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Extraction of Cyanobacterial Slime from Community Samples and Subsequent Analysis via GC-MS

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Kelsey Cremin[1], Jerko Rosko[1], Sarah J.N. Duxbury[1], Mary Coates[1], Lijiang Song[1], Orkun Soyer[1]

[1]University of Warwick

OSS Lab

Mary Coates
University of Warwick

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We use this protocol and it's working

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Abstract

This protocol details the extraction of cyanobacterial slime from community samples and subsequent analysis via GC-MS.

Attachments

DOCX

663-1393.docx

50KB

Guidelines

NOTE:

This protocol is for extraction of slime (a.k.a exopolysaccharides, EPS) from a filamentous cyanobacterial microbial community (from a freshwater environment) that forms extensive biofilms and granular structures.[1] For other cyanobacterial samples, the protocol can be adapted / simplified.

This protocol is based on the slime extraction method described in Plude *et al.* 1991[2], and the EPS extraction method described in Olenska *et al.* 2021[3] (therefore this protocol uses these original papers' definitions of EPS and slime). Both papers perform cyanobacterial slime/EPS extraction and perform GC-MS based analytical studies - via hydrolysis - on the extracts, however, details of some of the preparation and analysis steps are limited. Here, elements of hydrolysis method descriptions from the above two papers have been combined with a more rigorous and hopefully total hydrolysis method[4], so to further optimise this process.

The protocol also contains steps for preparation of a selection of monosaccharide standards for analysis, to be used as comparison against the samples and treated using the same GC-MS based analytical methods.

Associated protocols / media sheets:
DSMZ_Medium_BG11+_1593 (omitting Vitamin B12 and replacing with full vitamin mix supplement as described below in Table 1)

OSP_35: This protocol.

References

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(6) Zhu, R.; Lin, Y. S.; Lipp, J. S.; Meador, T. B.; Hinrichs, K. U. Optimizing sample pretreatment for compound-specific stable carbon isotopic analysis of amino sugars in marine sediment. *Biogeosciences Discuss* **2014**, *11*, 593–623.

Materials

Materials

- vitamin mix
- BG11+ growth media
- Nuclepore 5-µm pore size filter
- Gelman 0.45 µm membrane filter
- ddH_2O
- 18G needle
- syringe
- 25G needle
- $CaCl_2$
- H_2SO_4
- Na_2CO_3
- sodium hydroxide
- methanol
- pyridine
- 1-(Trimethylsilyl)imidazole
- heptane
- Phenyl Methyl Silox column
- Agilent 7890GC

Troubleshooting

PART A: Slime/EPS extraction

1 Use mature cultures (at least 35 days old, grown in BG11+ with full vitamin mix – see Table 1) and record culture origin and date of initiation.

Note

In our experience, an approximate volume of 200 mL of culture produces dried slime with a mass of 20-30 mg, therefore greater initial volumes of culture are advised.

Table 1. Full vitamin mix, prepared as a one thousand times concentrated stock, as referenced in Duxbury et al. (2023)[1]

	A	B	C	D	E
	Vitamins	**g/L (stock)**	**g/L (medium)**	**g/mol**	**Mol/l (medium)**
	Biotin	0.020	0.00002	244.31	8.2E-08
	Folic Acid	0.020	0.00002	441.40	4.5E-08
	Pyridoxin HCl	0.100	0.0001	205.63	4.9E-07
	Thiamine HCl	0.050	0.00005	337.26	1.5E-07
	Riboflavin	0.050	0.00005	376.26	1.3E-07
	Nicotinic Acid	0.050	0.00005	123.11	4.1E-07
	D-Ca-Panthotenate	0.050	0.00005	238.27	2.1E-07
	p-Aminobenzoic Acid	0.050	0.00005	137.14	3.6E-07
	Vitamin B12	0.001	0.000001	1355.37	7.4E-10
	Lipoic Acid	0.050	0.00005	206.33	2.4E-07

2 Once grown, centrifuge the cultures at 4000 x g, 4°C, 00:20:00 to pellet the cells. 20m

Note

Plude *et al.* believes the pellet will contain the adherent slime, whilst Olenska *et al.* work on the principle that the EPS exists in the supernatant - therefore, we suggest that both should be kept (from the same cultures) and analysed concurrently.

2.1 *Culture Supernatant slime/EPS.* Following centrifugation – in step 2 - remove the supernatant carefully into a separate container. Filter the supernatant through a Nuclepore 5-mm pore size filter, then through a Gelman 0.45 mm membrane filter and store frozen at -4 °C until lyophilised, and then use for analysis.

Note

=> this sample is called "Culture Supernatant" below.

3 Next, extract any slime/EPS from the remaining cell pellet, using an adapted method inspired by both Plude *et al.* (1991)[2] and Nakagawa et al. (1987)[5].

3.1 After centrifugation – in step 2 - approximately measure the culture pellet volume and resuspend in 30 times the pellet volume of ddH_2O (vortex vigorously). For this dilution step, we use a centrifuge tube which has volume markings, so if the pellet occupies 1 mL of the tube, then add 30 mL of ddH_2O. Store the suspension Overnight at 4 °C. The slime should separate from the cell pellet and enter the ddH_2O.

3.2 Next morning, centrifuge the cell pellet solution again 4000 x g, 4°C, 00:20:00, to pellet the cells. Remove this supernatant and pass into sterile glassware, store this at 4 °C whilst we extract the rest of the slime. 20m

Note

Note: The supernatant contains the slime according to Plude *et al.* (1991), while in our case we combine it with supernatants obtained from further treatment of the cell pellet, as explained next.

3.3 Suspend the cell pellet (which can measure approx. 5 mL for a 200 mL culture) in 25 mL of ddH_2O (or more, scale accordingly with the initial culture size). 1h 15m

1. Vortex the samples for 2 minutes max, split between twelve 2 mL centrifuge tubes and centrifuge at 15000 x g, 00:15:00 in a benchtop centrifuge. This sheds a portion of the slime and the fragments enter the supernatant. Extract the supernatant

from each tube, pool it together, and resuspend each pellet in fresh 1.5 mL of ddH_2O. (1/5)

2. Vortex the samples for 2 minutes max, split between twelve 2 mL centrifuge tubes and centrifuge at 15000 x g, 00:15:00 in a benchtop centrifuge. This sheds a portion of the slime and the fragments enter the supernatant. Extract the supernatant from each tube, pool it together, and resuspend each pellet in fresh 1.5 mL of ddH_2O. (2/5)
3. Vortex the samples for 2 minutes max, split between twelve 2 mL centrifuge tubes and centrifuge at 15000 x g, 00:15:00 in a benchtop centrifuge. This sheds a portion of the slime and the fragments enter the supernatant. Extract the supernatant from each tube, pool it together, and resuspend each pellet in fresh 1.5 mL of ddH_2O. (3/5)
4. Vortex the samples for 2 minutes max, split between twelve 2 mL centrifuge tubes and centrifuge at 15000 x g, 00:15:00 in a benchtop centrifuge. This sheds a portion of the slime and the fragments enter the supernatant. Extract the supernatant from each tube, pool it together, and resuspend each pellet in fresh 1.5 mL of ddH_2O. (4/5)
5. Vortex the samples for 2 minutes max, split between twelve 2 mL centrifuge tubes and centrifuge at 15000 x g, 00:15:00 in a benchtop centrifuge. This sheds a portion of the slime and the fragments enter the supernatant. Extract the supernatant from each tube, pool it together, and resuspend each pellet in fresh 1.5 mL of ddH_2O. (5/5)
6. Collect the supernatant with each iteration.

Note

By the third iteration, the supernatant – for our samples - had a slight blue colour, possibly from cellular photopigments being extracted as well.

4 *Further slime extraction from pellet*. Following on from the last cycle of step 3.3 collect the remaining pellets – using ddH_2O - into one falcon tube, and add 10 mL of ddH_2O to further loosen the pellet.

- Shear the cyano pellet solution, by pipetting up and down into a 10-mL syringe through a 18G needle 20 times, follow this by repeating the shearing steps with a 25G needle, also 20 times. Hope this removes the final remaining slime sheaths from cyanobacteria filaments.

- Centrifuge the cyano pellet again, and collect the supernatant and add to the other supernatant solutions collected in the previous steps 3.2 and 3.3.
- Keep the remaining cyano pellet for further analysis.

Note

=> this sample is called “Culture cyano pellet” below.

5 Following the iterations of slime extraction, which gives a final volume of 90 mL supernatant containing slime, to which add 45 mg of $CaCl_2$ (final conc. of 500 mg/L $CaCl_2$). Mix this and leave it Overnight in the fridge (4 °C).

6 On the next day, centrifuge the solution (from step 4) at 15000 x g, 00:30:00 , to pellet out the slime. Remove the supernatant - a green-tinged transparent gelatinous material in our case - and store in the fridge for further analysis if wished. 30m

Note

=> this sample is called “Culture slime supernatant” below.

7 Also, store the “slime pellet” from step 5 with a final volume of less than 1 mL .

Note

=> this sample is called “Culture slime pellet” below.

8 Prior to GC, lyophilise the Culture supernatant, Culture cyano pellet, Culture slime supernatant and Culture slime pellet fractions.

PART B: Preparation of samples (and known standards) for GC-MS

9

Note

Standards for known monosaccharides can be prepared, so that their GC-MS spectra can be compared to that of actual sample. Monosaccharide standards will all be of HPLC grade and will be prepared to 1 mg/L sample, through a serial dilution. Two control samples ('blanks'), one consisting of fresh BG11+ growth media, and one of ddH_2O were included in the GC-MS analysis.

Choice of monosaccharide standards should be project specific, but here we focus on the following monosaccharides based on previous studies of cyanobacterial slime/EPS:

- Glucose,
- Xylose,
- Galactose,
- Mannose,
- Rhamnose,
- Galacturonic acid,
- Galactonate
- Gluconarate
- D-Fructose,
- L-Fucose,
- Adonitol,
- Fumaric Acid,
- L-Aspartic Acid,
- L-Arabinose

The samples to analyse come from the different fractions resulting from the extraction step – part A – listed above:

- Media control (BG11+ with vitamin mix),
- ddH_2O water control,
- Culture Supernatant,
- Culture cyano cell pellet,
- Culture slime supernatant, and
- Culture slime pellet.

PART B: Sample/control prep for GC

4h 49m 30s

10 ***Hydrolysis (*see also Hydrolysis NOTE).*** Use acid hydrolysis over acidic methanolysis as total hydrolysis is desired, and a more thorough hydrolysis can be achieved with the harsher acid hydrolysis method.

Note

The hydrolysis method is based on Becker *et al.* 2021.[4] Hydrolysis consists of two stages.

10.1 Weigh 40 mg of each standard, control blank, and the lyophilised culture fraction samples are into separate clean glass vials. 2h

- In the first stage, add 1.5 mL of 72% *aq.* H_2SO_4 to each sample. This is left stirring under Room temperature for 02:00:00 .

10.2 In the second stage, add 2 mL of H_2O to each mixture and heat them in an oven at 80 °C for 01:00:00 . Cool down the hydrolysis solution in an ice bath and stored at 4 °C Overnight .

2h

10.3 Neutralise the hydrolysed sample to pH 7 , using Na_2CO_3 until CO_2 evolution subsides.

Note

This will require a considerable amount of Na_2CO_3 and will involve salt formation.

10.4 Filter the resulting pH 7 solutions through Gelman 0.45 mm filter into new test tubes. Then dry the hydrolysates under flowing nitrogen gas.

- In this set up, place a single branching capillaries from a line splitter into each test tube where it sits about the liquid level, then connect the line to a nitrogen cannister, which is set to allow a steady flow of nitrogen across the sample. In this set up the samples dry to completeness in 30-60 minutes.

Note

Hydrolysis NOTE
In our experience it is possible that some known monosaccharides fail to derivatise with the above hydrolysis method. Thus, an alternative hydrolysis method would be to use HCl instead of H_2SO_4. Such a method would be similar to the one used by Zhu *et al.* (2014) for amino sugar hydrolysis, where it was found to have high recovery rates.[6]

Alternative hydrolysis methods. *Weigh* 40 mg samples and standards into glass test tubes, to which add 1 mL of [M] 6 Molarity (M) HCl. Then heat this to 105 °C for 08:00:00 on the heat block, inside the chemical fume hood. Adjust this solution to pH 6.5–7.0 with [M] 1 Molarity (M) sodium hydroxide (NaOH), dry by evaporating it under N_2 as above, re-dissolved in 2 mL methanol (MeOH, 99+%). Samples collected in the supernatant after centrifugation, as above. The methanol step will remove the salt formed from neutralisation. Subsequent derivatisation steps after hydrolysis as the same as described from (step 11 onwards).

11 ***Trimethylsilylation.*** 30m 30s

Note

This trimethylsilylation method has been originated in Becker *et al.* 2013.[4]

Dissolve the dried hydrolysates in 1.5 mL of pyridine (Scientific Laboratory Supplies Ltd, W296600, 99+%), shake by hand for 00:00:30, and then incubate at Room temperature for 00:30:00.

11.1 Then add 0.5 mL of 1-(Trimethylsilyl)imidazole (1-TMS-imidazole, Merck, A12512.06, 97%), and incubate the samples in a shaking incubator at 60 °C.

11.2 Cool at Room temperature. Remove the pyridine fully by drying with nitrogen gas, as described above. Drying will again take 30-60 minutes.

Note

Due to safety concerns and the unpleasant odour of pyridine, it is highly encouraged to perform this in a chemical fume hood with outside extraction.

11.3 To the dried compounds, add 2 mL heptane (>99.3%, LC grade) and dry under nitrogen again. Repeat the addition and drying of heptane several times until the samples no longer smell of pyridine.

12 ***Concentrations for GC.*** Each sample contains 40 mg of the species of interest; therefore, dissolve each sample into 8 mL of hexane (>99%, LC grade, solvent used for GC), thereby creating 5 mg/mL master stocks.

12.1 From this master stock, take 1 mL and dilute into hexane to give a total volume of 1 mL in brown glass GC vials – resulting in final concentration of 5 mg/L.

Note

NOTE: There may be a precipitate left over from the silylation – step 11, above - at the bottom of the vials. This should be avoided and only the solution should be taken as the solution only contains the monosaccharides.

12.2 This creates a set of 5 mg/L working stocks, which can be taken directly to the GC. Smaller sample concentrations than this are difficult to interpret as the signal would be too low and it is feared that some compounds will fall within the noise level.

Note

NOTE: these samples should be produced close to the actual GC-MS date, if not, store the trimethylsilyl (TMS) derivatives moisture-sealed and in a -20 °C freezer where they will remain stable for several months.

13 ***GC-MS conditions.*** Perform all GC-MS experiments on an Agilent 7890GC coupled with 5977B MSD detector. Use an Agilent HP-5MS with 5% Phenyl Methyl Silox column (30 m × 250 mm × 0.25 mm). Set the initial column temperature to 150 °C, hold for 00:02:00, and increase at a rate of 8 °C /min to 250 °C, then hold for 00:17:00. Use Helium as a carrier gas (1.2 mL /min). Front inlet temperature is 275 °C, transfer line temperature is 280 °C, MS source temperature is 230 °C, and MS quad temperature is 150 °C. Use an injection volume of 1 mL, with an injection dispense speed of 6000 mL /min. The total run time is 32 minutes. Use electron ionisation, with a MS scan range 50-750 m/z. 19m