Water Research 126 (2017) 189–196

Contents lists available at ScienceDirect

Water Research

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

How myristyltrimethylammonium bromide enhances biomass harvesting and pigments extraction from *Synechocystis* sp. PCC 6803

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ARTICLE INFO

Article history:
Received 13 July 2017
Received in revised form
14 September 2017
Accepted 20 September 2017
Available online 21 September 2017

Keywords:
Synechocystis
Harvesting and extraction
Cationic surfactant
Mechanisms
Cyanobacteria

ABSTRACT

Myristyltrimethylammonium bromide (MTAB) is a cationic surfactant used to improve biomass harvesting and pigment extraction form microalgae, but the mechanisms underlying its effectiveness are poorly defined. We document the mechanisms for enhanced harvesting and pigment extraction for the cyanobacterium *Synechocystis* sp. PCC 6803 using measurements from flow cytometer, zeta potential, release of soluble components, and microscopy. Harvesting efficiency increased as the MTAB/Biomass dose increased from 0 to 40%. A low MTAB dose ($\leq$ 8%) mainly brought about coagulation and flocculation, which led to aggregation that improved harvesting, but 40% MTAB had the highest harvesting efficiency, 62%. Adding MTAB above a MTAB/Biomass dose of 8% also increased cell-membrane permeability, which allowed the solvent (ethyl acetate) to pass into the cells and resulted in a large increase in extraction efficiency of pigments: An MTAB/Biomass ratio of 60% for 180 min achieved the highest extraction efficiencies of chlorophyll and carotenoids, 95% and 91%, respectively. Combining harvesting and extraction performances with results from flow cytometry, zeta potential, release of soluble components, and microscopy lead to the following mechanistic understandings. MTAB dose from 8% to 40% solubilized EPS, which lowered the biomass's negative charge, but caused breakup of the large aggregates. An increase of cell permeability also in this stage allowed ethyl acetate to pass into the cells and achieve better pigment extraction. MTAB >40% led to cell lysis and a large increase in soluble organics, but complete cell lysis was not required to achieve the maximum extraction efficiency. The MTAB/Biomass % ratio for optimizing harvest efficiency and pigment extraction lay in the range of 40%–60%.

1. Introduction

Microalgae have enormous potential to be sustainable feedstock for numerous bioproducts (Ruiz et al., 2016), such as lipids, proteins, carbohydrates, and high-value compounds based on chlorophyll, carotenoids, antioxidants, and sterols (Gilbert-López et al., 2015; Oswald and Golueke, 1960; Pittman et al., 2011). Today, the production of high-value products is considered to be essential for making microalgae systems profitable (Ruiz et al., 2016). Major challenges for utilizing microalgae lie in harvesting the biomass and extracting intracellular products (Vandamme et al., 2013). Integrating biomass harvesting and product extraction into one simple step offers an opportunity to lower financial and energy costs in a major way (Lai et al., 2016b; Seo et al., 2016).

Coagulation and flocculation are important mechanisms in biomass harvesting (Lai et al., 2016a; Wan et al., 2015). Flocculation typically utilizes long-chain polyelectrolytes (usually cationic) to improve the biomass harvesting by linking the particles together (Hermansson, 1999; Magara et al., 1976). Coagulation promotes flocculation by decreasing the zeta potential of the suspended particles (Biggs et al., 2000; Ries and Meyers, 1968). Among the

Abbreviations: MTAB, Myristyltrimethylammonium bromide; EPS, Extracellular polymeric substances; FC, Flow cytometry; SG, SYTOX Green; NA, Nucleic acid; OD_{730}, The optical density at 730 nm; EA, Ethyl acetate; FI, Fluorescent intensity; DW, Dry weight; PCOD, Particulate chemical oxygen demand; BSA, Bovine serum albumin; SOC, Soluble organic compounds; SP, Soluble protein; SC, Soluble carbohydrate; SL, Slope; FSC, Forward scatter; SSC, Side scatter.

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https://doi.org/10.1016/j.watres.2017.09.036
0043-1354/

options are surfactants such as myristyltrimethylammonium bromide (MTAB, $C_{17}H_{38}NBr$), which has an alkyl-chain length of C14 and a quaternary-ammonium cation (Lai et al., 2016a, 2016b; Seo et al., 2016).

Extracellular polymeric substances (EPS) play important roles in biomass aggregation (Liu et al., 2007; Zhang et al., 2016). On the one hand, EPS contains carboxylic (≡X-COOH) and phosphoryl (≡X-PO_4H) groups (Schwarz and Rittmann, 2007; Zhou et al., 2016b, 2017a) that are negatively charged at slightly acidic to alkaline conditions (Zhou et al., 2017a). Un-neutralized, the negative functional groups prevent aggregation. Having a quaternary-ammonium cation, MTAB can neutralize the negative charges, and its long alkyl chain can serve as an inter-particle bridge; both aspects enhance aggregation (Sengco et al., 2001). On the other hand, if too much MTAB is added, its surfactant effect can dominate, resulting in the release of EPS from the cells and deflocculation (Lu et al., 2017; Schott et al., 1982).

Another feature of MTAB is that, because its linear hydrocarbon group is hydrophobic, MTAB can form micelles that are effective for extracting hydrophobic components from the cells and also can lead to cell lysis and release of intracellular compounds (Zhou et al., 2016c, 2017b). These effects can play important roles in the extraction of intracellular components, such as pigments.

The mechanisms affecting aggregation and product extraction depend on the MTAB concentration. For example, a previous study (Lai et al., 2016b) found that a 20% MTAB/Biomass ratio achieved the highest harvesting efficiency (due to aggregation) of *Chlorella*, but a 44% ratio achieved the highest extraction efficiency for fatty acid methyl esters (FAME).

Flow cytometry (FC) is a powerful tool to determine physical and chemical characteristics of single particles, including intact cells and cellular debris after lysis (Hyka et al., 2013). FC can be used to achieve two goals (Collier, 2000; Sheng et al., 2011; Vermes et al., 2000; Zhou et al., 2016a; Zipper et al., 2004): (1) characterizing cell features, such as cell size and granularity and cell membrane integrity; and (2) cell sorting according to size or a metabolic feature, such as autofluorescence emitted from chlorophyll, a pigment present in all photoautotrophic microalgae.

FC combined with SYTOX Green (SG) dye is commonly used for the characterization of cell features (Sheng et al., 2011; Zhou et al., 2016a; Zipper et al., 2004). SG is an unsymmetrical cyanine dye that binds strongly with nucleic acid (NA) (Lebaron et al., 1998). Because of its large molecular size, SG cannot penetrate an intact cell membrane (Roth et al., 1997; Zipper et al., 2004), and the emitted fluorescence is due only to NA in the EPS of intact biomass. However, when cells are compromised, such as by lysis, intracellular DNA can be complexed by SG. Since the concentration of intracellular NA is much higher than NA in EPS, the fluorescence emitted by SG complexed to released intracellular NA is much larger than SG complexed solely to extracellular NA (Roth et al., 1997; Sheng et al., 2011). Thus, FC with SG can sensitively differentiate whether or not cells have been lysed. As an example, previous study (Zhou et al., 2016a) used flow cytometry to evaluate thermal extraction of EPS from *Synechocystis* sp. PCC 6803 and showed that lysis was minimal during a 20-min thermal extraction as long as the temperature was less than 60 °C.

Although surfactants are used for biomass harvesting and pigment extraction, the mechanisms are not well understood. In this study, we use FC with SG, zeta-potential measurements, release of soluble components, and microscopy to comprehensively monitor how MTAB enhances biomass harvesting and pigments extraction. We show that adding just enough MTAB for complete EPS removal, but with minimal cell lysis, achieves the maximum harvesting efficiency by coagulation and flocculation. Furthermore, pigment extraction is enhanced by adding MTAB to a concentration that achieves an increase in cell-membrane permeability that allows solvent to diffuse into the cells, but does not lyse the cells completely.

2. Materials and methods

2.1. Chemicals and Synechocystis sp. PCC 6803 samples

Myristyltrimethylammonium bromide (MTAB) was obtained from Sigma-Aldrich (St. Louis, MO). A stock solution of MTAB with a concentration of 20 g/L was prepared by dissolving analytical grade of MTAB into deionized (DI) water.

Wild-type *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) was grown in 1-L Erlenmeyer flasks with a working volume of 500 mL, utilizing standard BG-11 medium (Rippka et al., 1979) and bubbled with air filtered through a 1.0-μm air filter (Pall, Port Washington, NY, USA) at a flow rate of about 0.1 L/min. The culturing conditions were: temperature of 30 °C, maintained by 3 × 12-W automated-air fans (Nguyen and Rittmann, 2016); incident light intensity of 276 $\mu E/m^2.s$, provided from T5 fluorescent plant grow lamps (Envirogro Hydrofarm, USA); and pH of 8.0 maintained using a pH-Stat that automatically sparged pure CO_2 when the pH was higher than 8.01 (Nguyen and Rittmann, 2015). Prior to inoculation, the flasks and the BG-11 medium were sterilized by autoclaving, and the pH probe was sterilized using 75% ethanol. After 5 days of cultivation, the optical density at 730 nm (OD_{730}) of the culture rose to ~ 2.3 and biomass dry weight to ~ 650 mg/L. After 5 days of growth, the biomass was collected for testing. In order to eliminate the effect of divalent metal ions (Ca^{2+} and Mg^{2+} in the standard BG-11) on biomass harvesting (Ayed et al., 2015; Li et al., 2017) or extraction, we centrifuged the culture at 4000 rpm at 4 °C for 10 min and then replaced the supernatant using the same volume of DI water. Table 1 summarizes characteristics of the *Synechocystis* samples used for the biomass harvesting and products extraction.

2.2. Biomass harvesting

The biomass-harvesting efficiency was evaluated by measuring culture OD_{730} over time. We mixed 10 mL of a *Synechocystis* sample with MTAB in 15-mL polypropylene centrifuge tubes (BD Falcon, VWR, USA), using the following mass ratios (MTAB mass/biomass dry weight, %): 0, 4, 8, 12, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70. We mixed the samples by hand until the contents were well-mixed. During the harvesting period (up to 240 min), we withdrew duplicate 0.15-ml samples from the middle of the tube (Salim et al., 2011) and diluted them to 1.5 mL using DI water for a final OD_{730} below 1.0. We calculated the harvest efficiency using the following equation (Salim et al., 2011):

$$\textit{Harvesting efficiency}\ (\%) = \frac{OD_{730}(t_0) - OD_{730}(t)}{OD_{730}(t_0)} \times 100$$

where $OD_{730}(t_0)$ is the OD of the sample at time zero, and $OD_{730}(t)$ is the OD of the sample after t min.

2.3. Pigments extraction and SOC separation

The effects of MTAB on the efficiency of pigment extraction were evaluated using the wet-biomass-extraction method (Lai et al., 2016a). We mixed 10 mL of the prepared *Synechocystis* with MTAB each in 15-mL polypropylene centrifuge tubes (BD Falcon, VWR, USA) with the following mass ratios (MTAB mass/biomass dry weight, %): 0, 4, 8, 12, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70. The slurries were mixed within an incubator (New Brunswick