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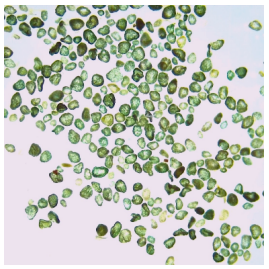
Isolation of cyanobacterial packets from *Azolla* ferns



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Dave Armitage[1]

[1]Rice University

Dave Armitage

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol for extracting intact cyanobacterial packets from leaves of living *Azolla* ferns using enzymatic digestion. This protocol is modified from: Uheda E. (1986) *Plant Cell Physiol*. 27: 1255-1261.

Image Attribution

David Armitage

Materials

MATERIALS

- D-Mannitol **Merck MilliporeSigma (Sigma-Aldrich)**
- Cellulase R-10 **Gold Biotechnology Catalog #**C8001
- Macerozyme R-10 **Gold Biotechnology Catalog #**M8002
- Polyvinylpyrrolidone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**PVP40
- Dithiothreitol (DTT) **Thermo Fisher Catalog #**D1532
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787
- Phosphate buffer solution (PBS)
- Pectinase (from Aspergillus niger) **Worthington Biochemical Corporation Catalog #**LS004298

1. 0.5 M D-mannitol solution

dissolve 91.09 grams D-mannitol into 1 L DI water and autoclave

2. 0.1 v/v% Triton X-100

in Erlenmeyer flask, add 200 uL Trition X into 200 mL sterile DI water and dissolve by swirling

3. Enzyme solution*

Dissolve the following into 50 mL 0.5M manntol solution:

- 1 g Cellulase
- 0.5 g Macerozyme,
- 0.05 g Pectinase24 g KH_2PO_4
- 0.077 g Dithiothreitol (DTT) (10 mM)
- 0.5 g Polyvinylpyrrolidone (PVP)

Pipet the solution into a sterile 50 mL centrifute tube and place on ice or in a refrigerator until use. It is probably OK to freeze, but I have always prepared it on the day it is used. I usually make up to 150 mL at a time - enough for processing three *Azolla* genotypes. More than that means you'll be pipetting 12 hours straight the next day.

4. Phosphate buffered saline (PBS)

For a 20x PBS stock solution, dissolve the following into 1 L of distilled water and autoclave:

- 160 g NaCl
- 4 g KCl
- 28.8 g Na2HPO4· 2H2O
- 4.8 g KH2PO4

For Adjust pH to 7.4 and autoclave. Working solutions can then be made by adding 50 mL 20x stock to 950 mL DI water and autoclaved.

*Pectinase can be substituted with Pectolyase Y-23, and Cellulase R-10 can be substituted with Cellulase RS. These enzymes are more expensive, but I have not noticed an improved packet yield when using them.

Troubleshooting

Day 1

1d

1 **SETUP:** 30m

- Prepare vacuum dessicator. Make sure it holds vacuum of 15 to 20 in Hg.
- Prepare enzyme solutions, dispense into separate 50 mL centrifuge tubes, and store on ice
- Prepare 200 mL Triton-X solution in erlenmeyer flasks
- For each *Azolla* strain that will be digested, prepare the following and label with ID markings:

1. one 50 mL sterile centrifuge tube containing enzyme solution
2. up to 3 empty wells in a 6-well tissue culture plate
3. one erlenmeyer flask containing 200 mL of Triton-X solution
4. one plastic weigh boat
5. one 50 mL serological pipet

2 ***AZOLLA* PREPARATION:** 20m

- Prior to starting, confirm *Azolla* genotype to be digested contains cyanobacteria by crushing some plants on a microscope slide and looking for filamentous cyanobacteria leaking from leaves.
- Weight out 4-8 g fresh *Azolla* tissue in weigh boat.
- With forceps, remove as much root tissue as possible from the plants (no need to remove all of it)
- Place plants in coarse mesh situated inside of funnel (we use Nitex mesh 500 uM mesh size), and rinse under DI water for 60 sec.
- Move rinsed plants into Erlenmeyer flask with Triton-X solution and vigorously swirl for 30 seconds.
- Dump flask's contents into clean mesh funnel and rinse under DI water for at least 2 minutes, making sure that no Triton-X remains on plants.
- Do a final rinse with sterile DI water and then, with clean forceps, move plants into wells of a 6-well tissue culture plate. Label each well with the *Azolla* genotype by

writing on the cover of the plate. I usually use 2 or 3 wells per genotype, depending on the amount of the material.

- Weight plants down to prevent floating. We use a custom-etched stainless steel mesh grid that can be inserted into tops of wells.

3 **VACUUM INFILTRATION OF *AZOLLA* LEAVES:** 45m

- Into each well of the 6-well plates, pipette cold enzyme solution to cover plant material (make sure to use the solution from each strain's individual centrifuge tube, since we'll be adding it back to the tube afterwards.)
- Place cover on culture plate and place, stacked, into vacuum dessicator. Carefully place cover on dessicator.
- Draw the vacuum in the chamber to between 15 and 20 in Hg and maintain this vacuum for at least 30, and up to 45 minutes.
- Slowly repressurize the chamber and **carefully** open and remove the plates.

4 **DIGESTION OF LEAVES:** 18h

- Using forceps, remove the mesh grid in the culture plate well.
- Remove plant material using weigh spoon or forceps and place into 50 mL centrifuge tube containing enzyme solution (wells containing the same genotype should all be added to a single centrifuge tube).
- If a significant amount of enzyme solution remains in wells, add it back to centrifuge tube with a serological pipette up to 35 or 40 mL total. There should be a small amount of headspace in each tube. **Note: use a different serological pipette for each plant genotype.**
- Move tubes into a heated shaker and tape in place horizontally, at a slight angle downwards. Shake at 80 rpm and 32° C for 18 - 22 hours.
- Before you leave, make sure plant material is being agitated and not just piling up on one end of the tube--- you might need to adjust the angle of the tube, amount of enzyme, and rpm of the shaker.

Day 2

5 **CLEANING THE DIGESTED PLANT MATERIAL:**

- For each genotype, prepare a sterile Erlenmeyer flask capable of holding at least 500 mL of fluid, and label with individual *Azolla* genotypes.
- Remove centrifuge tubes from shaker.
- Place a funnel with 350-500 um mesh (we use NITEX mesh) over the empty Erlenmeyer flask
- Gently agitate the tube containing the digested plant material by rocking back and forth a few times, and then dump its contents through the mesh and into the flask.
- Use 0.5 M mannitol solution to rinse the remaining plant material from the centrifuge tube.
- Using a serological pipettor, wash over the plant material suspended on the mesh funnel with 500 mL of mannitol solution. This will flush the separated cyanobacterial packets into the flask but keep the un-digested solids in the mesh.
- Cover the tops of these flasks with tinfoil and allow them to settle for 30 min.
- Using a 50 mL serological pipette, slowly and carefully remove the mannitol solution from the flask by taking it from the top of the water column. **Take care not to agitate the cellular contents on the bottom of the flask.**
- Once about 150-200 mL of fluid is left in the flask, turn it at an angle and allow it to settle for 10 min.
- Carefully remove all but 100 mL of the fluid by drawing from the top of the water column. Again, do not agitate the contents that have settled to the bottom.
- Once you have 80-100 mL of liquid remaining, swirl the flask and transfer the contents into wells of a sterile 6-well tissue culture plate. Label the plate with the plant genotype it contains.
- For each plant genotype, prepare a second 6-well plate containing sterile, phosphate buffer solution (1x PBS) in each well. Label this with the same plant genotype identifier and "PBS". This is the 'cleaning solution' that will be used to dilute any contaminants from outside of the packets.

6 **ISOLATING THE CYANOBACTERIAL PACKETS:**

- Using a dissecting (stereo) microscope, examine an individual 6-well plate containing digested plant material. The isolate cyanobacterial pockets should have sunk to the bottom of the plate and will be dark green and oval-shaped. They can also be flat, depending on the species of *Azolla.*
- Use a sterile 2 uL pipettor to remove individual packets from these wells and move them into the first well of the matching PBS plate.
- Repeat this for each well of the 6-well plate, moving all packets into the A1 well of the second plate.
- Once finished removing the packets, serially transfer them across wells of the PBS-containing plate to dilute any contaminants. I usually transfer them across all six wells using a 100 mL pipette tip.
- Once the packets reach the final well, transfer them directly into a sterile cryo-tube and store at -20C for DNA extraction.