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Testing a dual-fluorescence assay to monitor the viability of filamentous cyanobacteria

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ABSTRACT

Filamentous cyanobacteria are currently being engineered to produce long-chain organic compounds, including 3rd generation biofuels. Because of their filamentous morphology, standard methods to quantify viability (e.g., plate counts) are not possible. This study investigated a dual-fluorescence assay based upon the LIVE/DEAD® BacLight™ Bacterial Viability Kit to quantify the percent viability of filamentous cyanobacteria using a microplate reader in a high throughput 96-well plate format. The manufacturer's protocol calls for an optical density normalization step to equalize the numbers of viable and non-viable cells used to generate calibration curves. Unfortunately, the isopropanol treatment used to generate non-viable cells released a blue pigment that altered absorbance readings of the non-viable cell solution, resulting in an inaccurate calibration curve. Thus we omitted this optical density normalization step, and carefully divided cell cultures into two equal fractions before the isopropanol treatment. While the resulting calibration curves had relatively high correlation coefficients, their use in various experiments resulted in viability estimates ranging from below 0% to far above 100%. We traced this to the apparent inaccuracy of the propidium iodide (PI) dye that was to stain only non-viable cells. Through further analysis via microplate reader, as well as confocal and wide-field epi-fluorescence microscopy, we observed non-specific binding of PI in viable filamentous cyanobacteria. While PI will not work for filamentous cyanobacteria, it is possible that other fluorochrome dyes could be used to selectively stain non-viable cells. This will be essential in future studies for screening mutants and optimizing photobioreactor system performance for filamentous cyanobacteria.

1. Introduction

Microorganisms have been used for thousands of years to produce foods, beverages, and feeds. More recently, microbes are being developed to produce chemicals and fuels from biomass to replace petroleum. Cyanobacteria are capable of producing biofuels by using solar energy and CO_2 as their sole energy and carbon sources (Machado and Atsumi, 2012). Furthermore, many strains of filamentous cyanobacteria are capable of fixing N_2 with specialized cells called heterocysts. This ability makes these strains preferable for industrial application (Yoon and Golden, 1998). There is also increasing interest in engineering cyanobacteria to produce high-value products such as linalool (Gu et al., 2012), limonene (Halfmann et al., 2014a), farnesene (Halfmann et al., 2014b), sugars (Ducat et al., 2011), and 1-butanol (Lan and Liao, 2011). A key consideration for these engineered cyanobacteria is tolerance to the chemicals they are being engineered to produce, as well as their long-term stability in photobioreactor systems (Jin et al., 2014). Therefore, a method is needed to rapidly and accurately quantify the viability of filamentous cyanobacteria.

Because living microbes are responsible for converting substrates to desired end products, monitoring cell viability is an important requirement. For microbes that grow as single cells, methods such as plate counts (Harmsen et al., 1999), hemocytometer counts (Schaeffer et al., 1979), flow cytometry (Berney et al., 2007), and automated analyses of microscopic images such as Image J (Schulze et al., 2011) work effectively. Unfortunately, these methods do not work well for cyanobacteria that grow as filaments. Although Lee and Rhee (1999a) and Lee and Rhee (1999b) report accurate counts of the filamentous cyanobacteria *Anabaena flos-aquae* using epi-fluorescence microscopy, this approach would be far less accurate in conditions where cells aggregate, such as during production of chemicals that could be toxic to the cells.

To assess the tolerance of filamentous cyanobacteria to certain chemicals, a fast and reliable method is needed to quantify cell viability. The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Life Technologies™, Carlsbad, CA, USA) has been successfully used to estimate the viability of bacteria from a broad range of ecosystems (Filoche et al., 2007; Sato et al., 2004). For example, this technology has been used to assess the viability of over-wintering unicellular cyanobacteria (Zhu and Xu, 2013). The BacLight™ Kit utilizes dual fluorescence dyes to determine

Abbreviations: CLSM, confocal laser scanning microscopy; LB, Luria broth; LBA, Luria broth agar; PI, propidium iodide; PC, phycocyanin; U, intensity units.

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cell viability based on the differential ability of the nucleic acid stains SYTO® 9 and propidium iodide (PI) to penetrate viable versus non-viable cell membranes, respectively. This method is based upon the fact that cell membrane permeability is substantially higher in non-viable cells (Agustí et al., 2006; Llabrés and Agustí, 2008).

SYTO® 9 is a permeant intercalating green fluorescent dye capable of penetrating most plasma membranes and staining all cells containing nucleic acid. PI is a red fluorescent dye that is membrane impermeable, thus excluded by the intact cell membranes of living cells (Lee and Rhee, 2001; Shi et al., 2007). Therefore in the BacLight™ Kit, viable cells stain green. PI can pass through the porous membranes of dead cells, where it also intercalates into the nucleic acids, and displaces the SYTO® 9 dye due to intermolecular excitation energy transfer by the resonance mechanism (Samuilov et al., 2008). Thus, non-viable cells fluoresce in the red spectra at an excitation wavelength of 490 nm and an emission wavelength of 635 nm (Stocks, 2004; Alakomi et al., 2006). Thus this kit is designed to differentiate live cells from dead cells due to the ability of PI to displace SYTO® 9 from nucleic acids of cells with compromised membranes due to a higher affinity for nucleic acids (Stocks, 2004; Biggerstaff et al., 2006; Rath and Adhikary, 2014; Nybom et al., 2008; Llabrés and Agustí, 2008). The aim of this study was to determine if this kit was accurate in estimating the viability of filamentous cyanobacteria. Such a methodology would be broadly applicable to the study of gene function, biofuel tolerant mutant screening, and culture maintenance of filamentous cyanobacteria.

2. Materials and methods

2.1. Microbial strains, maintenance, and culture conditions

The filamentous cyanobacteria *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133 were obtained from their respective culture collections. *Escherichia coli* strain NEB 10-beta was obtained from New England Biolabs, Ipswich, MA, USA. Cyanobacteria strains were grown in BG11 medium at pH 7.2 (Allen and Stanier, 1968) supplemented with 20 mM HEPES buffer (Thermo Fisher Scientific™, Waltham, MA, USA). For a solid medium, BG11 was supplemented with 1% agar (Benton Dickinson, Franklin Lakes, NJ, USA). For short-term maintenance the cyanobacteria strains were grown on solid BG11 agar and incubated at room temperature under constant illumination of 24 μmol m^{-2} s^{-1}, and then stored at room temperature. For long-term culture storage, cyanobacteria were frozen at −80 °C in 5% v/v methanol. *E. coli* strain NEB 10-beta was grown in 500 ml Luria broth (LB) in 1 l Erlenmeyer flasks at 37 °C. For short-term maintenance the *E. coli* strain was grown on Luria broth agar (LBA) and stored under refrigeration. For long-term maintenance, the strain was frozen at −80 °C in 5% v/v glycerol.

Broth cultures of cyanobacteria were grown in 1 l Erlenmeyer flasks containing 500 ml of BG11 medium. Flasks were stoppered with a two-hole rubber stopper and a 2 ml disposable polystyrene serological pipet (Thermo Fisher Scientific™, Waltham, MA, USA) was inserted into one of the stopper holes to serve as the gas inlet port. The end of this pipet was submerged in the culture fluid. A section of another 2 ml pipet was inserted through the other stopper hole and was fitted with a sterile filter on the outside of the flask so exhaust gas could flow out. A mixture of 5% CO_2 in compressed air was sparged into the flasks at a constant rate of 0.25 l min^{-1}. Gas flow was regulated by flow meters (Cole-Parmer®, Vernon Hills, IL, USA). The flasks were incubated in a Lab-Line® Incubator-Shaker (Lab-Line® Instruments, Melrose Park, IL, USA) at 30 °C and 100 rpm under constant illumination of 19 μE m^{-2} s^{-1} using fluorescent lights. Light intensities were measured with a Heavy Duty Light Meter with PC Interface (Extech Instruments, Waltham MA, USA). *E. coli* strain NEB 10-beta was grown in 500 ml Luria Broth (LB) in 1 l Erlenmeyer flasks at 37 °C and 150 rpm.

2.2. Analysis for autofluorescence

Autofluorescence in the red spectrum occurs in cyanobacteria when excited in the green spectrum due to phycobiliproteins (Baier et al., 2004). PI also fluoresces in the red spectrum, but must be excited in the blue spectrum. Because the BacLight™ Kit uses a blue excitation spectrum, cyanobacteria autofluorescence was not anticipated to be a problem. However previous studies have suggested that red spectrum autofluorescence of cyanobacteria (Grilli Caiola et al., 1996; Dagnino et al., 2006) would interfere with attempts to use PI as a non-viable cell indicator (Sato et al., 2004). Therefore we assessed whether autofluorescence would interfere with the BacLight™ fluorescence assay by performing a lambda scan on viable cyanobacteria cultures. This test used a blue spectrum excitation wavelength (488 nm), and monitored emissions ranging from 500 to 680 nm in 10 nm segments using an Olympus® Fluoview FV1200 laser scanning confocal microscope system interfaced with an IX81 Microscope (Olympus® Corporation, Tokyo, Japan). Autofluorescence results were negative, but to confirm similar results in the Synergy 2 Multi-Mode Microplate Reader (BioTek®, Winooski, VT, USA), we repeated the test with the following fluorescence filters as recommended in the kit protocol: excitation wavelength 485 ± 20 nm; green emission 528 ± 20 nm and red emission 620 ± 40 nm. Two hundred microliters of unstained cyanobacteria that had been grown to mid-log phase was pipetted into a microplate well and incubated in the dark at room temperature for 15 min. Fluorescence was measured by the microplate reader.

2.3. Protocol development

Each cyanobacterial strain was grown to exponential phase under the conditions previously described, and then processed as shown in Fig. 1 as per instructions in the BacLight™ Kit. Viable and non-viable cells were mixed in ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. The SYTO® 9 and PI dyes were prepared following the manufacturer's instructions. Equal volumes of the viable:non-viable cell solutions were then mixed with an equal volume of the stain solutions. Plates containing the stained cells were wrapped in aluminum foil and incubated in the dark at room temperature for 15 min. After incubation, the fluorescence in each well was measured using the microplate reader equipped with the fluorescence filters as previously described. For each trial, an empty well was used for a control to ensure the microplate was working properly.

Linear regression was then performed to plot the ratio of green and red fluorescence versus cell viability percentage. The ratios used to generate linear calibration curves were calculated utilizing the equations in the protocol (Invitrogen Molecular Probes, 2004). The data was analyzed by dividing the green fluorescence (F) emitted (em) by the red fluorescence emitted ($Ratio_{G/R} = F_{cell,em1} / F_{cell,em2}$). This ratio was plotted as $Ratio_{G/R}$ versus percentage of viable cells in the suspension. Linear regression analysis was then performed to calculate the equation and assess the correlation coefficient. Stained and unstained BG11 were tested and acted as a control for the microplate reader to ensure it was working properly.

2.4. Microscopy

Confocal laser scanning microscopy (CLSM) was used to evaluate possible binding of PI to cell components other than nucleic acids. For these observations, 488 nm was used as the excitation wavelength and 619 nm as the emission wavelength, and individual scans were made throughout the width of the cells. The staining patterns of the SYTO® 9 and PI dyes on viable and non-viable cyanobacteria strains were observed with an Olympus® AX70 wide-field epi-fluorescence microscope (Olympus® Corporation, Tokyo, Japan). The filter cube (U-MNB) used for these observations contained an excitation filter with a wavelength maximum at 488 nm and a long-pass emission filter