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Evaluation of experimental conditions that influence hydrogen production among heterocystous Cyanobacteria

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ABSTRACT

The overall goal of this research was to systematically evaluate H_2 production among different heterocystous cyanobacteria in response to defined experimental variables including N_2 and O_2 concentration, carbon source, and light intensity. N_2 elicited an immediate reduction of H_2 production rates and the magnitude of the effect was strikingly similar across the diverse collection of heterocystous cyanobacteria that were tested. At the N_2:O_2 ratio found in air (4:1), N_2 was a much more potent inhibitor of H_2 production than O_2. Low levels of O_2 (1–5% headspace, vol:vol) were generally found to support optimal H_2 production. Glucose addition (10 mM) stimulated light-dependent H_2 production in 8 of 10 cyanobacteria examined, eliciting a 2–11 fold increase in production rates and 2–45 fold increase in yields. The addition of glucose also effectively lowered the intensity of light required for optimal H_2 production in 4 of 10 strains tested. H_2 production rates ranged from 1 to 50 μmol mg chl a^{-1} h^{-1}. The results from this study provide important benchmark phenotypes against which to evaluate newly discovered H_2-producing heterocystous cyanobacteria, and we discuss how these findings highlight the necessity of a multi-parameter approach to comprehensively screen for superior H_2-producing heterocystous cyanobacteria.

1. Introduction

H_2 generated by cyanobacteria is a highly attractive form of alternative energy that is a renewable resource, requiring only water, sunlight, air, and trace minerals for production. This process does not use or produce hazardous materials, is considered a carbon-neutral process, and could be coupled to the production of other commodities. Current research efforts aimed at understanding and producing biohydrogen directly from sunlight have largely focused on developing operational, hydrogen producing bioreactors or conducting detailed molecular analysis of genes and proteins from select cyanobacterial strains. Much progress has been made, yet an important element of the research continuum from genes to

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H_2-producing bioreactors has not received the same level of attention in recent years – the physiological diversity of naturally occurring, H_2-producing cyanobacteria.

In recent years a number of review articles have been published that consolidate and compare the results of three decades worth of cyanobacterial H_2 production research [1–7]. Cyanobacterial hydrogen production rates vary considerably among the 50–100 strains that had been tested, ranging from less than 1 to greater than 100 μmol mg chl a^{-1} h^{-1} [1,2]. Although the interplay between experimental conditions (nutrients, light intensity, cell density, temperature, pH, carbon source, gas composition, etc.) and H_2 production has been extensively examined for individual strains of cyanobacteria [8–15], it is difficult to critically compare H_2 production rates and yields from these studies because of the variability in experimental design.

Comparative analysis of H_2 production by multiple strains has been limited to just a handful of studies [6,12,16–19]. In a pioneering study, Lambert and Smith [12] examined H_2 production by five strains of heterocystous cyanobacteria obtained from culture collections. Berchtold and Bachofen [17] enriched 71 cyanobacterial cultures under diazotrophic conditions and subsequently screened them for hydrogen production under an argon atmosphere with 1% CO_2 in the light over a period of 48 h. Most strains were capable of hydrogen formation (presumably via nitrogenase), and this phenotype was identified among filamentous (with and without heterocysts) and unicellular strains. From a study comparing nine unicellular cyanobacteria that lacked nitrogenase activity, it was subsequently concluded that hydrogenase catalyzed H_2 production could also be widespread among cyanobacteria [18]. Yoshino et al. [19] compared acetylene reduction rates to H_2 evolution rates among 13 *Nostoc* and *Anabaena* strains and found a positive correlation between the two measurements, thus acetylene reduction assays could be used as a first step when screening for H_2-producing strains. Most recently, Allahverdiyeva et al. [16] screened 400 cyanobacterial strains from the Baltic Sea and Finnish lakes and identified 10 superior H_2-producing strains (this research greatly expands the total number of cyanobacterial strains screened for H_2 production). Comparative analysis of the effects of culture conditions on H_2 production was performed with just 2 of the strains isolated in that study.

From these studies performed to date, several generalizations can be made concerning H_2 production among cyanobacteria. First, rates of nitrogenase-catalyzed H_2 production typically exceed that of hydrogenase- or fermentation-based H_2 generating mechanisms in cyanobacteria [2,6,8,20]. Second, H_2 production via nitrogenase is susceptible to inhibition by the major components of air – oxygen and nitrogen [11,12,14,21–23]. Third, addition of exogenous carbon to cyanobacterial cultures can increase H_2 production rates and yield (researchers typically utilize CO_2 but addition of organic carbon has also been found to stimulate cyanobacterial H_2 production) [14,24–27]. Finally, most cyanobacteria harbor one or more uptake hydrogenases that oxidize evolved H_2 to initiate an oxyhydrogen (Knallgas) reaction (presumably to recoup energy/reductant expended during H_2 production), which can significantly decrease net H_2 production rates [19,28–30]. The schematic shown in Fig. 1 provides a simplified overview of H_2 production by heterocystous cyanobacteria. Yet, to our knowledge, no single study has been conducted that systematically analyzes the effects of culture conditions on H_2 production in more than 2–3 cyanobacterial strains.

Considering that many thousands, or even millions, of microbial species are capable of photobiological hydrogen production, it certainly makes sense to explore (and potentially harness) this untapped diversity. The overall goal of the research described here was to systematically evaluate the H_2-producing characteristics of diverse heterocystous cyanobacteria. Specifically we aimed to: 1) assess several of the environmental parameters (N_2 concentration, O_2 concentration, carbon source, and light intensity) that control H_2 production among different unialgal cultures (both axenic and non-axenic) of heterocystous cyanobacteria, and 2) establish benchmark activities/phenotypes against which to evaluate newly discovered H_2-producing strains.

2. Materials and methods

2.1. Cyanobacterial strains and culture conditions

Anabaena cylindrica B629 and *Tolypothrix* sp. B379 were purchased from the Culture Collection of Algae at the University of Texas at Austin. *Tolypothrix* sp. B379 is also referred to as *Calothrix membranacea* B379, but because of strain morphology and 16S rRNA analysis, we feel that this strain is more aptly classified as a *Tolypothrix* sp. *Nostoc punctiforme* PCC 73102 and *Anabaena* sp. PCC 7120 were purchased from the Pasteur Culture Collection. *Fischerella muscicola* PCC 7414 was purchased from the American Type Culture Collection (ATCC 29161™). *Nostoc commune* MFG-1, *Spirirestis rafaelensis* LQ-10, *Scytonema hylanium* NCC-4B, and *Calothrix* sp. MCC-3 were isolated from biological soil crusts collected in southeastern Utah, USA [31]. *Fischerella* sp. Dx-SRS was isolated from a reservoir receiving thermal effluent from a nuclear reactor at the Savannah River Site, South Carolina, USA (unpublished results). All strains purchased from PCC and *Fischerella* sp. Dx-SRS were grown and tested as axenic cultures (contaminant testing was performed periodically by streak plating on R2A agar plates), the other strains were grown and tested as unialgal, non-axenic cultures (bacterial contaminants). All strains isolated from biological soil crusts had been through at least 10 passages on BG11 medium and harbored 1–4 bacterial contaminants as determined by streaking on R2A agar plates. Fungal contaminants were not observed. In this study we purposefully examined axenic and unialgal, non-axenic cyanobacterial cultures because large scale, high throughput screening of environmental strains or samples will necessitate working with non-axenic cultures. Although a number of the cultures tested were non-axenic, the data strongly indicate that cyanobacterial nitrogenase systems were responsible for most of the observed H_2 production: 1) H_2 production was almost undetectable for each strain when incubated in the dark (data not shown), 2) each strain was extremely sensitive to N_2 inhibition, and 3) diazotrophic cyanobacteria were greatly enriched in all cultures by using $BG11_0$ to grow biomass for the experiments.