Algal Research 34 (2018) 250–255

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Cell disruption by cationic surfactants affects bioproduct recovery from *Synechocystis* sp. PCC 6803

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ARTICLE INFO

Keywords:
Synechocystis
Harvesting and extraction
Cationic surfactants
Flow cytometry
Cyanobacteria

ABSTRACT

Cationic surfactants can be used to improve extraction of high-value products from microalgae, but the best way to apply cationic surfactants has not been established. We evaluated the impacts of contact time and concentration for two cationic surfactants – dodecyltrimethylammonium bromide (DTAB) and hexadecyltrimethylammonium bromide (CTAB) – on extraction of lipids and nonpolar pigments, carotenoids and chlorophyll *a*, from *Synechocystis* sp. PCC 6803. For the most dose-effective concentration, pigment extraction was better with the longer alkyl-chain length, i.e., CTAB > DTAB, because the longer alkyl chain of CTAB increased cell membrane permeability, causing cell lysis that allowed solvent-pigment contact. A CTAB dose of 25 mM and treatment for 5 h gave the highest pigment extraction, nearly 30 and 12 mg/g dried biomass for chlorophyll *a* and carotenoids, respectively. In contrast, lipid recovery was better with the shorter alkyl-chain length, DTAB > CTAB. DTAB disrupted the cells less, which allowed the solvent to access more lipids held in the cells. Thus, the selection of surfactant and dosing strategy depends on which products are targeted. With moderate cell disruption, a moderate dose of CTAB (10 mM) was effective for recovering pigments and lipids simultaneously.

1. Introduction

Microalgal biomass has the potential to be a renewable energy source, and it also can contain numerous high-value bioproducts, such as proteins, carbohydrates, lipids, chlorophyll, carotenoids, and antioxidants [1,2]. Major challenges for utilizing microalgae lie in biomass pretreatment before extraction of lipids and other bioproducts [3].

Cationic surfactants, such as hexadecyltrimethylammonium bromide (CTAB), and dodecyltrimethylammonium bromide (DTAB), contain a long alkyl chain and a quaternary-ammonium cation [4], and they can be used to improve biomass harvesting and bioproduct extraction [5–7]. When adsorbed to the cells, the quaternary-ammonium cation reduces the cell's negative charge, which enhances the onset of aggregation [8]. The long alkyl chain also is an inter-particle bridge that links the cells together, which further enhances aggregation and leads to more efficient harvesting. When enough surfactant is added to form micelles, it can disrupt the cell's membrane and help extract hydrophobic components from the cell's interior [7,8]. Thus, cationic surfactants offer a means to integrate harvesting (at low doses) and extraction (at higher doses) [5,6]. Among the commonly used cationic surfactants, CTAB has the longest alkyl chain (16C) and the lowest critical micelle concentration (CMC; 0.9 mM); DTAB has a smaller alkyl chain (12C) and a CMC of 10 mM. Thus, CTAB ought to be the most effective surfactant to disrupt the cells, while also neutralizing the cells' negative charge [5,6]. In addition, the selected cationic surfactants in this study were biodegradable [9,10]. The previous study has demonstrated [11] that an oxygen-based membrane biofilm reactor could sustain up to ≥1 mM CTAB biodegradation performance. Thus, given that novel approach it could effectively prevent surfactant discharged to aquatic environments and lays the foundation of the future industrial application for biofuel and bioproduct extraction.

Although cell disruption ought to enhance target-to-solvent contact and lead to high product recovery, this positive effect will only be realized when the target and solvent are compatible in terms of polarity [12]. For example, chlorophyll *a* ($C_{55}H_{72}MgN_4O_5$) and carotenoids (e.g., fucoxanthin, $C_{42}H_{58}O_6$) [13,14] are closely associated with or

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https://doi.org/10.1016/j.algal.2018.08.010
Received 14 April 2018; Received in revised form 6 August 2018; Accepted 9 August 2018
Available online 11 August 2018

bound to the thylakoid membrane of cyanobacteria. To extract these compounds, the solvent must be able to disrupt the polar membrane lipids composed of diacylglycerides (DAGs), which contain polar heads (galactosyl, phospho, and sulfoquinovosyl) that prevent strongly non-polar solvents (e.g., hexane) from disrupting the membrane and entering the cell [15]. In contrast to non-polar solvents, the cationic surfactants contain a polar functional group, which allows them to disrupt the lipid bilayer, allowing for nonpolar solvents to diffuse into the cell and extract the desired products. In this work, we use ethyl acetate, a non-polar solvent, because it is a nontoxic solvent that may be a viable alternative for pigment and lipid extraction, since it has similar polarity to chloroform.

A good method to quantify cell disruption combines flow cytometry (FC) with the SYTOX Green (SG) dye [16,17]. FC characterizes properties of individual cells, such as size and granularity [17,18]. In particular, particle size is linearly correlated to the ratio between side scatter (SSC) and forward scatter (FSC) in the region with the highest density of points [7,17,19]. SG binds strongly with nucleic acid (NA) [20], but cannot penetrate an intact cell membrane due to its large molecular size [21]; therefore, emitted fluorescence is only ascribed to NA in extracellular polymeric substances (EPS) for intact biomass. However, SG can bind with intracellular NA when the cell's membrane becomes permeable or lysed. Thus, FC with SG can sensitively quantify cell lysis by a sharp increase in fluorescence intensity [16].

The over-arching goal of this study is to select a surfactant and dose for effective recovery of lipids and pigments. We conducted studies using *Synechocystis* sp. PCC 6803, a spherical, unicellular cyanobacterium with a peptidoglycan layer and an outer membrane that is thicker than in most Gram-negative bacteria. This feature allows *Synechocystis* to resist disruption and product recovery. We evaluated two cationic surfactants with different alkyl-chain lengths for their ability to disrupt the *Synechocystis* cells and enable extraction of lipids and pigments. The results of this study offer guidelines of how to apply cationic surfactants to enhance recovery of desired products.

2. Materials and methods

2.1. Chemicals and Synechocystis sp. PCC 6803

Hexadecyltrimethylammonium bromide (CTAB, $C_{19}H_{42}NBr$, alkyl-chain length of C16) and dodecyltrimethylammonium bromide (DTAB, $C_{15}H_{34}NBr$, alkyl-chain length of C12) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Wild-type *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis)* was obtained from Dr. Willem Vermaas's laboratory in the School of Life Sciences at Arizona State University. It was grown in a 14-L flat-panel photobioreactor as described by Straka [22], except that pure CO_2 was sparged separate from air when pH exceeded 8.01 to maintain a pH around 8.0 (providing an excess of inorganic carbon [23]). Briefly, we utilized standard BG-11 medium [24], bubbled the culture with ambient air filtered through a 1.0-μm air filter (Pall, Port Washington, NY, USA) at a flow rate of about 0.1 L/min, maintained the temperature at 30 °C, and provided incident light from T5 fluorescent plant grow lamps (Envirogro Hydrofarm, USA) at an average of 120 $\mu E/m^2.s$ from each side of the reactor. After one to two weeks of cultivation, the optical density at 730 nm (OD_{730}) of the culture rose to ~3.6 (biomass dry weight ~1.15 g/L). Biomass was then collected and concentrated by cross-flow membrane filtration [25] to a dry weight concentration of ~9.4 g/L of biomass for testing.

2.2. Wet-biomass extraction

To evaluate wet-biomass extraction, we mixed 30 mL of concentrated *Synechocystis* with either surfactant in a 50-mL polypropylene centrifuge tube (BD Falcon, VWR, USA) with the following surfactant concentration (mM): 25, 10, 5, 1, 0.5 and 0. Samples at time = 0 were taken after the contents were gently mixed by vortexing until all surfactant powder was fully dissolved. The slurries were mixed in a shaker incubator (New Brunswick, Scientific, Enfield, CT, USA) at 210 rpm and at room temperature (23.8 °C). After 5 h and 1 day, we withdrew a 1-mL sample and mixed it with 3 mL of ethyl acetate (EA) solvent in 7.5-mL Pyrex disposable screwcap culture tubes (13 × 100 mm). The mixtures of EA and biomass were vortexed at 3200 rpm for 1 min on Vortex-Genie 2 (Scientific Industries, USA) and then centrifuged at 3600 *g* and 23 °C for 5 min to separate solids. We removed 2 mL of clear supernatant for the assay of extractable chlorophyll *a* and carotenoid (i.e., Fucoxanthin), and 1 mL for the assay of fatty acid methyl esters (FAME).

2.3. SYTOX green staining and flow cytometry

We adapted published methods for SG-staining and FC [7,16,17] to identify cell size and cell lysis after surfactant treatment. After treatment with each surfactant for 5 h, we withdrew a 2-mL sample and applied the fluorescent dye SG according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA), mixing it with 1 μL SG, and then allowing the reaction to proceed for 15 min in the dark on a rocker mixer (Lab-Line, TX, USA). We used *Synechocystis* biomass without treatment or SG stain to zero the fluorescent intensity (FI). After staining, we performed FC using a FACSAria flow cytometer (BD Biosciences, CA, USA) having an air-cooled 20-mW argon ion laser with an excitation wavelength of 488 nm. We used a fluorescein isothiocyanate (FITC) filter with a wavelength band of 510–550 nm to detect the SG emission. We diluted the samples stained with SG to a concentration suitable for the instrument's counting speed of 300 to 400 events/s, and we counted 10,000 events for each sample. We performed the data analysis and graphical outputs with FlowJo 7.6.1 software (Treestar, Inc., San Carlos, CA, USA).

2.4. Analytical methods

The concentrations of chlorophyll *a* and carotenoid were quantified by a spectrophotometer (Bio Cary 50 – Varian, USA) based on the characteristic absorbances of the pigments [14]: 470 and 665 nm for chlorophyll *a* and carotenoid, respectively. Standard curves are provided in Fig. S1.

FAME was quantified for the control and surfactant-treated samples. Direct transesterification (DT) was carried out for the control with a 1-mL slurry sample of freeze-dried biomass (FreeZone Benchtop instrument, Labconco, MO, USA). Samples were amended with 2 mL of 3 N methanolic HCl (Sigma-Aldrich, MO, USA) and incubated at 85 °C in the oven for 2.5 h. Transesterified compounds were extracted by 1.55 mL hexane solvent. DT allowed us to define the maximum FAME content FAME content obtained from solvent extraction could be compared against the total FAME obtained from DT.

Following Sheng et al. [16], FAME components for DT and solvent-extracted samples were quantified using a gas chromatograph (Shimadzu GC 2010, Japan) equipped with a Supelco SP-2380 capillary column (30 m × 0.25 mm × 0.20 μm) and flame ionization detector (FID) against a 37-Component FAME Mix standard (Supelco, PA, USA).

We adapted the TEM approach of Lai et al. [5] and Sheng et al. [16] to identify morphology changes for *Synechocystis* with surfactant treatment. The microalgal samples (control or surfactant-treated) were incubated with 2% glutaraldehyde in 50 mM sodium phosphate at pH 7.2 for 1 h, washed with buffer, and fixed with 1% osmium tetroxide for 1 h. Fixed cells were fully dehydrated with an ascending series of acetone solutions. Spurr's epoxy resin was used to infiltrate and embed the samples, which were polymerized at 60 °C for 36 h. Finally, samples were cut into 60-nm sections using a Leica Ultracut-R microtome (Wetzlar, Germany) and post-stained in uranyl acetate and lead citrate. Images were generated using a transmission electron microscope (TEM, model CM12, Phillips, USA) operated at 80 kV with a Gatan model 791