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Optimizing cyanobacteria growth conditions in a sealed environment to enable chemical inhibition tests with volatile chemicals

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ABSTRACT

Cyanobacteria are currently being engineered to photosynthetically produce next-generation biofuels and high-value chemicals. Many of these chemicals are highly toxic to cyanobacteria, thus strains with increased tolerance need to be developed. The volatility of these chemicals may necessitate that experiments be conducted in a sealed environment to maintain chemical concentrations. Therefore, carbon sources such as $NaHCO_3$ must be used for supporting cyanobacterial growth instead of CO_2 sparging. The primary goal of this study was to determine the optimal initial concentration of $NaHCO_3$ for use in growth trials, as well as if daily supplementation of $NaHCO_3$ would allow for increased growth. The secondary goal was to determine the most accurate method to assess growth of *Anabaena* sp. PCC 7120 in a sealed environment with low biomass titers and small sample volumes. An initial concentration of 0.5 g/L $NaHCO_3$ was found to be optimal for cyanobacteria growth, and fed-batch additions of $NaHCO_3$ marginally improved growth. A separate study determined that a sealed test tube environment is necessary to maintain stable titers of volatile chemicals in solution. This study also showed that a SYTO® 9 fluorescence-based assay for cell viability was superior for monitoring filamentous cyanobacterial growth compared to absorbance, chlorophyll α (chl a) content, and biomass content due to its accuracy, small sampling size (100 μL), and high throughput capabilities. Therefore, in future chemical inhibition trials, it is recommended that 0.5 g/L $NaHCO_3$ is used as the carbon source, and that culture viability is monitored via the SYTO® 9 fluorescence-based assay that requires minimum sample size.

1. Introduction

Fossil fuels are a finite resource, and it is well established that the massive use of fossil fuels has led to pollution and detrimental health effects in many organisms (Chen et al., 2011). Beyond the long-recognized negative environmental impacts of smog formation and ozone depletion, global warming is a more recently recognized effect of fossil fuel use (von Blottnitz and Curran, 2007). Due to these environmental concerns, it is urgent to develop efficient, clean, and secure systems for the production of biofuels from sustainable sources (Becerra et al., 2015; Gu et al., 2012).

One potential source of renewable biofuels is the photoautotrophic, diazotrophic cyanobacterium *Anabaena* sp. PCC 7120 (herein referred to as *Anabaena* sp. 7120). This microbe is capable of being genetically engineered to produce next-generation biofuels and high-value chemicals such as linalool (Gu et al., 2012), limonene (Halfmann et al., 2014b), and farnesene (Halfmann et al., 2014a). These chemicals are insoluble or have low solubility in water, and this hydrophobic nature leads to high bio-concentration in aquatic organisms such as cyanobacteria, making these chemicals quite toxic at low concentrations (Mayer et al., 2000). Thus it is important to increase the microbe's tolerance to these chemicals to improve productivity and the industrial potential of these photoautotrophs.

The chemicals previously mentioned are highly volatile, thus chemical inhibition tests must be conducted in a sealed environment with minimal headspace. This will enable maintenance of desired titers of the chemical of interest during the incubation period. Unfortunately, this means that sparging with CO_2 enriched air, or even simple exposure to atmospheric CO_2 cannot be used to supply carbon for cell growth. An alternative carbon source for cyanobacteria is $NaHCO_3$. Many cyanobacterial species are capable of taking up HCO_3^- from the environment via transport across the plasma membrane into the cytosol. There, CO_2 is derived from HCO_3^- by carbonic anhydrase maintaining a steady flux to ribulose-1,5-bisphosphate carboxylase/oxygenase for photosynthesis (White et al., 2013).

Studies have been conducted on algal chemical inhibition tests with volatile chemicals, but to the best of our knowledge no studies have used cyanobacteria. Mayer et al. (Mayer et al., 2000)) used 0.3 g/L $NaHCO_3$, while Herman et al. (Herman et al., 1990)) used 4 g/L $NaHCO_3$ for algal chemical inhibition tests. Mayer et al. (Mayer et al., 2000)) also supplemented the medium with 2% CO_2 which was adapted

Abbreviations: ANOVA, analysis of variance; Chl a, Chlorophyll α; MRT, Duncan's new multiple range test; U, fluorescence intensity units.

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from a study by Hailing-Sørensen et al. (Hailing-Sørensen et al., 1996)). CO_2 was used both for carbon enrichment and to act as a pH buffering agent. However, supplementing the medium with a physiological buffer, such as HEPES could also suit this purpose.

While Herman et al. (Herman et al., 1990) did evaluate different $NaHCO_3$ concentrations, the vessels used had a significant volume of headspace. Also, both Herman et al. (Herman et al., 1990) and Mayer et al. (Mayer et al., 2000) performed these trials with algal rather than cyanobacterial strains. Thus it is necessary to determine the optimal concentration of $NaHCO_3$ for growth of a cyanobacteria strain (*Anabaena* sp. 7120), and if supplementing with $NaHCO_3$ in a fed-batch manner would further increase growth in a sealed environment.

An additional challenge with chemical inhibition tests with hydrophobic chemicals is that the chemicals can have sorption interactions with the cyanobacterial biomass itself and/or the walls of the culture vessel, thereby altering the effective concentration exposed to the cells (Mayer et al., 2000). To minimize this problem, it was recommended that trials are conducted with low biomass levels (Mayer et al., 1997; Nyholm and Peterson, 1997; Peterson and Nyholm, 1993). However, at low biomass levels, classical methods of monitoring culture biomass are less accurate. For example, optical density is considered to have borderline sensitivity and precision at the biomass levels of standard algal toxicity tests (Mayer et al., 1997). Another issue to consider is that optical density and chlorophyll α (chl a) content can be easily affected by biomass debris formation (Robertson et al., 1998).

A fluorescence viability assay has previously been shown by Johnson et al. (Johnson et al., 2016) to be a superior method of monitoring viability of *Anabaena* sp. 7120 at low biomass titers when compared to optical density and chl a content. Determining if there is a strong correlation between the viability assay and absorbance, chl a content, and biomass content would provide further evidence that the viability assay is an accurate means of monitoring cell viability.

For next-generation biofuels and high-value chemical production from cyanobacteria to become industrially feasible, it is essential to develop strains with increased tolerance to the chemicals that they will be engineered to produce. Because many of these compounds are highly volatile, a sealed environment will be necessary to maintain the chemical titer in solution. Biomass levels must also be minimized to ensure constant chemical-to-biomass concentrations. Therefore, the objectives of this study were to: 1) determine the most accurate and reproducible methods to monitor cyanobacterial growth and viability in a sealed environment, 2) determine the optimal initial concentration of $NaHCO_3$ and if fed-batch addition of $NaHCO_3$ would enhance growth, and 3) compare cyanobacterial growth in the sealed test tube environment optimized in the previous objective to growth in test tubes that are not sealed.

2. Materials and methods

2.1. Microbial strains, maintenance, and culture conditions

Anabaena sp. PCC 7120, a model species for filamentous cyanobacteria (Bryant, 2006; Rippka et al., 1979), was obtained from the Pasteur Culture Collection of Cyanobacteria (Paris, France). For long term storage, strains were frozen at −80 °C in 5% v/v methanol. For short term maintenance the cyanobacteria were grown on BG11 agar (Allen and Stanier, 1968) (1.5% agar) at pH 7.1, incubated at room temperature under constant illumination of 24 μmol m^{-2} s^{-1}, and then stored at room temperature. Light intensity was measured with a Heavy Duty Light Meter with PC Interface (Extech Instruments, Waltham MA, USA).

Inoculum for the experiments described below was grown in 250 mL Erlenmeyer flasks containing 100 mL of BG11 medium at pH 7.1 supplemented with 20 mM HEPES buffer. Flasks were stoppered with a foam stopper and then covered with aluminum foil. 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 μmol m^{-2} s^{-1} using fluorescent lights.

In the sealed test tube experiments cyanobacterial cultures were grown in 27 mL screw capped test tubes. The 27 mL test tubes had an open top cap sealed with a PTFE/silicone septum to allow inoculation and sampling via a syringe and needle, and yet prevent the loss of volatile chemicals that were being tested. Tubes were filled with ~27 mL BG11 with 20 mM HEPES buffer and various concentrations of $NaHCO_3$ for a carbon source. The tubes were incubated at ~22 °C under constant illumination of approximately 24 μmol m^{-2} s^{-1} while rotating at 8 rpm in a Thermo Fisher Scientific™ Labquake™ Tube Rotator (Thermo Fisher Scientific™, Waltham, MA, USA).

2.2. Correlation of growth parameters in sealed test tubes

To assess the accuracy and reproducibility of various methods of monitoring biomass levels, 27 mL sealed test tubes containing ~27 mL BG11 with 20 mM HEPES and 0.5 g/L $NaHCO_3$ were inoculated with 270 μL (1%) of a mid-log phase culture of *Anabaena* sp. 7120. The initial starting concentration of 0.5 g/L $NaHCO_3$ was chosen as it was previously shown to support cyanobacterial growth in sealed test tubes (Johnson et al., 2016). Sufficient test tubes were inoculated so that 3 tubes could be sampled and then discarded each day. This was necessary, because the sample volume removed would have altered the headspace volume and introduced variability. Test tubes were incubated under the conditions previously described in Subsection 2.1, and the trials continued for 1 day after the maximum viability was reached as determined by the fluorescence assay. Daily samples of 14 mL were taken for determination of absorbance (OD_{700}), viability via a fluorescence assay, chl a content, and biomass content. These methods are described in detail in the analytical methods Subsection 2.6.

2.3. Optimizing the initial $NaHCO_3$ concentration in sealed test tubes

To determine the optimal initial concentration of $NaHCO_3$, 27 mL sealed test tubes containing ~27 mL BG11 with 20 mM HEPES and 0, 0.25, 0.5, 0.75, or 1.0 g/L $NaHCO_3$ were inoculated with 270 μL (1%) of a mid-log phase culture of *Anabaena* sp. 7120. The tubes were incubated in conditions previously described. Daily, 100 μL samples were collected via a 1 mL syringe and a 21-gauge needle. Fluorescence was measured as described below in Subsection 2.6. Maximum fluorescence reached during a trial (U), increase in fluorescence from the initial time point to the maximum fluorescence reached (%), and fluorescence rate of change during the trial (U/d) were calculated from these data. The trials continued for 1 day after the maximum viability was reached, which was typically 5–7 days.

2.4. Fed-batch trials optimizing $NaHCO_3$ concentrations in a sealed test tube system

To determine if supplementing $NaHCO_3$ in a fed-batch manner would further increase biomass levels in a sealed environment, 27 mL sealed test tubes containing ~27 mL BG11 with 20 mM HEPES and 0.5 g/L $NaHCO_3$ were inoculated with 270 μL (1%) of a mid-log phase culture of *Anabaena* sp. 7120. The tubes were incubated, sampled, and measured for fluorescence as previously described in Subsections 2.1 and 2.6. On a daily basis, 0, 8.0×10^{-4}, 2.4×10^{-3}, or 4.8×10^{-3} g $NaHCO_3$ was added to the tubes via a 1 mL syringe and a 21-gauge needle. This was accomplished by adding 0, 100, 300, or 600 μL of an 8 g/L $NaHCO_3$ stock solution, respectively. The volume of fluid added to the tube was removed after manual mixing to maintain a constant headspace volume. Maximum fluorescence reached during a trial (U), increase in fluorescence from the initial time point (%), and fluorescence rate of change during the trial (U/d) were calculated from the data. The trials continued for 1 day after the maximum viability was reached, which was typically 3–6 days.