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Increasing the tolerance of filamentous cyanobacteria to next-generation biofuels via directed evolution

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ABSTRACT

Renewable biofuels can lessen our reliance on fossil fuels. Cyanobacteria are being investigated for the production of biofuels directly from carbon dioxide, thus eliminating the steps of biomass production, harvest, logistics, and conversion required for 1st and 2nd generation biofuels. This study determined the initial tolerance of *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133 to four potential biofuels: farnesene, myrcene, linalool, and limonene. These cyanobacteria were then subjected to three rounds of directed evolution where the strains were exposed to increasing titers of each chemical. This led to a library of 12 putative mutants with higher tolerance to individual chemicals. These mutants were assessed for growth performance at chemical titers higher than the wildtype strains could tolerate. Power analysis was used to determine the necessary sample size to achieve statistical power of ≥0.95. Student's *t*-tests were performed to determine if the putative mutants had significantly improved growth parameters compared to the wildtype, thereby establishing whether an inheritable genetic change or possibly an epigenetic change had occurred. Three mutants were confirmed as having higher tolerance to individual chemicals. This work serves as proof-of-concept that directed evolution is a valid methodology to increase the tolerance of filamentous cyanobacteria to biofuels.

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1. Introduction

Replacements for petroleum-derived fuels and chemicals are becoming increasingly necessary due to the adverse effects of fossil fuel-generated greenhouse gases, including ozone depletion, global warming, and smog formation [84]. The extensive use of fossil fuels has led to pollution, global climate change, and detrimental effects on many organisms [13]. One solution to this problem is to explore the utilization of cyanobacteria to manufacture next-generation biofuels.

Cyanobacteria are photosynthetic prokaryotes present in a wide variety of ecosystems [31,40,54]. They have morphologies ranging from unicellular to filamentous, and utilize the same type of photosynthesis as higher plants [48]. Cyanobacteria have garnered significant attention from industrial microbiologists due to their potential to be genetically engineered to produce high-value chemicals and next-generation biofuels [27–29,42,46] from CO_2 and solar energy [49]. The strains of filamentous cyanobacteria utilized in this study also have the ability to fix atmospheric nitrogen in specialized cells termed heterocysts, located along the filament. Thus, these strains have an advantage in biofuel production, as supplying nitrogen is a major cost in large scale biofuel strategies [29,57,61].

Next-generation biofuels derived from cyanobacteria are considered to be a technically viable alternative energy resource devoid of the major drawbacks associated with 1st and 2nd generation biofuels [8]. At this time, cyanobacteria appear to be the only current biorenewable resource capable of meeting the global demand for transportation fuels [15,70,77]. While there are several companies with the capability to produce fuel from photosynthetic organisms [16], the development of cyanobacteria for mass production of fuels and chemical is still in its infancy [59].

Biofuels and high-value chemicals are often toxic to the microbe that produces them [17,22,35,41,87]. Thus for commercial deployment of cyanobacterial fuel/chemical production, it will be necessary to develop strains able to withstand these toxic effects. One strategy to achieve this goal is use of directed evolution, a process in which a microbe is grown under a selective pressure that forces rapid evolution to tolerate that pressure. Directed evolution has been used to improve production of a range of microbial products [45]. The process of directed enzyme evolution begins with creation of a library of strains with mutated genes. Strains that show improvement in the desired property are identified by screening, and are subjected to further cycles of mutation and screening to further enhance performance [44,83].

Abbreviations: LCIC, Lowest-complete-inhibition concentration; MIC, Minimum inhibitory concentration; PI, Propidium iodide; TEM, Transmission electron microscopy; U, Fluorescence intensity units.

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In this study we sought to enhance the tolerance of three species of cyanobacteria to four biofuels which are toxic to cyanobacteria (Fig. 1). Farnesene is a sesquiterpene with commercial applications in lubricants, cosmetics, fragrances, and biofuels [9,28]. Myrcene, a monoterpene, has applications as a starting material for high-value compounds such as geraniol/linalool, and (−)-menthol [41]. Linalool, an energy dense long-chain alcohol has the potential to become a 'drop-in' biofuel [27]. It is also used in perfumes, cosmetics, and flavoring agents [90]. Limonene, a cyclic monoterpene, has potential as a biodiesel and a biofuel [29]. The objectives of this study were to: 1) create a library of putative mutant strains of biofuel tolerant filamentous cyanobacteria via directed evolution, and 2) screen the putative mutants in a spiked culture medium to determine if an inheritable genetic or epigenetic change occurred.

2. Materials and methods

2.1. Microbial strains, maintenance, and culture conditions

The filamentous, diazotrophic cyanobacterial strains *Anabaena* sp. PCC 7120 (herein referred to as *Anabaena* sp. 7120), *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133 were obtained from their respective culture collections. 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 (1.5% agar) [3] 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 sterile BG11 media 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 μE m^{-2} s^{-1} using fluorescent lights.

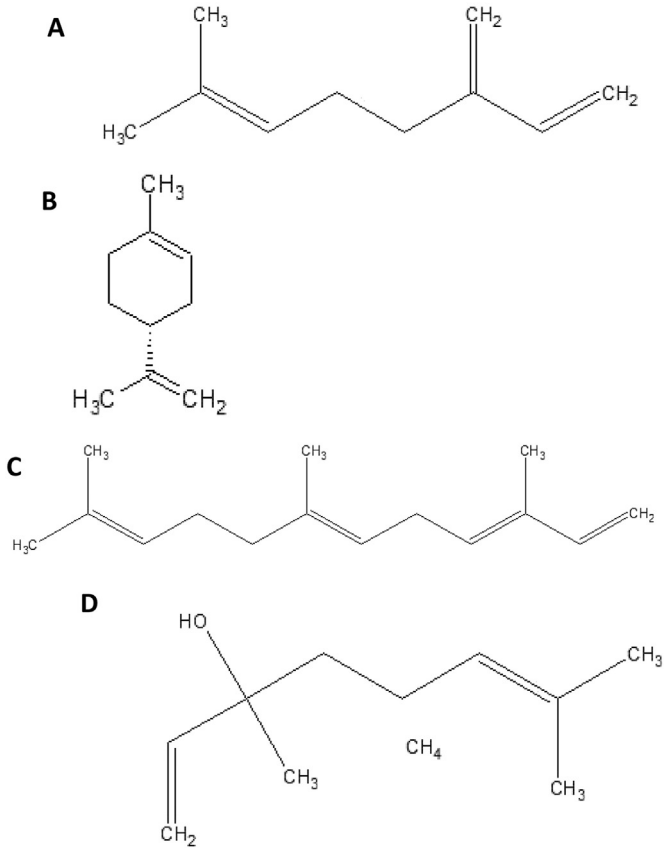

Fig. 1. Molecular structure of the chemicals utilized in this study: myrcene (A), limonene (B), farnesene (C), and linalool (D). (Structures drawn with ACD/ChemSketch Freeware).

In the experiments described below, cyanobacteria were grown in either 20 or 27 mL, sealed test tubes. The sealed test tube system was needed to prevent evaporative loss of the volatile chemicals being tested. In directed evolution trials, cyanobacteria were grown in 20 mL screw capped test tubes. Tubes were filled with ~20 mL BG11 with 20 mM HEPES buffer and 0.5 g/L $NaHCO_3$ for a carbon source. Cyanobacteria are capable of taking up HCO_3^- via transport across the plasma membrane into the cytosol. There CO_2 is derived from HCO_3^- via carbonic anhydrase, which maintains a steady state flux to ribulose-1.5-bisphosphate carboxylase oxygenase for photosynthesis [85]. It has been determined by our research group that 0.5 g/L $NaHCO_3$ is the optimal concentration for cultivating filamentous cyanobacteria in a sealed environment [37]. This method was adapted from previous studies that used a sealed environment for chemical inhibition tests with algae [33,52]. In mutant screening trials, cyanobacteria were grown in 27 mL screw capped test tubes that had an open top cap sealed with a PTFE/silicone septa to allow inoculation and sampling via a syringe and needle. Tubes were filled with ~27 mL BG11 with 20 mM HEPES buffer and 0.5 g/L $NaHCO_3$. After inoculation, tubes were incubated at room temperature 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. Determination of wildtype cyanobacteria tolerance to biofuels

Tolerance of the wildtype strains of *Anabaena* sp. 7120, *N. punctiforme*, and *A. variabilis* to farnesene, linalool, myrcene, and limonene was established by determining the titer of the chemical that each strain could survive after a 3 d incubation period. An initial inoculum of a mid-log culture was transferred to test tubes to achieve an initial OD_{700} of 0.5. The test tubes were spiked with specific titers of the biofuel of interest. For each trial, a positive control containing the cyanobacterial strain and no chemical was used. The test tubes were incubated under the conditions previously described.

After 3 d, cultures were centrifuged and washed repeatedly to remove any residual biofuel. This protocol involved: 1) centrifuging at 4000 rpm at room temperature for 10 min, 2) re-suspending cell pellets in 1 mL BG11 and transferring to a 1.5 mL centrifuge tube, 3) centrifuging at 6000 rpm at room temperature for 5 min, 4) re-suspending cell pellets in 1 mL BG11, 5) centrifuging at 6000 rpm at room temperature for 5 min, and 6) re-suspending in 1 mL BG11. This cell suspension was then spread onto BG11 plates containing 1.5% agar, and incubated at 30 °C with constant illumination at 30 μmol/m^2/s until colonies were present (~1–2 weeks). The highest titer of chemical that the strain could survive was determined to be the initial tolerance level.

2.3. Mutant library creation via directed evolution

To create a library of putative mutants that potentially had increased biofuel tolerance, filamentous cyanobacteria strains were exposed to serially increasing titers of the biofuel of interest. Inoculum was mid-log cultures that were transferred into test tubes containing BG11 medium to achieve an OD_{700} of 0.5. The test tubes were spiked with specific titers of the biofuel, and were incubated under the conditions previously described. After 3 d, the cultures were centrifuged and washed as previously described. Cell suspensions were then inoculated onto BG11 plates containing 1.5% agar and incubated as described previously until colonies were present (~1–2 weeks). This represented one round of directed evolution.

Colonies that survived the highest titer of biofuel were selected from plates, and were then inoculated into 250 mL Erlenmeyer flasks

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