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Aniline blue and Calcofluor white staining of callose and cellulose in the streptophyte green algae *Zygnema* and *Klebsormidium*

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Abstract

Plant including green algal cells are surrounded by a cell wall, which is a diverse composite of complex polysaccharides and crucial for their function and survival. Here we describe two simple protocols to visualize callose (β -1 $\rightarrow$ 3-glucan) and cellulose (β -1 $\rightarrow$ 4-glucan) and related polysaccharides in the cell walls of streptophyte green algae by using standard dyes and epifluorescence microscopy.

Materials and Reagents

1. Algal cells or filaments (Figure 1A)
2. Culture medium (BBM, MBBM)
3. Sodium hydroxid pellets (Merck, catalog number: 1310-73-2)
4. Sørensen's phosphate buffer (preparation see below)
5. Aniline blue (Sigma-Aldrich, catalog number: 415049)
6. Calcofluor white (Sigma-Aldrich, catalog number: 18909)
7. *A. bidest.*

Equipment

1. Glassware: 25 mL beaker, 1000 mL and 500 mL volumetric flask, 100 mL Schott flask
2. Balance
3. Pair of fine-pointed tweezers
4. Glass Pasteur pipettes
5. 2 mL Eppendorf tubes
6. Metal rack

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7. Water bath or heat plate and beaker
8. Tabletop centrifuge
9. Microscopic slides and cover slips
10. Epifluorescence microscope (Zeiss Axiovert 200M microscope (Carl Zeiss AG), equipped with a 63×1.4 NA objective, a Zeiss Filter Set 01 (excitation: band pass (BP) 365/12 nm; emission: long pass (LP) 397 nm) and connected to a Axiocam MRc5 camera).

Procedure – Aniline blue staining

1. Prepare 20 mL of 8N NaOH from sodium hydroxid pellets in double distilled water (*A. bidest*) in a 25 mL beaker.
2. Prepare 50 mL of Sørensen's phosphate buffer (0.1 M, pH=8.0).
Note: Prepare the following two stock solutions (0.2 M): (A) Add 15.60 g sodium phosphate monobasic dehydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; Merck, catalog number: 1063420250) to 500 mL of A. bidest. (B) Add 35.61 g sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; Merck, catalog number: 1065800500) to 1000 mL of A. bidest. Both stock solutions can be stored at 4 °C for several months. To prepare the buffer (0.1 M, pH=8.0), mix 2.7 mL of stock solution (A) with 47.3 mL of stock solution (B) (Sørensen 1909) in a 100 mL Schott flask.
3. Transfer algae to 2 mL Eppendorf tubes (Figure 1B) and wash them once with culture medium.
Note: Washing can be omitted if algae from lab cultures are investigated. Avoid mechanical stress or damage to the cells by using a pair of fine-pointed tweezers. This prevents additional short-term incorporation of callose in the cell walls. Single-celled organisms or cells surrounded by a sticky mucilage layer can be transferred easily by using glas pipettes.
4. Decant and discard culture medium and add 1.5 mL 8N NaOH in the tube (Figure 1C).
Note: Centrifuge at $\sim 1000 \times g$ to sediment biomass in order to remove liquid.
5. Incubate the tubes at 60 °C for 20 min.
Note: An easy way to do this is fixing the tubes in a metal rack in a pre-heated water bath or in a beaker on a heat plate. Maintain bath level at same level as the liquid in the tubes.
6. Decant and discard the 8N NaOH and wash algae 3x with A. bidest. for at least 5 min.
7. Add 1 % Aniline blue (w/v) to freshly prepared Sørensen's phosphate buffer.

8. In the following, it is recommended to work under low light intensities ($\sim 5 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) to prevent degradation of the dye.
9. Stain algae in 1.5 mL of 1 % Aniline blue for 1 h (Figure 1D).
Note: Incubate in darkness under gentle shaking.
10. Wash algae 2x with Sørensen's phosphate buffer for 5 min. and transfer to microscopic slides (mount in buffer).
11. Visualize Aniline blue with an epifluorescence microscope (excitation: 365 nm; Figure 1E).

Note: Aniline blue staining allows a quick and easy-to-use visualization of callose in plant's cell walls. Furthermore, Aniline blue can be used to quantify the callose content in plant material spectrofluorimetrically (Köhle et al. 1985). For a more specific detection of callose at the confocal laser scanning and transmission electron microscopy level, commercially available monoclonal antibodies can be applied (Biosupplies, catalog number: 400-2; Meikle et al. 1991, Herburger and Holzinger 2015).

Procedure – Calcofluor white staining

1. Add 1 % (v/v) Calcofluor white to 50 mL of *A. dest.*
2. Transfer algae to 2 mL Eppendorf tubes and wash once with culture medium (Figure 1B).
Note: Washing can be omitted, if algal cultures are investigated. Single-celled organisms or cells surrounded by a sticky mucilage layer can be transferred easily by using plastic pipettes.
3. Stain algae with 1.5 mL 1 % Calcofluor white for 20 s (Figure 1F).
Note: Krishnamurthy (1999) describes protocols using 0.01 – 0.7 % (v/v) Calcofluor white in buffered or unbuffered solutions and incubation times up to 2 min. Although our plant material was rather small (algal filaments with a max. diameter of $\sim 24 \mu\text{m}$), a 1 % aqueous solution of Calcofluor white produced the best results. Lower concentrations yielded very low fluorescence signals despite incubation times up to 10 min.
4. Wash algae twice in *A. dest.* for 30 sec. and transfer immediately to microscopic slides (mount in *A. dest.*).
Note: To immobilize entire algal filaments in one optical plane, polylysine-coated microscopic slides (Thermo Scientific, catalog number: ER-208B-AD-CE 24) can be used. This allows focusing the central area of algal filaments and reduces blur effects by filament's top or bottom side (Figure 1 G).
5. Visualize Calcofluor white with an epifluorescence microscope (excitation: 365; Figure 1G).

Note: Excitation at 400 – 410 is also fine. Calcofluor white may not specifically bind to cellulose and can also stain callose, chitin, mixed-linkage glycans or other cell wall polysaccharides (Krishnamurthy 1999).

Acknowledgements

Aniline blue staining as applied by Herburger and Holzinger (2015) and described in this protocol is based on the fluorescence method of Currier et al. (1955), previously used for pollen tube staining (Linskens and Esser 1957). Calcofluor white staining follows the protocol of Krishnamurthy (1999). The study was funded by Austrian Science Fund (FWF) projects P 24242-B16 and I 1951-B16 to A.H.

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Figure 1.
 (A-G) Summary of the experimental procedure for Aniline blue (C-E) and Calcofluor white (F, G) staining. Aniline blue (E) and Calcofluor white (G) fluorescence is shown in blue. Bright field and corresponding fluorescence images in (E) and (G) are reprinted from Herburger and Holzinger (2015). Scale bars = 10 μ m.