EXTRACTION of EXTRACELLULAR POLYMERIC SUBSTANCES

1. Important Factors

There is a couple of factors influencing the EPS extraction efficiency and accuracy. Please refer to Chapter 3 in "*Microbial Extracellular Polymeric Substances: Characterization, Structure and Function*" by J. Wingender *et al.* (1999) for details. The most important thing though is to be consistent within and between the experiments.

Factors	Notes
MLVSS / DOWEX ratio	**0.5 g MLVSS / 35 g washed and filtered DOWEX**
Homogenization of the biomass	Ultra-Turrax (IKA, T-25), speed level (1), 4 min. (Loc: 4130)
Temperature	**+4oC** (keep everything on ice or at +4oC)
Applied shear	• Use the same rpm while stirring the samples • Use same type of magnetic stirrers, with the same dimensions, shape and performance • Use same type of glassware with same shape and volume • Always extract for the same duration (4 hours)

2. MATERIALS

*Cation Exchange Resin : DOWEX Marathon C (strongly acidic, Na+ form, 20-50 mesh size). (Fluka 91973, from Sigma-Aldrich, see protocol folder for material properties and Material Safety Data Sheet)

*Buffer : PBS (Phosphate Buffer Saline): Its advised to use an extraction buffer with an ionic strength/conductivity similar to that of the sample collected (Frolund *et al*., 1996; Wingender *et al.* 1999). So you might need to change the composition of the extraction buffer in accordance with your sample.

*Glassware : Same shape, same volume extraction vessels (500 mL flasks), etc.

*Stirring magnet bars : Same shape and dimensions for each sample

*Stir-plate : Same brand for each sample, with knobs displaying rpm

3. Recipe for PBS (for 1 L)*:

Chemical	Amount to weigh (g)	Mol. weight (g/mol)	Molarity	Location
$Na_3PO_4.12(H_2O)$	0.328	163.94	2 mM	4130
$NaH_2PO_4.(H_2O)$	0.552	137.99	4 mM	4130
NaCl	0.526	58.44	9 mM	4130
KCl	0.0746	74.56	1 mM	4130

* Dissolve the chemicals in dI water to a final volume of **1 L**, then adjust the **pH to 7** (w/ NaOH).

4. Measure MLVSS

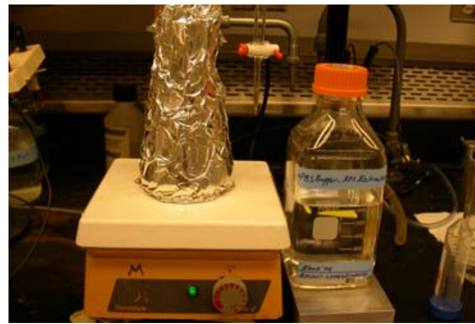
Measure the MLVSS value of the sample and estimate the volume required to get 0.5 g MLVSS.

5. Rinsing the DOWEX

The DOWEX is not totally clean; it's a bit dusty when it's purchased. It should be washed with the buffer used for extraction, prior to use.

- Wear gloves, do not touch DOWEX with bare hands (DOWEX is a cation exchange resin and strongly acidic; it damages the skin).
- Weigh the amount of DOWEX required for the experiment (35 g per SAMPLE, plus 35 g for the BLANK).
- You can weigh a little bit less than 35 g of DOWEX (per sample), because when it is washed and filtered it gets heavier.
- Be careful while weighing: once the chemical is spilled, it gets hard to clean because the spherical DOWEX beads tend to bounce and roll around.

- Add 100-150 mL of the PBS to the DOWEX
- Close the flask, wrap it with alu-folio (to keep in dark) and stir for 1 hour.



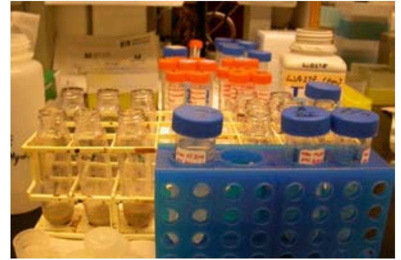
(………….while waiting, you can proceed with centrifuging your samples: step 6……………)

6. Separating Supernatant and Biomass

The soluble portions of the saccharides and proteins are present at the bulk liquid of the sample. You need to separate the biomass and the liquid as much as possible, while not disturbing the cells: So, centrifuge at high speed (Sorvall RC58 Plus centrifuge, Sorvall SA600 rotor) and at +4oC (check the "rpm vs g" conversion sheet on the lid of the centrifuge: approx. 12,000 xg=9000rpm).

- Divide the mixed liquor sample (that you estimated the volume to give 0.5 g MLVSS / 35 g DOWEX) into centrifuge tubes (do not use the 50 mL Croning (orange-capped) or Falcon (blue-capped) tubes since these cannot withstand the high centrifugational forces. Use the Nalgene tubes which hold approx. 35 mL sample).

- Balance the tubes+caps on a coarse scale. Centrifuge samples at **12,000xg, +4oC, for 15 min**.



- Collect a sample from the supernatant avoiding any biomass particles (1 tube 50 mL and 2 tubes of 15 mL supernatant sample are enough. You can decrease the amount of sample you collect; according to the amounts required in the protocols you'll use to measure the polysaccharides and proteins, etc.)

- Put these samples to -20oC and discard the rest.

- If you need to centrifuge more mixed liquor sample, pour your sample into the same Nalgene tubes with the biomass pellets in them and centrifuge.

- After centrifuging all of your sample, storing some supernatant and discarding the rest; try to combine the pellets in 2 in Nalgene tubes, via either pouring directly or adding little amounts of dI water to each pellet (5 mL), vortexing and resuspending the pellets and combining them. When you end up with combined pellets in 2 Nalgene tubes, fill the tubes up to 30 mL with dI water and vortex-shake to mix. This is the washing step.

POINT OPEN FOR DISCUSSION: In my original protocol, biomass is resuspended and washed with dI water. But, discussing with Amiliy and Adrienne, and also reading the comments at the references (Wingender *et al.* 1999), I'm thinking that it might be better to resuspend and wash the biomass pellets with the appropriate buffer (PBS, for this protocol) to keep the ionic strength constant as much as possible.

- Repeat centrifugation at 12,000xg, +4oC, for 15 min.

- While waiting, filter the DOWEX, washed for 1 hour (may use coarse filter- I even use coffee filters as you see at the picture). Always filter for the same duration prior to weighing the washed DOWEX. Filtering duration influences the degree of wetness, thus the weight of the washed DOWEX, which might change the actual amount of DOWEX that you contact with 0.5 g MLVSS.

- Weigh 35 g of washed and drained DOWEX directly into the extraction flasks. Wear gloves!!!!

7. Homogenizing the Biomass

- Get your samples from the centrifuge and discard the supernatant.
- Add approx. 5 mL of the PBS to your biomass pellets and vortex to re-suspend
- Combine the re-suspended biomass, in a 50 mL Croning tube (orange-capped).
- Rinse the Nalgene centrifugation tubes with some PBS (5-10 mL) to get the remaining parts of the biomass in the tubes and add these to the 50 mL Corning tube with re-suspended biomass.
- Repeat rinsing the Nalgene tubes until you have approx. 25 mL of sample.
- Go to 4130 and clean the steel rod of the Ultra-Turrax (T-25 basic, IKA) via wiping the exterior with 70% EtOH and also operating the instrument for couple of seconds while immersing the rod into 25 mL EtOH. Don't forget to rinse the instrument thoroughly (at least 2 times) with ddI water (otherwise the remaining EtOH will interfere). Please read the manual of the homogenizer before use.

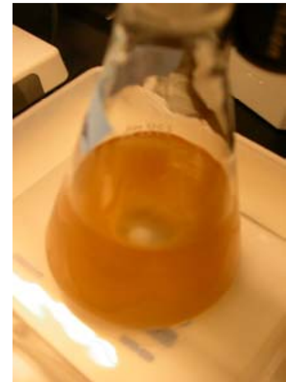
- Do not exceed 25 mL, since the Ultra-Turrax might splash your sample out.

- **Homogenize** your biomass, re-suspended in PBS, for 4 minutes using the Ultra-Turrax, at the lowest speed (orange dial: speed 1: 8,000 rpm[1])".
- Keep the sample in an ice-bucket while homogenizing (since the Ultra-Turrax produces a lot of heat when its operating)

- Pour the homogenized biomass into the extraction flask that includes the washed-filtered 35 g of the DOWEX.

- The **final volume**[2] of the re-suspended and homogenized sample in the extraction flask will be **100 mL**, so use some PBS to rinse the rod of the Ultra-Turrax (turn the instrument on while immersing the rod into some PBS) and all the tubes you used, and add this to your sample in the flask to a final volume of 100 mL.

- Run a BLANK: add 100 mL PBS to a second extraction flask with 35 g DOWEX.



- Add a magnetic stirrer bar to the extraction flask and cap the flask.

- Adjust the mixing speed to 800 rpm (700-900 rpm, but use always the same rpm)

- Check the vortex created by the mixing and be sure to balance the stirrer to prevent it going out of the center during extraction.

[1] You migth prefer to use a gentler homogenizier at a lower speed, but for a longer time: You can homogenize your sample at 2000 rpm, for 10 min.
[2] The alternative to fixing the final extraction volume to 100 mL is, to use 100 mL of PBS (in addition to the volume of your biomass pellet). But if so, use this much (volume of biomass pellet+100 mL PBS) of PBS for the blank. This gets tricky though, when you have more than one biomass sample with different pellet volumes!!

8. Extraction

- Wrap the extraction flasks with alu-folio
- Place them in buckets full of ice and put these on the stirring plates
- Fix the speed to **800 rpm** and start extraction

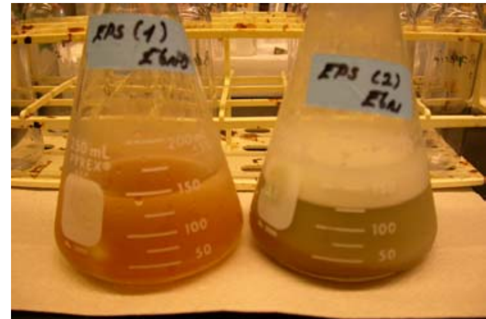
- **Mix for 4 hours (@ +4oC, in dark, 800 rpm)**

- Check once in a while (each 45-60 min) to be sure about the mixing and add ice if it melts.

- By the end of 4 hours your samples will look like this:

BLANK : Clear supernatant + clean DOWEX

SAMPLE : Turbid bulk liquid
+some foam
+DOWEX contaminated with biomass



- To spin down and get rid of the relatively heavy and dense DOWEX particles, you can centrifuge the samples for a short time. You can also use the Nalgene tubes you used before the extraction step.

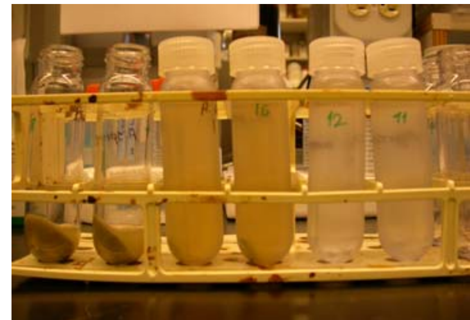
- Aliquot the extracts into Nalgene centrifuge tubes, balance them (with their caps) and spin for a short time. (**1 min, 12,000xg, +4oC**).

- **Transfer** the samples, except the DOWEX beads at the bottom, from the previous step to **CLEAN Nalgene** tubes and balance.

- To separate the bulk liquid from the biomass, centrifuge the samples at **12,000xg, +4oC, for 15 min**.

- The sample will look like those in the picture:

2 tubes at the very left: Pellet of contaminated DOWEX and some biomass after short spin.
2 tubes in the middle: Sample after final centrifugation
2 tubes at the very right: liquid phase from the BLANK



9. Sample Preservation

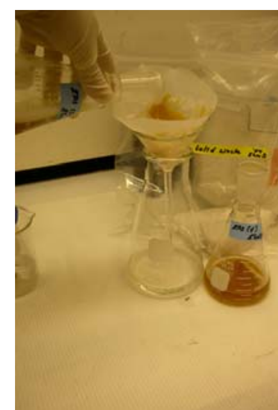
The extractable portions of the Extracellular Polymeric Substances-EPS- extracted by DOWEX from the outer surrounding of the biomass are now in the liquid phase

- Combine the supernatants from the final centrifugation in a clean flask and shake well to mix (to get a homogenous sample). Avoid getting any biomass particles into the flask.
- Aliquot the well-mixed liquid to tubes for long-term preservation (1 tube 50 mL and 2 tubes of 15 mL sample are enough. You can decrease the amount of sample you collected; according to the amounts required in the protocols you'll use to measure the polysaccharides and proteins, etc.)
- **Preserve** the samples at **-20oC** until performing measurements.

CONGRATULATIONS, you're almost done for the day, except cleaning-up ☺

10. Clean-up

- Wear gloves
- Combine all DOWEX-including wastes via pouring them to the funnel with the filter paper you used to filter the washed DOWEX at step 6.
- Let the waste drain overnight and discard the liquid.
- Collect the solid waste and put it in the plastic bag labeled "DOWEX Solid Waste-EPS extraction" sitting inside the fume hood at 4127. Inform Xu Li about the solid waste you generated. This waste will be picked up by the guys from "ChemTrek" and discarded accordingly. Check the MSDS of DOWEX present at the protocols folder.



- Clean all the glassware and centrifuging tubes with soapy-water (preferably antibacterial soap or even better some mucasol) and use a brush to clean the sticky biomass pellets. Rinse them well and let dry.

Now you can proceed with the measurement of the EPS constituents, like total carbohydrates and proteins.

11. Additional NOTES

A. Ideas on EPS extraction for the ones operating membrane systems

If there is a probability that the membranes used in the membrane bioreactor systems are retaining the soluble- or bound-EPS due to the surface charges of these biomolecules, then it may not be sufficient to measure the EPS components (total carbs and proteins) in the supernatant (the soluble portions) and in the DOWEX-extract (loosely- and tightly-bound biomass-EPS). Actually the chemical for EPS extraction (DOWEX MarathonC) is a strongly acidic one in the Na+ form, with a mesh size of 20-50, which basically extracts the loosely- and tightly-bound EPS from the surrounding of the cells. So, if the surface charges of the membranes you're using in your systems are suitable, these bound-EPS could be retained by the membranes you're using, leaving no (or at least not as much as we expect to see) EPS around the biomass that we're treating with DOWEX.

So, here is what I think:

1. The phenomenon of EPS being possibly retained on the membrane depends on the surface charges of the membranes you're using

2. If EPS is retained on the membrane, it should be measured.

3. OK, here is the tough part: how to measure the membrane-bound EPS?: Since consistency (of temperature of +4oC, shear due to 800 rpm mixing with same hydraulics, extraction time of 4hrs, VSS/DOWEX ratio of 0.5g/35g) is the key point in comparative EPS work, it should be kept in mind. I think it's easier to do this with batch runs (like Adrienne's work). As long as you know the amount of biomass getting in touch with the membrane you're using, you can calculate the surface area of the membrane corresponding to 0.5g VSS, and take that much of the membrane, cut into pieces (not to disturb the mixing hydraulics during extraction) and contact them with DOWEX like the way you do for your biomass samples. Of course in order for this to work, the membrane itself should not give any reactions with the extraction buffer and the DOWEX beads. Then the total extractable bound-EPS will be the sum of the part from the biomass and the part from the membrane (minus the supernatant value and the blank).

4. I don't know how this could be applied directly to continuously operated membrane systems. But there might be some info at the literature, or you guys might come up with an idea.

This was a small note from me, which might be helpful with your EPS measurements.

B. The amount of PBS to be used

The golden ratio for the EPS extraction is:

0.5 g MLVSS + 35 g DOWEX + 100 mL extraction buffer

Though the amount of buffer can be adjusted depending on the number of measurements to be made and the protocols to be followed.

For example, I'm using max 3 mL of the extract (1 mL/measurement, triplicates) for total carbs, and 1.5 mL (0.5 mL/measurement, triplicates) for proteins. If you want to measure TOC also, you'll need some more sample (amount depending on the instrument you're using for TOC)

These values depend on the sample and the concentration of the EPS.

You can adjust the amount of buffer for you case,
but do not change the MLVSS or DOWEX amount.

C. Checking the extent of cell lyses during extraction

Glucose-6-phosphate dehydrogenase test (at + 4°C)

Use the G-6-PDH Kit to measure the extent of cell lyses that occurred during extraction.
The kit is purchased from Sigma and costs approx. 100 euro for 20 measurements.

Preparations

- G-6-PDH reagent + 1 mL dI Water

Test procedure

- measure the room temperature (18-26°C) - write it down!
- mix 0.5 mL of sample and 1 mL of G-6-PDH reagent - incubation for 5-10 min.
- add 2 mL of G-6-PDH substrate solution - mix it very carefully!
- put this solution into the tray/vessel - immediately measure the absorbance at 340 nm against water (= abs. at beginning)
- precisely after 5 min. and 10 min. of the incubation, repeat the measurement of the absorbance at 340 nm (= abs. at the end)

Estimation

- Delta abs./min. = (abs.end -abs. begin) /5
- G-6-PDH (U/mL) = [(abs./min. x 3.5 x TCF)/(0.5 x 6.22)] x 2

12. Additional Documents

DOWEX MARATHON C Cation Exchange Resin

A uniform particle size, high capacity cation exchange resin for softening and demineralization applications.

Product Name	Type	Matrix	Functional Group
DOWEX MARATHON C	Strong Acid Cation	Styrene-DVB Gel	Sulfonic Acid

Guaranteed Sales Specification	Units	Na^+ Form	H^+ Form
Total Exchange Capacity, min.	eq/l	2.0	1.8
Total Exchange Capacity, min.	kgr/ft^3 as $CaCO_3$	43.7	39.3
Water Retention Capacity	%	42 - 48	50 - 56
Uniformity Coefficient		1.1 max.	1.1 max.

Typical Physical and Chemical Properties	Units	Na^+ Form	H^+ Form
Whole Uncracked Beads	%	95 - 100	95 - 100
Mean Particle Size	µm	585 ± 50	600 ± 50
Total Swelling ($Na^+ \rightarrow H^+$)	%	8	8
Particle Density, approx.	g/ml	1.28	1.20
Shipping Weight, approx.	g/l	820	800
Shipping Weight, approx.	lbs/ft^3	51	50

Additional Information on DOWEX MARATHON C:

Datasheet: English (19KB) | Deutsch (109KB) | Françasi (143KB) | Japanese (00KB)
Engineering Information: English (113KB) | Japanese (213KB)
MSDS (Na+ Form) - United States: English (319KB)
MSDS (H+ Form) - United States: English (309KB)

For additional particle size information, please refer to the Particle Size Distribution Cross Reference Chart (40KB PDF).

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Related Information and Products
DOWEX® Ion Exchange Resins: Product List

See the PDF file named "DOWEX_SafetyDataSheet" at the protocol folder for the info and MSDS of the DOWEX Marathon C we have in the lab.

13. REFERENCES

Frolund, B.; Palmgren, R.; Keiding, K.; Nielsen, P.H. (1996). "Extraction of extracellular polymers from activated sludge using a cation exchange resin", *Water Res* **30**(8), pp. 1749-1758.

Wingender, J.; Neu, T.R.; Flemming, H.-C. (1999). "Microbial Extracellular Polymeric Substances: Characterization, Structure and Function", Springer-Verlag, NY, ISBN 3-540-65720-7 (present at the UIUC Grainger Engr. Library)

Dulekgurgen, E.; McSwain, B. S., Artan, N.; Wilderer, P.A. (2005). *In prep*