Video Article

Looking Outwards: Isolation of Cyanobacterial Released Carbohydrate Polymers and Proteins

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Abstract

Cyanobacteria can actively secrete a wide range of biomolecules into the extracellular environment, such as heteropolysaccharides and proteins. The identification and characterization of these biomolecules can improve knowledge about their secretion pathways and help to manipulate them. Furthermore, some of these biomolecules are also interesting in terms of biotechnological applications. Described here are two protocols for easy and rapid isolation of cyanobacterial released carbohydrate polymers and proteins. The method for isolation of released carbohydrate polymers is based on conventional precipitation techniques of polysaccharides in aqueous solutions using organic solvents. This method preserves the characteristics of the polymer and simultaneously avoids the presence of contaminants from cell debris and culture medium. At the end of the process, the lyophilized polymer is ready to be used or characterized or can be subjected to further rounds of purification, depending on the final intended use. Regarding the isolation of the cyanobacterial exoproteome, the technique is based on the concentration of the cell-free medium after removal of the major contaminants by centrifugation and filtration. This strategy allows for reliable isolation of proteins that reach the extracellular milieu via membrane transporters or outer membrane vesicles. These proteins can be subsequently identified using standard mass spectrometry techniques. The protocols presented here can be applied not only to a wide range of cyanobacteria, but also to other bacterial strains. Furthermore, these procedures can be easily tailored according to the final use of the products, purity degree required, and bacterial strain.

Video Link

The video component of this article can be found at https://www.jove.com/video/59590/

Introduction

Cyanobacteria are widely recognized as prolific sources of natural products with promising biotechnological/biomedical applications. Therefore, understanding cyanobacterial secretion mechanisms and optimization of the extraction/recovery methods are essential to implement cyanobacteria as efficient microbial cell factories.

Many cyanobacterial strains are able to produce extracellular polymeric substances (EPS), mainly formed by heteropolysaccharides, that remain associated to the cell surface or are released into the medium as polysaccharides[1]. These released carbohydrate polymers have distinct features compared to those from other bacteria, which make them suitable for a wide range of applications (e.g., antivirals[2], immunostimulatory[3], antioxidant[4], metal-chelating[5], emulsifying[6], and drug delivery agents[7,8]). Methodology for the isolation of these polymers has largely contributed not only to improved yield but also to increased purity and the specific physical properties of the polymer obtained[9]. A vast majority of these methods for isolation of the polymers rely on precipitation strategies from the culture medium that are easily accomplished due to the polymer's strong anionic nature[9,10]. In addition, removal of the solvents used in the precipitation step can be rapidly achieved by evaporation and/or lyophilization. Depending on the foreseen application, different steps can be coupled either after or before polymer precipitation in order to tailor the final product, which include trichloroacetic acid (TCA) treatment, filtration, or size exclusion chromatography (SEC) column purification[10].

Cyanobacteria are also able to secrete a wide range of proteins through pathways dependent on membrane transporters (classical)[11] or mediated by vesicles (non-classical)[12]. Therefore, analysis of the cyanobacterial exoproteome constitutes an essential tool, both to understand/manipulate cyanobacterial protein secretion mechanisms and understand the specific extracellular function of these proteins. Reliable isolation and analysis of exoproteomes require the concentration of the extracellular milieu, since the relative abundance of secreted proteins is relatively low. In addition, other physical or chemical steps (e.g., centrifugation, filtration, or protein precipitation) may optimize the quality of the exoproteome obtained, enriching the protein content[13], and avoiding the presence of contaminants (e.g., pigments, carbohydrates, etc.)[14,15] or the predominance of intracellular proteins in the samples. However, some of these steps may also restrict the set of proteins that can be detected, leading to a biased analysis.

This work describes efficient protocols for the isolation of released carbohydrate polymers and exoproteomes from cyanobacteria culture media. These protocols can be easily adapted to the study's specific objectives and user needs, while maintaining the basic steps presented here.

Protocol

1. Cyanobacterial released carbohydrate polymer isolation

1. **Polymer isolation and removal of contaminants**
    1. Cultivate the cyanobacterial strain under standard conditions [e.g., 30 °C under a 12 h light (50 µE $m^{-2}s^{-1}$)/12 h dark regimen, with orbital shaking at 150 rpm]. Measure growth using standard protocols [e.g., optical density at 730 nm (OD_{730nm}), chlorophyll *a*, dry-weight, etc.], then measure the production of released polysaccharides according to the phenol-sulfuric acid method[16].
    2. Transfer the culture into dialysis membranes (12-14 kDa of molecular weight cut-off) and dialyze against a minimum of 10 volumes of deionized water for 24 h with continuous stirring.
    NOTE: Depending on the volume of culture to dialyze and medium composition, it may be necessary to change the dialysis water.
    3. Centrifuge the culture at 15 000 x *g* for 15 min at 4 °C. Transfer the supernatant to a new vial and discard the pellet (cells).
    4. Centrifuge again at 20 000 x *g* for 15 min at 4 °C to remove contaminants such as cell wall debris or lipopolysaccharides (LPS).
    5. Transfer the supernatant to a glass beaker and discard the pellet.

2. **Precipitation of the polymer**
    1. Add 2 volumes of 96% ethanol to the supernatant.
    2. Incubate the suspension at 4 °C, at least overnight.
    3. Polymer recovery
        1. For small or not visible amounts of precipitated polymer: centrifuge the suspension at 13,000 x *g* for 25 min at 4 °C. Discard the supernatant and resuspend the pellet in 1 mL or 2 mL of autoclaved deionized water. Transfer the aqueous suspension to a vial.
        CAUTION: The supernatant should be discarded gently, as it can become easily resuspended.
        2. For visible/large amounts of precipitated polymer: collect the precipitated polymer with sterile metal forceps to a vial. Squeeze the polymer and discard the excess ethanol.
    4. Optional: depending on the degree of polymer purification required, repeat the precipitation step with 96% ethanol after resuspension of the polymers in deionized water.

3. **Lyophilization of the polymer**
    1. Keep the vials with the precipitated polymer at -80 °C, at least overnight.
    2. Freeze-dry (lyophilize) the polymer for at least 48 h (do not let the suspension defrost before freeze-drying).
    3. Store the dried polymer at room temperature (RT) until further use.
    NOTE: Storing the polymer in a desiccator is advisable, as it can absorb water over time.

2. Cyanobacterial exoproteome isolation

1. **Medium concentration**
    1. Cultivate cyanobacteria under standard conditions [e.g., 30 °C under a 12 h light (50 µE $m^{-2}s^{-1}$)/12 h dark regimen, with orbital shaking at 150 rpm]. Monitor the cyanobacterium growth using standard procedures (e.g., OD_{730nm}, chlorophyll *a*, dry-weight, etc.).
    2. Centrifuge the cultures at 4 000 x *g,* for 10 min at RT.
    3. Transfer the supernatant to a flask and discard the cell pellet.
    4. Filter the decanted medium through a 0.2 µm pore size filter.
    NOTE: The protocol can be paused here for a short time period, if the medium is kept at 4 °C.
    5. Concentrate the medium approximately 500x (considering the initial volume of filtered medium), using centrifugal concentrators with a nominal molecular weight cut-off of 3 kDa. Centrifugation should be operated at 4 000 x *g* (maximum 1 h per centrifugation round) at 15 °C.
    CAUTION: For the majority of concentrator brands, the filter device needs to be rinsed by centrifugation with ultrapure water before use. Once the filter is wet, do not let it dry out. Leave enough fluid on the reservoir when the device is not being used.
    NOTE: Reducing the centrifugation temperature to 4 °C may be helpful if the aim is to study protein activity, though it will increase the time necessary for sample concentration. The protocol can be paused between centrifugation steps for short time periods if the medium is kept at 4 °C.
    6. Rinse the walls of the filter device sample reservoir with the concentrated sample and transfer the content to a microcentrifuge tube.
    7. Perform an additional washing step of the filter device sample reservoir with autoclaved culture medium to ensure maximal exoproteome recovery.
    NOTE: To quantify the percentage of recovery, follow the specific manufacturer's instructions.
    8. Store the exoproteome samples at -20 °C until further use.
    NOTE: Addition of protease inhibitors is recommended for long-term storage.

2. **Analysis of the exoproteome**
    1. Quantify the protein content by BCA protein assay in 96-well plate according to the manufacturer's instructions.
    2. Separate the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using standard staining protocols (e.g., Coomassie blue, silver staining).
    3. Cut out the bands/gel regions of interest and collect them in different microcentrifuge tubes containing appropriate volumes of ultra-pure water.

4. Proceed with identification and analysis of the proteins by mass spectrometry.

Representative Results

A schematic representation of the method described to extract released carbohydrate polymers from cyanobacterial cultures is depicted in **Figure 1**. Precipitated polymers from the moderate EPS producer cyanobacterium *Synechocystis* sp. PCC 6803 and the efficient EPS producer *Cyanothece* sp. CCY 0110 are shown in **Figure 2**. In **Figure 3**, lyophilized polymers with different degrees of contamination are shown, highlighting the importance of the centrifugation steps for the final product purity. **Figure 4** depicts the isolation method for the cyanobacterial exoproteome. Distinct cell-free medium concentrated samples (i.e., obtained from cultures in different growth phases and from a cyanobacterial strain with lower carotenoid production) are shown in **Figure 5**. Exoproteome samples from two morphologically distinct cyanobacterial strains, the unicellular cyanobacteria *Synechocystis* sp. PCC 6803 and the filamentous cyanobacteria *Anabaena* sp. PCC 7120, separated by SDS-PAGE, are shown in **Figure 6**.

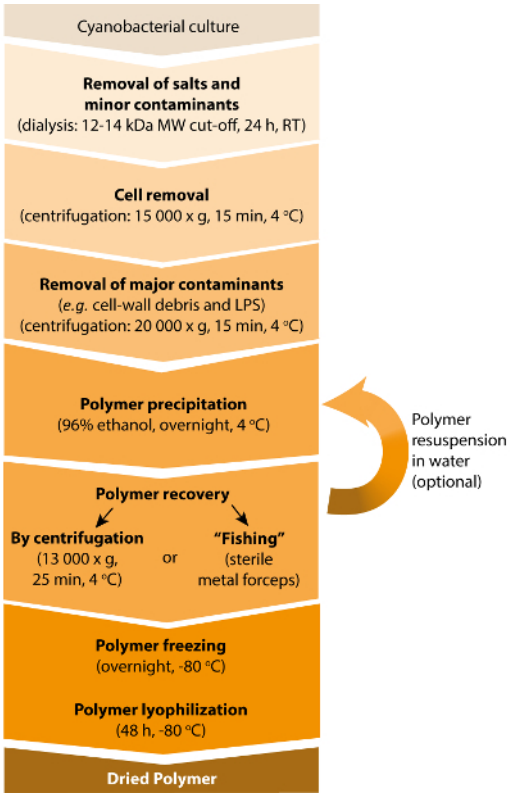

Figure 1: Workflow for the isolation of cyanobacterial released carbohydrate polymers. Shown is the workflow for isolation, starting from the cyanobacterial culture and removal of contaminants and ending with polymer isolation and lyophilization. Please click here to view a larger version of this figure.

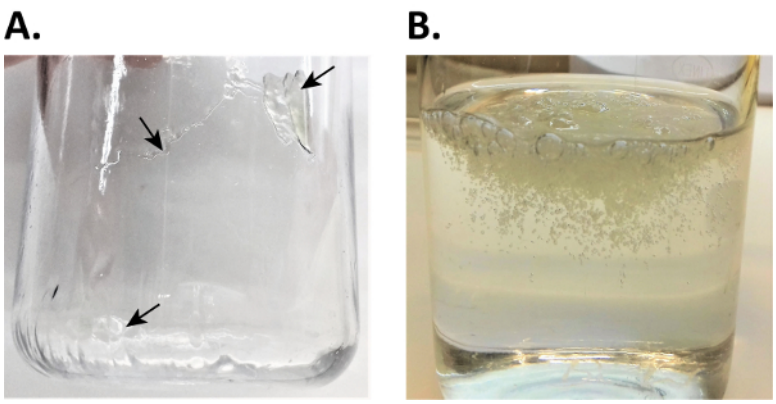

Figure 2: Cyanobacterial polymers after precipitation. (A) Polymer from the moderate EPS producer *Synechocystis* sp. PCC 6803 on the wall of the centrifuge flask after precipitation and centrifugation (arrows). **(B)** Polymer clumps from the efficient EPS producer *Cyanothece* sp. CCY 0110 floating in the glass beaker after precipitation. Please click here to view a larger version of this figure.

Figure 3: Lyophilized cyanobacterial polymers. (A) Three independent batches of polymers isolated from *Synechocystis* sp. PCC 6803: without visible contamination **(AI)**, and with pigmentation indicative of contamination with carotenoids **(AII)** or cell debris **(AIII)**. **(B)** Lyophilized polymer from *Cyanothece* sp. CCY 0110. Please click here to view a larger version of this figure.

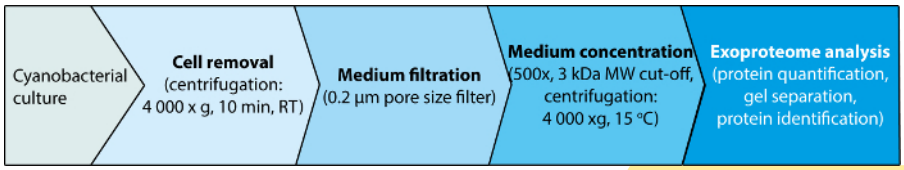

Figure 4: Workflow for cyanobacterial exoproteome isolation. Shown is the workflow for isolation, from cyanobacterial culture to medium separation and concentration, ending with exoproteome analysis. Please click here to view a larger version of this figure.

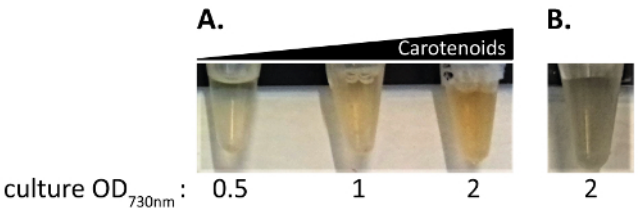

Figure 5: Microcentrifuge tubes with concentrated cell-free medium samples. (A) Concentrated medium samples from *Synechocystis* sp. PCC 6803 wild-type, collected at different OD_{730nm} (0.5, 1, and 2). **(B)** Concentrated medium sample from *Synechocystis* Δ*sigF*, a mutant with impaired carotenoids production[15]. Please click here to view a larger version of this figure.

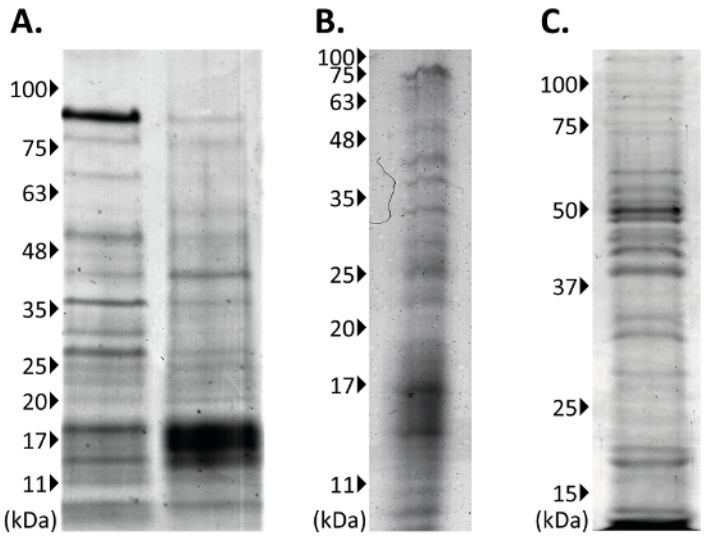

Figure 6: Coomassie blue-stained SDS-PAGE gels showing the proteins accumulated in cyanobacterial cell-free concentrated medium. (A) Exoproteome from the unicellular cyanobacteria *Synechocystis* sp. PCC 6803 wild-type and Δ*sigF* mutant. **(B)** Exoproteome from *Synechocystis* Δ*sigF* contaminated with high levels of polysaccharides. **(C)** Exoproteome from the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Please click here to view a larger version of this figure.

Discussion

To better understand bacterial secretion mechanisms and study the products released, it is of extreme importance to demonstrate the efficient isolation and analysis of the biomolecules present in the extracellular bacterial environment (such as released carbohydrate polymers and proteins).

Cyanobacterial extracellular carbohydrate polymers are extremely complex, mainly due to the number and proportion of different monosaccharides that constitute their composition[1]. The conventional methods used for isolation of these polymeric substances rely on the simple concept that these sugar-rich substances are soluble in aqueous solutions and can be precipitated by the addition of organic solvents (such as acetone or ethanol)[9,17,18]. This occurs due to the extraction of water molecules from the polymers' hydration shells, and efficiency of the process depends intrinsically on the polymer's molecular weight (more efficient at higher molecular weight fractions), chemical structure, and concentration[9,18]. In addition to the precipitation step, the method described here includes the critical steps of dialysis and centrifugation. Dialysis will efficiently remove salts and other compounds from the medium, which may appear after lyophilization as powder-like structures, while the centrifugation steps will remove major contaminants and cell debris.

Failures during these steps may lead to polymers with high contamination levels and different characteristics, depending on the isolation batch. Some contaminations can be easily detected macroscopically after polymer lyophilization, since they will alter the polymer pigmentation (usually to white or light brown). For example, lyophilized polymers that are green or orange are generally highly contaminated with cell debris/chlorophyll or carotenoids. This is related to insufficient time or *g* force in centrifugation steps. However, some cyanobacterial strains can release pigments and secrete proteins that remain naturally associated with the polymers, and this needs to be considered when analyzing the final product[19]. Additional steps can be added to further purify the polymers (e.g., TCA treatments)[10]. Nevertheless, these purification steps could also have a negative impact in the final product, as removing proteins and other components can alter the polymer properties (e.g., viscosity, hydrophobicity, etc.)[1,20]. Even repeating the precipitation/lyophilization steps can negatively affect polymers, mainly due to the freeze-thawing cycles that easily modify their physicochemical properties[21]. To improve the polymer yields, heating treatments can be applied to whole cultures prior to precipitation. This extra step releases the polymer associated with the cell surface, but it may also lead to depolymerization[18,20]. In summary, it is important to notice that the choice of protocol will influence both the amount and quality of isolated polymers[9,20].

Regarding the cyanobacterial proteins identified in the extracellular milieu, they display a wide range of molecular weights and isoelectric points and can be either soluble or membrane-associated. This diversity of physicochemical properties represents an issue for the selection of the most suitable method for exoproteome isolation. The method presented here depends heavily on the concentration of the biomolecules in the extracellular milieu. This method isolates not only proteins that are secreted into the medium but also proteins present in outer membrane vesicles (OMVs) and derived from cell lysis. Therefore, centrifugation steps should be gently performed in order to avoid cell disruption, but at the same time collect OMVs. In cyanobacterial strains that are efficient OMVs producers, the exoproteome preparations are usually orange due to the presence of carotenoids associated with these lipidic structures[14,15]. However, this feature can vary considerably depending on the cyanobacterial strain and growth phase. In order to assess the contribution of proteins by OMVs, ultracentrifugation steps should be added to the procedure[22].

Furthermore, proteins that reach the extracellular space due to cell lysis may be detected by collecting samples in different growth phases and increasing the number of replicates. As aforementioned, since many cyanobacterial strains produce extracellular carbohydrate polymers (EPS), the exoproteome preparations could also have EPS in their composition. The filtration step should retain more complex and large EPS, but simpler EPS fractions may eventually pass through. Consequently, contamination with large amounts of carbohydrates can interfere with exoproteome analysis. For example, this contamination may cause a delay in protein separation in polyacrylamide gels, as well as mask less abundant proteins. Alternative protocols have been proposed for exoproteome isolation aiming to remove contaminating biomolecules present in

the extracellular medium, but they have been shown to be very selective, which may lead to biased exoproteome profiles[13]. On the other hand, a certain amount of proteins may be trapped in more complex EPS fractions if they are stuck in the filter. In this case, analyzing exoproteome preparations from different growth phases/experimental conditions as well as analysis of the proteins in EPS fractions may help identify the entrapped proteins.

Overall, the protocols described here embody the crucial steps for efficient isolation of cyanobacterial released carbohydrate polymers and exoproteomes. Most importantly, they can be easily tailored according to specific user needs and include other bacterial strains.

Disclosures

The authors have nothing to disclose.

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