

Apr 03, 2023

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

DOI

dx.doi.org/10.17504/protocols.io.5qpvorw8dv4o/v1

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Protocol Citation: Kelsey Cremin, Jerko Rosko, Sarah J.N. Duxbury, Mary Coates, Lijiang Song, Orkun Soyer 2023. Extraction of Cyanobacterial Slime from Community Samples and Subsequent Analysis via GC-MS. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.5qpvorw8dv4o/v1>

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Protocol status: Working

We use this protocol and it's working

Created: March 04, 2023

Last Modified: April 03, 2023

Protocol Integer ID: 78088

Keywords: Slime/EPS extraction, Preparation of samples for GC-MS, Trimethylsilylation

Funders Acknowledgement:

**Gordon and Betty Moore
Foundation**
Grant ID: 9200

Abstract

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

Attachments



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.* 1991², and the EPS extraction method described in Olenska *et al.* 2021³ (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 method⁴, 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

- (1) Duxbury, S. J. N.; Raguideau, S.; Rosko, J.; Cremin, K.; Coates, M.; Quince, C.; Soyer, O. S. Reproducible spatial structure formation and stable community composition in the cyanosphere predicts metabolic interactions. *bioRxiv* **2022**.
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- (3) Olenska, E.; Malek, W.; Kotowska, U.; Wydrych, J.; Polinska, W.; Swiecicka, I.; Thijs, S.; Vangronsveld, J. Exopolysaccharide Carbohydrate Structure and Biofilm Formation by *Rhizobium leguminosarum* bv. *trifolii* Strains Inhabiting Nodules of *Trifolium repens* Growing on an Old Zn-Pb-Cd-Polluted Waste Heap Area. *Int J Mol Sci* **2021**, 22(6).
- (4) Becker, M.; Ahn, K.; Bacher, M.; Xu, C.; Sundberg, A.; Willfor, S.; Rosenau, T.; Potthast, A. Comparative hydrolysis analysis of cellulose samples and aspects of its application in conservation science. *Cellulose (Lond)* **2021**, 28(13), 8719-8734.

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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
- ddH₂O
- 18G needle
- syringe
- 25G needle
- CaCl₂
- H₂SO₄
- Na₂CO₃
- sodium hydroxide
- methanol
- pyridine
- 1-(Trimethylsilyl)imidazole
- heptane
- Phenyl Methyl Silox column
- Agilent 7890GC

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-Panhotenate	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 ddH₂O (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 ddH₂O. Store the suspension Overnight at 4 °C. The slime should separate from the cell pellet and enter the ddH₂O.



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

1h 15m

25 mL of ddH₂O (or more, scale accordingly with the initial culture size).



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 ddH₂O. (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 ddH₂O. (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 ddH₂O. (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 ddH₂O. (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 ddH₂O. (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 ddH₂O - into one falcon tube, and add 10 mL of ddH₂O 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 CaCl₂ (final conc. of 500 mg/L CaCl₂). 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 ddH₂O 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
- Gluconate
- 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),
- ddH₂O 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.* H₂SO₄ to each sample. This is left stirring under  Room temperature for  02:00:00.

- 10.2 In the second stage, add  2 mL of H₂O 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  7, using Na₂CO₃ until CO₂ evolution subsides.

Note






This will require a considerable amount of Na₂CO₃ and will involve salt formation.

- 10.4 Filter the resulting  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 H₂SO₄. 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 N₂ 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