Algal Research 30 (2018) 94–100

Contents lists available at ScienceDirect

Algal Research

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

Quantification of heterotrophic bacteria during the growth of *Synechocystis* sp. PCC 6803 using fluorescence activated cell sorting and microscopy

Yun Zhou[a,b], Everett Eustance[a], Levi Straka[a], YenJung Sean Lai[a], Siqing Xia[b,*], Bruce E. Rittmann[a,*]

[a] Biodesign Swette Center for Environmental Biotechnology, Arizona State University, Tempe, AZ 85287-5701, United States
[b] State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, China

ARTICLE INFO

Keywords:
Heterotrophic bacteria
Quantification
Synechocystis sp. PCC 6803
Flow cytometry
Cyanobacteria

ABSTRACT

The presence of heterotrophic bacteria in microalgal cultures can dilute the microalgal content of the harvested biomass, compete for nutrients, and be associated with culture crashes. Being able to detect and quantify heterotrophic bacteria would be of high value for monitoring culture health and reducing deleterious effects. Here, we developed and applied a new method that combines flow cytometry (FC) and fluorescence activated cell sorting (FACS) for the quantification of heterotrophic bacteria in cultures of the cyanobacterium *Synechocystis* sp. PCC 6803. Particles not containing chlorophyll – heterotrophic bacteria and cell debris – were separated from mixed cultures using FACS based on autofluorescence of *Synechocystis*. Heterotrophic bacteria were differentiated from cell debris using FC with SYTOX green fluorescence. Using microscopy, we verified that FACS was able to quantify heterotrophic bacteria in *Synechocystis* cultures effectively. Applying these methods to batch cultures of *Synechocystis* showed that the count proportions of heterotrophic bacteria were significant (3–13%) and that depletion of inorganic P in the culture favored *Synechocystis* over heterotrophic bacteria, but led to more cell lysis.

1. Introduction

Cyanobacteria are valuable sources of bioproducts that span energy feedstock, cosmetics, and nutraceuticals [1–3]. One of the largest concerns in scaling microalgal cultures is the presence of microbial "contaminants" that can reduce bioproduct productivity or quality [4, 5]. Heterotrophic bacteria are one type of "contaminant" of concern, because they can dilute the cyanobacterial content of the harvested biomass, compete for nutrients, or be associated with culture crashes [4, 6]. Cyanobacteria are the primary producers during cultivation, while the heterotrophic bacteria are secondary consumers of materials generated by the cyanobacteria. The cyanobacteria contain the valuable outputs, such as lipids and pigments.

All microorganisms produce extracellular polymeric substances (EPS) and soluble microbial products (SMP), which are sources of organic carbon and electrons. During phototrophic cultivation of cyanobacteria, the cyanobacteria generate EPS and SMP, which become food for heterotrophic bacteria [3, 7, 8]. While most heterotrophic bacteria have little effect on cyanobacterial growth [4, 9], they still compete for nutrients and can change the quality of the harvested biomass [5, 10]. Stress to the phototrophs can lead to increased release of microbial products [7, 11], and increases in heterotrophs are expected. Additionally, some heterotrophic bacteria can cause lysis of cyanobacteria cells through enzymatic or antibiotic mechanisms leading to drastic declines in yield or "culture crashes" [3, 12]. Quantification of heterotrophic bacteria during the culturing of cyanobacteria is, therefore, valuable for assessing culture health.

Traditionally, microscopy has been applied for counting heterotrophic bacteria [13, 14], but it is reliable only when the heterotrophic bacteria are phenotypically distinct from the phototrophs [15]. While this approach may work with eukaryotic algae, it is not as effective for cyanobacteria. In any case, microscopy is tedious and time-consuming.

The limitations of microscopy can be overcome by cell sorting, which permits differentiation and subsequent isolation of single cells based upon their cell size and autofluorescence [16]. Flow cytometry (FC) and fluorescence activated cell sorting (FACS) are powerful tools that enable rapid detection of differences among cells according to size and physiological characteristics [14], and this includes differentiating intact cells from cellular debris after lysis [17, 18].

FC sorts particles according to the characteristics detectable by multi-dimensional and quantitative measurement of light scattering and fluorescence emission [14]. In microbial cultures, it can be used to

* Corresponding authors.
E-mail addresses: siqingxia@tongji.edu.cn (S. Xia), Rittmann@asu.edu (B.E. Rittmann).

https://doi.org/10.1016/j.algal.2018.01.006
Received 18 August 2017; Received in revised form 30 November 2017; Accepted 8 January 2018

achieve two goals [19–21]: (1) characterizing cell features, such as cell size, granularity, and membrane integrity; and (2) cell sorting according to size or metabolic features that can be identified by staining. FC can also be used to differentiate heterotrophic bacteria from eukaryotic algae based on the significant difference in cell size [14].

FACS separates cells based on fluorescence derived from photosynthetic pigments (autofluorescence) or applied fluorescent probes [14]. Photosynthetic pigments in cyanobacteria include chlorophylls, carotenoids, and phycobilins [22–24], which autofluorescence with an intensity that is linearly related to the pigment concentration [25, 26]. Being the primary photosynthetic pigment, chlorophyll *a* is the only pigment present in all photosynthetic microalgae, and it emits UV-blue light (< 450 nm) and far-red light (> 650 nm) [27, 28]. Thus, FACS can be used to differentiate cyanobacteria and heterotrophic bacteria based on cyanobacteria's autofluorescence, which the heterotrophic bacteria do not have. FACS also has been used to separate heterotrophic bacteria from a mixed culture to provide an axenic culture of *Chlorella vulgaris* [14]. It should be noted, however, that the cell size for *Chlorella* is larger than 5 μm, and the authors did not attempt to quantify and detect low levels of heterotrophs. The average cell size of *Synechocystis* is approximately 3 μm, and the fraction of the biomass that is heterotrophic can be substantial [29, 30], making their differentiation from heterotrophic bacteria using FACS a much greater challenge. Another challenge is differentiating cell debris due to cell lysis from heterotrophic bacteria in a mixed culture because neither autofluorescence. Cell debris does not have the same potential deleterious effects as heterotrophic bacteria.

In this study, we develop and apply a fluorescence method for the quantification of heterotrophic bacteria in a culture of *Synechocystis* sp. PCC 6803 (*Synechocystis* from here). FACS is used to separate particles not containing chlorophyll (the heterotrophic bacteria and cell debris) from autofluorescencing *Synechocystis* cells. The non-fluorescing particles are then separated into heterotrophic bacteria and cell debris using a fluorescence stain, SYTOX green, that is better absorbed by the heterotrophs. We use light microscopy to verify the accuracy of the divisions using FC and FACS. Finally, we apply the method to evaluate how the availability of phosphorus (P) in *Synechocystis* cultures affects the quantity of heterotrophic bacteria present at the end of batch experiments.

2. Materials and methods

2.1. Synechocystis sp. PCC 6803 cultures and growth experiments

Wild-type *Synechocystis* sp. PCC 6803 was maintained in 500-mL Erlenmeyer flasks with a working volume of 300 mL. The medium was standard BG-11 [31] bubbled with air filtered through a 1.0-μm air filter (Pall, Port Washington, NY, U.S.). An aliquot from a flask culture was diluted to an optical density (OD) of 0.6 ± 0.02 to initiate each batch growth experiment.

1-L Erlenmeyer flasks with working volumes of 700 mL were used for batch growth experiments. *Synechocystis* was cultivated at a constant temperature of 30 ± 0.8 °C maintained by 3 12-W automated-air fans (Minebea-Matsushita Motor Corp., Japan) [32], and sparging with humidified air (bubbled through deionized water) filtered through a 1.0-μm air filter (Pall, Port Washington, NY, USA). The incident light intensity was 276 μmol photons $m^{-2} s^{-1}$ using T5 fluorescent plant-grow lamps (Envirogro Hydrofarm, USA). The pH of the culture was maintained at 8.0 using a pH-Stat that initiated pure CO_2 sparging when the pH rose above 8.01 [33].

In batch growth experiments, NO_3-N and PO_4-P concentrations of 120 mg N/L (8.6 mM) and 12 mg P/L (0.39 mM) served as the baseline concentrations, consistent with the stoichiometric ratio of 22 mol N/mol P in standard BG-11 medium [11], although the N concentration we used was lower than in standard BG-11. To evaluate the effects of P limitation, we decreased the starting concentrations of P from 0.39 mM (High P) to 0.055 mM (Low P) while keeping the NO_3-N concentration at 8.6 mM. We augmented the starting alkalinity by adding 6.0 mM of bicarbonate (as $NaHCO_3$). All constituents other than N, P, and alkalinity were the same as standard BG-11. Prior to inoculation, the flasks and the BG-11 medium were sterilized by autoclaving, and the pH probe was sterilized using 75% ethanol.

2.2. Autofluorescence detection

Autofluorescence, a unique biomarker for photosynthetic organisms, enabled us to set a FC gate that separated *Synechocystis* from non-fluorescing particles [34]. For autofluorescence analysis, we withdrew a 2-mL sample and diluted it to a particle concentration with the OD_{730} of about 0.08, which is suitable for counting in the FACSAria flow cytometer (BD Biosciences, CA, U.S.), and the FC had an air-cooled 20-mW argon ion laser. FC settings were: counting speed, 300 to 400 events/s; counted events for each sample, 10,000; excitation and emission wavelengths, 488 nm and > 650 nm, respectively, to detect the autofluorescence from chlorophyll [27, 28].

2.3. SYTOX Green staining and fluorescence detection

We adapted SYTOX Green (SG) (Invitrogen, Carlsbad, CA) staining and flow cytometry (FC) to differentiate heterotrophic bacteria from cell debris in the non-chlorophyll particles. At the noted time, we withdrew a second 2-mL sample, mixed it with 1 μL SG, and then allowed them to react in a rocker mixer (Lab-Line, TX, U.S.) for 15 min in the dark. We used non-chlorophyll particles directly (without SG) to zero the fluorescent intensity (FI). After staining, we detected the biomass's fluorescence using FC. The FC settings were the same as for FAC, except that we changed the emission wavelength to a band of 510–550 nm to detect SG's emission. We performed data analysis and generated graphical outputs using FlowJo 7.6.1 software (Treestar, Inc., San Carlos, CA, U.S.).

2.4. Procedures to develop and verify the method

Fig. 1 illustrates the fluorescence-based protocol for developing and verifying the method to detect heterotrophic bacteria in a culture of *Synechocystis*. In Step I, we applied FC to characterize the autofluorescence spectrum of a non-axenic culture of *Synechocystis* withdrawn from the 500-mL Erlenmeyer flasks after incubation for 3 days. Based on the spectrum from Step I, we used Step II to separate particles having low Fluorescence Intensity (FI) (i.e., putative heterotrophs) based on autofluorescence. We set the FACS cutoff at the following cumulative percentages from the low-FI end of the spectrum: 40%, 35%, 30%, 25%, 20%, 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2% and 1% of the total distribution.

In Step III, we used light microscopy to observe the morphology of particles for each of the low-FI fractions. Large particles containing pigmentation were *Synechocystis*, while smaller non-pigmented particles were heterotrophic bacteria or cell debris. One goal was to identify the fraction below which *Synechocystis* cells were no longer detectable, which provided the threshold for detecting *Synechocystis*. Fractions below this point contain only heterotrophic bacteria or cell debris. We define the fraction of non-chlorophyll particles as R_{LP} (%). Using sorting results from a higher-FI fraction, we verified that the proportion of the non-pigmented particles in the mixed culture (i.e., R_{LP}).

In Step IV, we differentiated heterotrophic bacteria from cell debris using SG and FC. SG binds to nucleic acids (NA). The extracellular polymeric substances (EPS) of intact heterotrophic bacteria contain a small amount of DNA that binds with SG and emits enough fluorescence that they can be detected by FC [17, 18]. The DNA content of cell debris was much lower than that of intact bacteria [35], which led to a much lower fluorescence intensity from cell debris. Thus, after staining with SG dye, the high-fluorescence peak in the fluorescence spectra