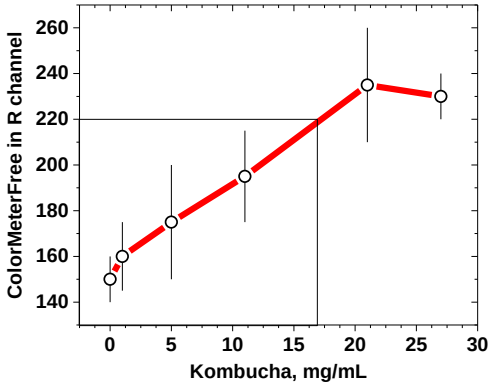
STAINED BIOFILMS ASPECT AFTER WASHING THE EXCESS OF CONGO-RED WITH DILUENT

BOUND CONGO-RED EXTRACTED FROM THE STAINED BIOFILMS WITH 1mL per WELL OF METHANOL DURING 4 HOURS



CALIBRATION OF THE ABSORBANCES OF BOUND CONGO-RED TO DIFFERENT KOMBUCHA BIOFILM WEIGHTS (WASHED AND DRIED)





Important note. To calculate the equivalences between calibration curves and measurements (previous slide), they have to be compared at the same dilutions and only in the linear part of the curve (between 0 and 21 mg) before saturation. Each sample may be stained at several dilutions if increased accuracies are required.

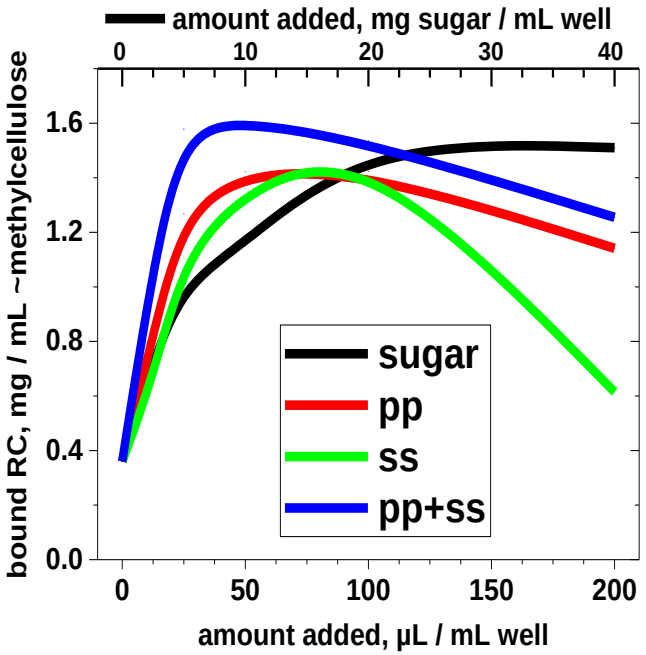
EXAMPLE OF CALIBRATION CURVE AND INTERPOLATION OF ONE OF THE ABSORBANCES WITH GUILLE'S COLORMETER FREE APP IN THE R CHANNEL: MEANS ± STANDARD DEVIATIONS

QUANTIFICATION OF THE CONGO-RED BOUND TO BIOFILMS

1. Extract the bound stain with methanol during 4-6 hours.

2. Compare the color of the extracts with the color of the calibration with known amounts of kombucha cellulose

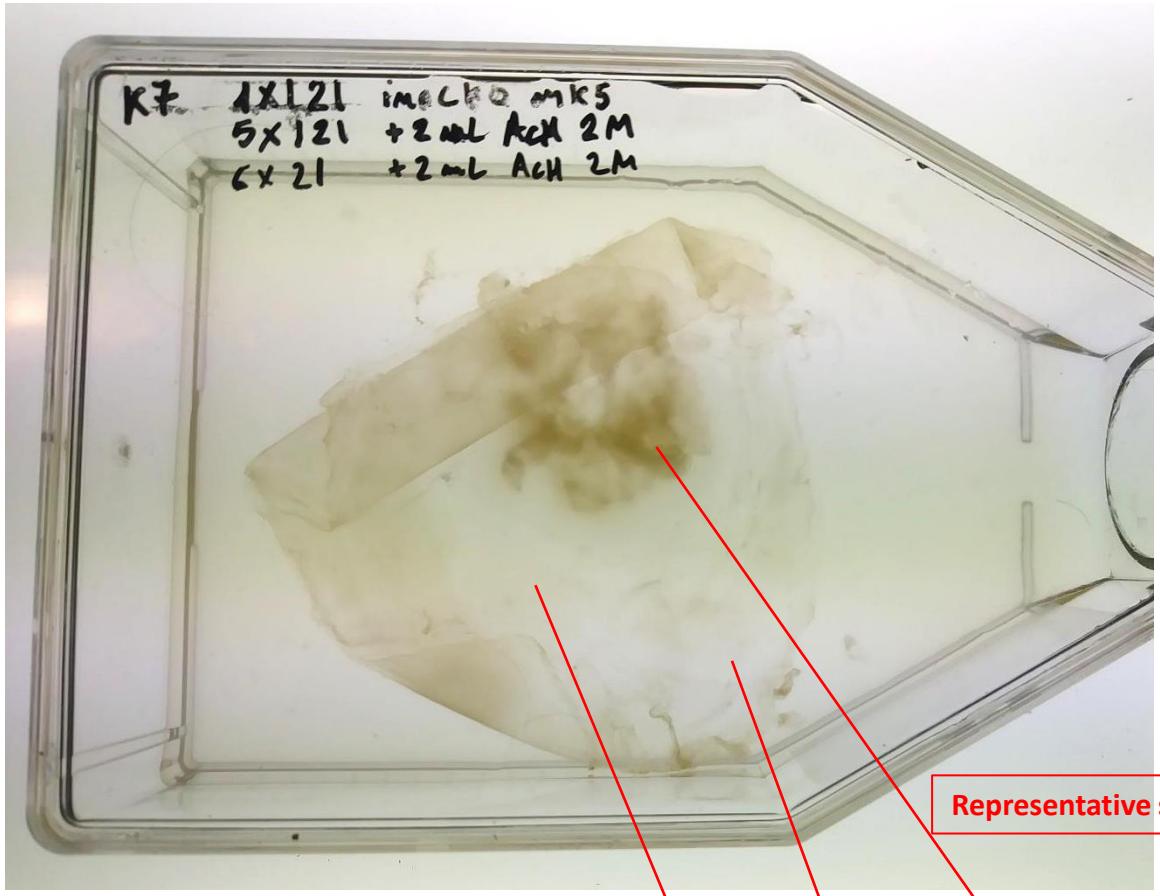
3. Draw and fit the raw data



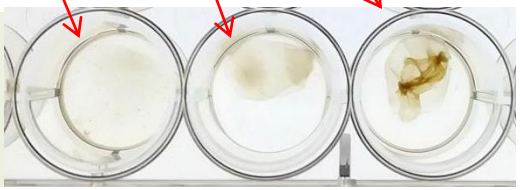
<u>TO QUANTIFY LARGER BIOFILMS, REPRESENTATIVE SAMPLING IS REQUIRED</u>:

For instance, weight the whole biofilm either wet or after drying

Take 2-3 small samples, weight them and stain them with Congo-red



Representative samplings



1) Weight each sample to know how much we are staining relative to the total kombuchas biofilm
2) Stain for cellulose
3) Interpolate and calculate

FINALLY: Compare your results with the published data to see if you have improved their results

Maximal cellulose productions reported:
Revin, 2018: 6mg/mL
Asuini, 2020: 17 mg/mL

CONGRATULATIONS !!!
YOU HAVE QUANTITATED THE PRODUCTION OF KOMBUCHA'S CELLULOSE WITH A SCIENTIFICALLY GREATER ACCURACY THAN JUST BY WEIGHTING !!