

Improving Cyanobacterial Hydrogen Production through Bioprospecting of  
Natural Microbial Communities

by

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A Dissertation Presented in Partial Fulfillment  
of the Requirements for the Degree  
Doctor of Philosophy

Approved November 2013 by the  
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ARIZONA STATE UNIVERSITY

December 2013

## ABSTRACT

Some cyanobacteria can generate hydrogen (H<sub>2</sub>) under certain physiological conditions and are considered potential agents for biohydrogen production. However, they also present low amounts of H<sub>2</sub> production, a reaction reversal towards H<sub>2</sub> consumption, and O<sub>2</sub> sensitivity. Most attempts to improve H<sub>2</sub> production have involved genetic or metabolic engineering approaches. I used a bio-prospecting approach instead to find novel strains that are naturally more apt for biohydrogen production. A set of 36, phylogenetically diverse strains isolated from terrestrial, freshwater and marine environments were probed for their potential to produce H<sub>2</sub> from excess reductant. Two distinct patterns in H<sub>2</sub> production were detected. Strains displaying Pattern 1, as previously known from *Synechocystis* sp. PCC 6803, produced H<sub>2</sub> only temporarily, reverting to H<sub>2</sub> consumption within a short time and after reaching only moderately high H<sub>2</sub> concentrations. By contrast, Pattern 2 cyanobacteria, in the genera *Lyngbya* and *Microcoleus*, displayed high production rates, did not reverse the direction of the reaction and reached much higher steady-state H<sub>2</sub> concentrations. *L. aestuarii* BL J, an isolate from marine intertidal mats, had the fastest production rates and reached the highest steady-state concentrations, 15-fold higher than that observed in *Synechocystis* sp. PCC 6803. Because all Pattern 2 strains originated in intertidal microbial mats that become anoxic in dark, it was hypothesized that their strong hydrogenogenic capacity may have evolved to aid in fermentation of the photosynthate. When forced to ferment, these cyanobacteria display similarly desirable characteristics of physiological H<sub>2</sub> production. Again, *L. aestuarii* BL J had the fastest specific rates and attained the highest H<sub>2</sub> concentrations during fermentation, which proceeded via a mixed-acid

pathway to yield acetate, ethanol, lactate, H<sub>2</sub>, CO<sub>2</sub> and pyruvate. The genome of *L. aestuarii* BL J was sequenced and bioinformatically compared to other cyanobacterial genomes to ascertain any potential genetic or structural basis for powerful H<sub>2</sub> production. The association *hcp* exclusively in Pattern 2 strains suggests its possible role in increased H<sub>2</sub> production. This study demonstrates the value of bioprospecting approaches to biotechnology, pointing to the strain *L. aestuarii* BL J as a source of useful genetic information or as a potential platform for biohydrogen production.

## ACKNOWLEDGMENTS

This work would have not been possible without the mentoring of Dr Ferran Garcia-Pichel. I owe him my foundation and core values in science. His passion for science and critical thinking has inspired me tremendously. I am very thankful to him for his belief in me all through my PhD. I would like to express my deepest gratitude to my committee members; Dr Willem F. J. Vermaas, Dr Bruce Rittmann and Dr Cesar I. Torres, for their immense support and guidance in helping me shape my project. I am also grateful to Dr Marty Wojciechowski and Dr Anne Jones for their comments and guidance on my project.

I would like to thank all the past and present members of the Garcia-Pichel lab, who have played an important part in my scientific development, as peers, mentors and friends. I would like to specially thank Ipsita Dutta, my friend and part of the Biohydrogen project, who has been extremely helpful throughout the project. I would like to thank people associated with the Biohydrogen project, Cosmin Sicora, Doerte Hoffmann, Daniela Ferreira, Prathap Parmeswaran, Michael Vaughn and Juan Maldonado with whom I could discuss specific aspects of my project. I am also thankful to my labmates, Estelle Couradeau, Brandon Guida, Ana Giraldo, Ruth Potrafka, Yevgeniy Maruseko, Natalie Myers and Edgardo Ramirez who have been of great help with my project and also made the work atmosphere a lot of fun. I would like to thank Yvonne Delgado and Wendi Simonson for administrative help. And to all those that I have failed to mention, who made the many years of graduate school a gratifying journey, thank you!

I am grateful to Brian Swette and his family for supporting this research through the ASU President's Fusion Fund. I would also like to thank Dr Garcia-Pichel, Dr Rittmann and School of Life Sciences for financial support.

Above all, I am thankful to my father, Dr Sushil Kumar Kothari, whose hardwork and dedication to science continues to inspire me, my mother, Shashi Kothari, and sister, Snehita Kothari, for their immense love and support. I am thankful to all my friends who have been my pillar of support through my PhD, especially Nilotpal Chakravarty, Sriya Sanyal, Mayur Agarwal and Arpan Deb.

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## ABBREVIATIONS

|             |                                                                                                  |
|-------------|--------------------------------------------------------------------------------------------------|
| ATCC        | American Type Culture Collection                                                                 |
| DDBJ        | DNA Database of Japan                                                                            |
| EMBL        | European Molecular Biology Laboratory                                                            |
| $[H_2]_M$   | Maximal steady-state hydrogen concentration                                                      |
| <i>hcp</i>  | Gene coding for hybrid cluster protein                                                           |
| <i>hoxH</i> | Gene coding for the large hydrogenase subunit of the cyanobacterial<br>bidirectional hydrogenase |
| FAD         | Flavin Adenine Dinucleotide                                                                      |
| $K_M$       | Michaelis constant                                                                               |
| MEC         | Microbial Electrolysis Cells                                                                     |
| NAD(P)      | Nicotinamide Adenine Dinucleotide (Phosphate)                                                    |
| PCC         | Pasteur Culture Collection                                                                       |
| $R_H$       | Initial maximal rate of hydrogen production                                                      |
| $T_R$       | Time after which hydrogenase reverts in direction from H <sub>2</sub> consumption to<br>uptake   |

## I. INTRODUCTION

### **1. Biological Hydrogen Production**

Hydrogen ( $H_2$ ) offers great potential as a versatile energy carrier (i.e., a substance that stores energy which can be released later by means of a chemical reaction).  $H_2$  is a viable alternative to conventional carbon based fossil fuels, because on combustion with oxygen ( $O_2$ ) it releases energy and water, thus avoiding release of green house gases such as carbon dioxide ( $CO_2$ ). Compressed  $H_2$  has very high specific energy (i.e., energy released per unit mass upon combustion with  $O_2$ ). On a mass basis, aside from fissile uranium (specific energy = 83,140,000 MJ/kg),  $H_2$  (specific energy = 123 MJ/kg) is the most energy dense fuel available, followed by gasoline and propane (specific energy = 46 MJ/kg).

Apart from its use as an energy carrier,  $H_2$  also has application in industries where it is used as a chemical reactant for hydrogenation of products, wherein hydrogen atoms are inserted to saturate a molecule or to cleave a molecule (Ramachandran and Menon, 1998). The process of hydrogenation is employed in the production of petrochemicals (e.g., methanol is produced by the reaction of carbon monoxide ( $CO$ ) and  $H_2$ ), ammonia-based fertilizers (e.g.,  $H_2$  reacts with nitrogen ( $N_2$ ) making ammonia ( $NH_3$ )) and oil/fat hydrogenation (decreasing the degree of unsaturation in fats and oils).  $H_2$  is employed as an  $O_2$  scavenger in certain metallurgic processes, to prevent corrosion of metals by oxidation (Ramachandran and Menon, 1998). It also has application in fuel cells (a cell

producing an electric current directly from a chemical reaction) wherein H<sub>2</sub> and O<sub>2</sub> are combined to produce electricity, heat, and water.

### **1.1 Methods for hydrogen production**

Hydrogen production methods are broadly classified into three major categories depending on the nature of the process and/or energy input (Chaubey et al., 2013).

#### **1.1.1 Electrochemical**

In this process the production of H<sub>2</sub> occurs from water by the passage of an electric current resulting in a chemical reaction along with the absorption or liberation of heat.

This offers the potential to be a clean technology (no carbon-based emissions); however, it has the major drawback of being financially expensive (Armor, 1999).

#### **1.1.2 Thermochemical**

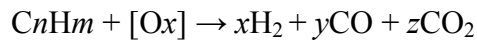
In this process the degradation of the feedstock (i.e., the hydrocarbon raw material) requires thermal energy inputs for driving chemical reactions for the separation of H<sub>2</sub>.

This method involves reaction of the feedstock in oxidative (in presence of O<sub>2</sub>/air/steam) or non-oxidative (using sources of energy like heat/plasma/radiation) processes to yield H<sub>2</sub> (Muradov, 2009).

##### ***1.1.2.1 Oxidative process***

In this process the degradation of feedstock occurs in the presence of oxidants such as O<sub>2</sub>/air/steam at high temperatures (>700 °C). The oxidative processes include established

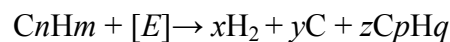
industrial methods of H<sub>2</sub> generation such as steam reformation (reaction of a methane (CH<sub>4</sub>) containing gas mixture, such as natural gas or biogas, with steam to produce H<sub>2</sub> and CO<sub>2</sub>), and partial oxidation (incomplete combustion of a hydrocarbon generating H<sub>2</sub> and CO). The oxidative process for H<sub>2</sub> production can be represented by the equation below.



where  $C_nH_m$  is a hydrocarbon ( $n \geq 1, m \geq n$ ) and  $[Ox]$  is an oxidant such as O<sub>2</sub>, air or steam (Chaubey et al., 2013).

#### ***1.1.2.2 Non-oxidative process***

In this process the degradation of feedstock occurs by direct splitting of C–H bond in presence of the energy sources like heat, plasma or radiation. The non-oxidative process for H<sub>2</sub> production can be represented by the equation below.



where  $C_nH_m$  is the hydrocarbon feedstock ( $n \geq 1, m \geq n$ ),  $[E]$  is the input energy and  $C_pH_q$  represents relatively stable products of the feedstock cracking ( $z \geq 0, p \geq 1, q \geq p$ ; in most cases  $C_pH_q$  is CH<sub>4</sub> or C<sub>2</sub>H<sub>2</sub>) (Muradov, 2009).

#### **1.1.3 Biological**

This avenue involves the use of microbes for H<sub>2</sub> production and shall be discussed in further detail later. The major advantages of this avenue are that it relies on the usage of



renewable energy sources and is less energy intensive (most processes are conducted at moderate temperatures and pressures)(Das and Veziroglu, 2001).

Of the three methods, only the thermochemical method is currently employed by industries for large scale H<sub>2</sub> production. Although extremely popular, the major drawback associated with the thermochemical method is the release of carbon-based emissions. Large-scale H<sub>2</sub> production by the electrochemical method is limited by the high operating costs. Both thermochemical and electrochemical methods are energy intensive processes.

Consequently, to address these shortcomings, efforts have been directed towards biological means of producing H<sub>2</sub>.

## **1.2 Methods for biological hydrogen production**

Biological H<sub>2</sub> production (Biohydrogen) involves the use of microorganisms (such as algae and bacteria). The various avenues to produce biohydrogen are described in detail in the following sections.

### **1.2.1 Microbial electrolysis cells**

The microbial electrolysis cells (MECs) combine electrochemistry with bacterial metabolism to generate H<sub>2</sub> (Liu et al., 2005). The MEC consists of anode-respiring bacteria (of the genera *Geobacter*, *Shewanella*, *Pseudomonas*, *Clostridium*, *Desulfuromonas*, *Escherichia*, and *Klebsiella*) on the anode of an electrochemical cell. The anode-respiring bacteria oxidize organic compounds like acetate, ethanol, lactate, butyrate, or propionate transferring electrons to the anode, which then reaches the

cathode. At the cathode, the electrons react with water producing H<sub>2</sub>(Reguera et al., 2005; Bond and Lovley, 2003; Kim et al., 2002; Gorby et al., 2006; Pham et al., 2008; Qiao et al., 2008). Since the final H<sub>2</sub> generating step is not catalyzed by a microbial enzyme, this avenue is inherently different from the other avenues to produce biohydrogen.

The high H<sub>2</sub> yields (moles of H<sub>2</sub> produced per mole of substrate, usually glucose) is the biggest advantage of using MEC (Lee et al., 2010). The H<sub>2</sub> yield ranges from 67% to 91% from various donor substrates (e.g., cellulose, glucose, butyrate, lactate, propionate, ethanol or acetate) (Segura et al., 2008; Call and Logan, 2008). Fermentative-product-rich effluent systems from food and beverage industries can be coupled to MECs decreasing the input costs (Angenent et al., 2004; Oh and Logan, 2005; Wenet et al., 2009).

One of the challenges associated with MECs is the low rate of H<sub>2</sub> production (Lee et al., 2010). The highest reported H<sub>2</sub> production rate in MECs is 0.13 l H<sub>2</sub> l<sup>-1</sup>h<sup>-1</sup> at an applied voltage 0.8 V (Call and Logan, 2008) that is lower than that seen in dark fermentation (7.9 l H<sub>2</sub> l<sup>-1</sup>h<sup>-1</sup>) (Lo et al., 2009). Another challenge associated with MECs is the requirement of external voltage. An external voltage needs to be applied to the MEC so the electrons reaching the cathode have sufficient energy to reduce H<sub>2</sub>O to H<sub>2</sub>.

Additionally, the decomposition of acetate (commonly used substrate in MECs) is not spontaneous under standard conditions and often requires 0.6–1.2 V of actual applied voltage (Tartakovsky et al., 2009; Call et al., 2009). Additional energy losses may also occur in the MEC (Lee et al., 2010). To achieve an energy benefit out of an MEC the applied voltage must be less than 0.6 V (Lee and Rittmann, 2010). If the amount of

applied voltage is too high, the energy value of the produced H<sub>2</sub> will be lower than the energy value of the input for its production (Lee et al., 2010). Thus, to effectively produce H<sub>2</sub> via MECs, research needs to be directed towards developing reactor designs and materials minimizing energy losses throughout the MEC (Lee et al., 2010).

### **1.2.2 Nitrogenase-mediated**

The nitrogenase enzyme is capable of evolving H<sub>2</sub> while fixing atmospheric N<sub>2</sub> (Phelps and Wilson, 1941; D'Eustachio and Hardy, 1964). Nitrogenase can be used as means to produce biohydrogen in certain strains of cyanobacteria and purple non-sulfur bacteria.

#### **a. *Cyanobacteria***

The nitrogenase harboring cyanobacterial strains can fix atmospheric N<sub>2</sub> into ammonium releasing H<sub>2</sub> as a byproduct. Since the nitrogenases are O<sub>2</sub> sensitive, this approach requires temporal or spatial separation of the O<sub>2</sub> and H<sub>2</sub> production processes. The temporal separation involves N<sub>2</sub> fixation in the nighttime whereas the spatial separation involves formation of specialized cells known as heterocysts where microaerobic conditions are maintained. Although most N<sub>2</sub>-fixing strains are not known to produce H<sub>2</sub> at significant rates (Kumar and Kumar, 1992), recent work on *Cyanothece* sp. ATCC 51142 displayed H<sub>2</sub> production rates of 150 nmol H<sub>2</sub> (mg Chl *a*)<sup>-1</sup> h<sup>-1</sup> in aerobic and 373 nmol H<sub>2</sub> (mg Chl *a*)<sup>-1</sup> h<sup>-1</sup> in anaerobic (sparged with argon) conditions (Bandyopadhyay et al., 2010).

Several factors appear to limit nitrogenase dependent H<sub>2</sub> production, including O<sub>2</sub> sensitivity, low turnover number (amount of H<sub>2</sub> produced by the enzyme per unit time), a requirement for ATP, as well as the additional energy costs involved in heterocyst differentiation and maintenance. Cyanobacterial nitrogenases shall be discussed in greater detail in section 2.2.1.

**b. Purple non-sulfur bacteria**

Purple non-sulfur bacteria can decompose organic acids by using light energy and nitrogenase in a process known as “photofermentation”. Since they do not evolve O<sub>2</sub>, the O<sub>2</sub>-sensitivity of nitrogenase is not an issue. Few examples of purple non-sulfur bacteria known to produce H<sub>2</sub> via photofermentation are *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sulidophilus*, *Rhodopseudomonas capsulata*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, *Chlorobium limicola*, *Thiocapsa roseopersicina*, *Halobacterium halobium* and *Chromatium* sp. Miami PSB 1071 (Das and Veziroglu, 2001; Tao et al., 2007).

The major disadvantages associated with photofermentation are the high energy requirements of the nitrogenase and the presence of an uptake hydrogenase that consumes majority of the H<sub>2</sub> produced by the nitrogenase (Kars and Gündüz, 2010). As expected, efforts have been directed towards increasing H<sub>2</sub> production by deleting the uptake hydrogenase. The uptake hydrogenase mutantsof seven *Rhodobacter* strains resulted in zero to three-fold increase in the total amount of H<sub>2</sub> produced per litre of culture (Kars and Gündüz, 2010).

### 1.2.3 Dark fermentation

Many bacteria are capable of fermenting exogenous organic substrates in dark anaerobic conditions. They use protons as an electron sink to evolve  $H_2$ , a typical fermentation end product. Dark fermentation involves two main routes for  $H_2$  production. The first route involves the enzymes pyruvate-ferredoxin oxidoreductase, ferredoxin NADP oxidoreductase, and a hydrogenase, whereas the second route involves pyruvate-formate lyase and hydrogen formate lyase. Strict anaerobes (*Clostridium*, *Ethanoligenens* and *Desulfovibrio*) use the first route (Thauer et al., 1977; Jungermann et al., 1973; Petitdemange et al., 1976), whereas the facultative bacteria (*Enterobacter*, *Citrobacter*, *Klebsiella*, *Escherichia coli* and *Bacillus*) primarily use the second route (Axley et al., 1990; Yoshida et al., 2006; Li and Fang, 2007).

A variety of substrates can be metabolized to yield  $H_2$  via dark fermentation. Some pure cultures of *Enterobacter* and *Clostridium* can degrade starch (Taguchi et al., 1996). Several species of *Clostridium* are also capable of degrading xylulose (Taguchi et al., 1996; Taguchi et al., 1995). Certain mixed cultures of fermenting bacteria are also capable of utilizing external complex organic substrates such as cellulose, food wastes, paper wastes, or municipal wastes (Li and Fang, 2007).

The advantage of producing  $H_2$  via dark fermentation is that the rates of  $H_2$  production are orders of magnitude higher than those achieved by other means. For instance, the rates of  $H_2$  production by dark fermentation in *Enterobacter cloacae* DM 11 ( $75.60 \text{ mmol } H_2 \text{ l}^{-1} \text{ h}^{-1}$ )

<sup>1</sup>) and *Clostridium* sp. strain No. 2 (64.50 mmol H<sub>2</sub> l<sup>-1</sup> h<sup>-1</sup>) are higher than that reported by direct biophotolysis in *C. reinhardtii* (0.07 mmol H<sub>2</sub> l<sup>-1</sup> h<sup>-1</sup>), cyanobacterial nitrogenase in *Anabaena variabilis* (0.36 mmol H<sub>2</sub> l<sup>-1</sup> h<sup>-1</sup>) and photofermentation in *Rhodobacter spheroides* (0.16 mmol H<sub>2</sub> l<sup>-1</sup> h<sup>-1</sup>) (Kotay and Das, 2008). Also dark-fermentation-based bioreactors have the advantage of relatively simple construction and low energy input demands.

The major challenge associated with fermentative biohydrogen production is the low H<sub>2</sub> yields (moles of H<sub>2</sub> produced per mole of substrate). If there were 100% conversion of the electron equivalents of glucose to H<sub>2</sub>, it should produce about 12 moles of H<sub>2</sub> per mole of glucose.



However, when fermented, the maximum theoretical H<sub>2</sub> yield from glucose is four moles when the only other products are acetate and CO<sub>2</sub> (Logan, 2004; Thauer et al., 1977).



In fact, it is also difficult to achieve the maximum theoretical H<sub>2</sub> yield (four moles of H<sub>2</sub> per glucose), given that fermentation also results in formation of other products (such as butyrate, propionate, ethanol and lactate), which act as competing electron sinks (Lee et al., 2009; Li and Fang, 2007; Fang et al., 2002; Fang et al., 2006; Chang et al., 2006). The

production of these additional products of fermentation lowers the observed maximum to about 1-2 moles of H<sub>2</sub> per mole of glucose (Fang et al., 2002;2006;Li and Fang, 2007;Lee et al., 2008; 2009;Lee and Rittmann, 2009).

#### **1.2.4 Direct biophotolysis**

Algae and cyanobacteria perform oxygenic photosynthesis wherein the light reaction generates reducing equivalents (NAD(P) and ferredoxin). These reducing equivalents can be employed to produce H<sub>2</sub> via the hydrogen-generating enzymes (hydrogenase). Since this process does not involve carbon fixation and its subsequent breakdown, for the supply of reducing equivalents, it is referred to as direct biophotolysis. This method is highly desirable because it offers direct coupling of light energy to the generation of H<sub>2</sub>.

In cyanobacterium *Synechocystis* sp. PCC 6803 and eukaryotic alga *Chlamydomonas reinhardtii* adapted to dark anaerobic conditions, a brief period of H<sub>2</sub> production (photohydrogen) was observed when illuminated. The photohydrogen production lasted only a few seconds and was followed by H<sub>2</sub> uptake in both strains (Cournac et al., 2002). This direct photolysis is strongly limited by the O<sub>2</sub> sensitivity of the hydrogenase enzymes (Gutthann et al., 2007). Thus, there is need to improve the O<sub>2</sub> tolerance of the hydrogenases to couple H<sub>2</sub> production directly to oxygenic photosynthesis for any significant photohydrogen production. Although the concept of direct biophotolytic processes appears to be inherently attractive, it has the practical drawbacks of transitory H<sub>2</sub> production and O<sub>2</sub> sensitivity of the process (given lack of temporal/spatial separation

of H<sub>2</sub> and O<sub>2</sub> generating processes) (Hallenbeck and Benemann, 2002). This avenue shall be discussed in greater detail in section 3.2.8.2.1.

In *Chlamydomonas reinhardtii*, selection pressure (ability to survive by utilizing H<sub>2</sub> and/or ability to evolve H<sub>2</sub> in presence of O<sub>2</sub>) was applied to select for O<sub>2</sub> tolerant hydrogenase harboring mutants (Ghirardi et al., 1997; Seibert et al., 2001). However, most of the selected mutants displayed only an increased respiratory activity, thus providing just an appearance of enhanced O<sub>2</sub> resistance (Hallenbeck and Benemann, 2002).

Simultaneous production of H<sub>2</sub> and O<sub>2</sub> has also been exhibited while using inert gas sparging to reduce the O<sub>2</sub> concentrations (Lindblad et al., 2002). O<sub>2</sub> absorbers such as hemoglobin A (Rosenkrans and Krasna, 1984) or sodium dithionite reduced methylviologen, or glucose, glucose oxidase and catalase (Gutthann et al., 2007) can be employed to scavenge O<sub>2</sub>. However, it is not practical to use argon sparging or O<sub>2</sub> absorbers in scaled-up long-term processes given the high operating costs involved.

In eukaryotic algae, sulfur deprivation can be employed to deal with the problem of O<sub>2</sub> sensitivity of the direct-photolysis-mediated H<sub>2</sub> production process (Hallenbeck and Benemann, 2002). When deprived of inorganic sulfur, the rates of O<sub>2</sub> synthesis and CO<sub>2</sub> fixation in *Chlamydomonas reinhardtii* decrease drastically within 24 h (in the light). This decrease occurs because sulfur depletion stops the synthesis of the D1 polypeptide chain (comprising sulfur-containing amino acids, such as cysteine and methionine),



which needs to be frequently replaced in the PSII reaction centre. With time, the photosynthetic capability decreases whereas the respiration remains unaffected, leading to establishment of anaerobic conditions and H<sub>2</sub> production (Ghirardi et al., 2000; Melis et al., 2000). When deprived of sulfur, cells can only grow photoheterotrophically, in presence of acetate in the media (due to reduced carbon fixation). Sulfur deprivation of algae is a very promising avenue that displays very high rates of H<sub>2</sub> production (200 nmol H<sub>2</sub>(mg Chl *a*)<sup>-1</sup> h<sup>-1</sup> in *C. reinhardtii* and about 150 nmol H<sub>2</sub>(mg Chl *a*)<sup>-1</sup> h<sup>-1</sup> in *Scenedesmus obliquus* and *S. vacuolatus*)(Winkler et al., 2002).

In any production system the one critical factor for low cost generation of H<sub>2</sub> is the light utilization efficiency of the microorganism (Polle et al., 2002). In a dense microalgal bioreactor, the algal cells near the illuminated surface capture bulk of the incident light and dissipate the excess. As a result, the cells deeper in the bioreactor face light limitation and acclimatize to low light conditions. These light-limited cells accumulate a large number of chlorophyll molecules and have a large chlorophyll antenna in association with their photosystems. Therefore, even in a mixed bioreactor system at high levels of light, about 80–95% of the absorbed photons are dissipated by the large light-harvesting antenna complexes (LHCs) proteins as heat and fluorescence, via photoprotective mechanisms (Polle et al., 2002). The light energy that cannot be dissipated causes photo-damage to the cells. To effectively distribute light, the algal cell density in the bioreactor needs to remain low, making eukaryotic algae inefficient for H<sub>2</sub> production in large bioreactors. Efforts have been directed towards silencing the LHC protein isoforms resulting in 290% higher light transmittance in the culture. However, these mutants were

not able to reach a high cell density, an unfavourable trait for application in bioreactors (Borowitzka and Moheimani, 2013; Mussgnug et al 2007). Thus, the low photon-to- $H_2$  conversion efficiency presents a major challenge associated with this process (Hankamer et al., 2007).

### **1.2.5 Indirect biophotolysis**

The indirect biophotolysis processes involve separation of the  $H_2$  and  $O_2$  evolution reactions, temporally, coupled with  $CO_2$  fixation (Hallenbeck and Benemann, 2002). Indirect biophotolysis occurs in two stages. The first stage involves the photosynthesis and accumulation of organic compounds along with  $O_2$  evolution. The second stage involves fermentative degradation of the stored organic compounds releasing  $H_2$  in dark anaerobic conditions (Melis and Melnicki, 2006). Predominantly, the endogenic organic compound accumulated during photosynthesis is starch in eukaryotic algae and glycogen in cyanobacteria. Using a temporal separation, these substrates can be fermented in algae (Ueno et al., 1998; Gfeller and Gibbs, 1984; Miura et al., 1986; Klein and Betz, 1978) and cyanobacteria (Stal and Moezelaar, 1997) producing  $H_2$  as one of the products of fermentation. As described earlier, the enzymatic pathway for  $H_2$  production involves two main routes, either via the pyruvate-ferredoxin oxidoreductase, ferredoxin-NADP oxidoreductase and hydrogenase route or the pyruvate-formate lyase and hydrogen formate lyase route (Ueno et al., 1998; Stal and Moezelaar, 1997).

This method is attractive because it does not require input of exogenous carbon substrates and the temporal separation solves the problem of  $O_2$  sensitivity of the hydrogen-

generating enzymes. In fact, *Synechocystis* sp. PCC 6803 displayed much higher rates and concentrations of H<sub>2</sub> via the indirect biophotolysis than with direct biophotolysis (Cournac et al., 2002). The major challenge associated with the indirect biophotolysis process is the low H<sub>2</sub> yield. The observed H<sub>2</sub> yields (0.5-2 moles of H<sub>2</sub> per mole of glucose) (Troshina et al., 2002; Moezelaar and Stal, 1994; Oost et al., 1989) are much lower than the theoretical maximum (4 moles of H<sub>2</sub> per mole of glucose).

### **1.3 Promising methods: Rationale for choosing suitable method**

It is difficult to adequately compare the H<sub>2</sub> produced via the various methods described above given the differences in the experimental set ups, the parameters being measured, and the units employed for measuring the parameters. In spite of the efforts geared towards developing biological platforms for H<sub>2</sub> production, so far none have reached commercial large-scale stages. All the methodologies discussed come with their own set of advantages and challenges (Table 1). Based on the H<sub>2</sub> yield per unit glucose, the MEC is highly desirable. However, it is difficult to compare this method with others due to the difference in the parameters used to quantify the H<sub>2</sub> production. Efforts have also been directed towards developing a 2-stage process involving dark fermentation (releasing organic acids, H<sub>2</sub> and/or CO<sub>2</sub>) followed by photofermentation (organic acids used as substrate for further H<sub>2</sub> production) to achieve high yields of H<sub>2</sub>. Using this method, H<sub>2</sub> yields as high as seven moles of H<sub>2</sub> per mole of glucose have been attained (Chen et al., 2008).

The cyanobacteria- and algae-based methods tap the atmospheric CO<sub>2</sub>, whereas the fermentative heterotrophic bacteria and purple non-sulfur-bacteria-based methods use organic acids (byproduct of several industries) for H<sub>2</sub> production. Hence both sets of processes are desirable in their own ways. Amongst the cyanobacteria- and algae-based methods, N<sub>2</sub> fixation (specifically using *Cyanothece* sp. ATCC 51142) and sulfur deprivation are highly lucrative given the high rates and concentrations of H<sub>2</sub> produced. In comparison, the rates and H<sub>2</sub> concentrations reported for indirect biophotolysis using cyanobacteria are lower than that reported by the N<sub>2</sub> fixation and sulfur deprivation methods. Of all the methods, the lowest rates and H<sub>2</sub> concentrations are associated with direct photolysis (photohydrogen).

This dissertation involves assessing the unexplored biodiversity in cyanobacterial strains for their potential and actual (indirect biophotolysis) capacities of H<sub>2</sub> production. This work is based on cyanobacteria because

1. They do not require external organic sources of carbon (unlike MECs, photofermentations and dark fermentations)
2. They have smaller LHCs thus minimizing light energy losses. They host [NiFe]-hydrogenases that are less sensitive to O<sub>2</sub> than the [FeFe]-hydrogenases (unlike eukaryotic algae based methods).

Among cyanobacterial H<sub>2</sub> producing enzymes, this work is based on bidirectional hydrogenase rather than the nitrogenase, given energy intensive nature of the nitrogenase-mediated processes. This study focuses on the fermentation capacities (via indirect

biophotolysis) rather than the photohydrogen production capacity (via direct biophotolysis) given the low rates,  $H_2$  concentrations, and transitory nature of  $H_2$  produced by the latter process. The rationale for selecting the cyanobacterial indirect biophotolysis method is summarized in Figure 1.

## **2. Cyanobacterial Hydrogen Production**

Cyanobacteria (blue-green algae) are photolithoautotrophic bacteria that harvest energy from the sun to split water for electrons (producing O<sub>2</sub> as a byproduct), thus fixing the atmospheric CO<sub>2</sub>. Cyanobacteria constitute the most phylogenetically diverse and widely distributed group of prokaryotes capable of oxygenic photosynthesis. Cyanobacterial photosynthesis was crucial in the initial formation of atmospheric O<sub>2</sub>, a fundamental step in the creation of the Earth's biosphere (Berman-Frank et al., 2003; Tomitani et al., 2006). Additionally, cyanobacteria were also central in the evolution of novel organisms via an endosymbiotic event leading to the origin of chloroplasts (McFadden, 2001; Bergman et al., 2008). The unique features of cyanobacteria in terms of their ability to perform oxygenic/anoxygenic photosynthesis, CO<sub>2</sub> (and sometimes N<sub>2</sub>) fixation and stress adaptations have allowed them to succeed both evolutionarily and ecologically. They have been regarded as important players in biological soil crusts, fresh water lakes, oceans, hot springs as well as microbial mats.

### **2.1 Cyanobacteria as model for hydrogen production**

Cyanobacteria are extremely promising microorganisms for biohydrogen production because they have minimum nutritional requirements- air, water, mineral salts and sunlight (Hansel and Lindblad, 1998). Certain strains also have the ability to tap the atmospheric N<sub>2</sub> for growth. They are simple model organisms from evolutionary and compartmentalization perspectives. Some strains of cyanobacteria can be genetically modified with ease. They are endowed with an ability to use the photosynthetically generated reductants (directly or indirectly) for making hydrogen. H<sub>2</sub> constitutes an

important intermediate in the world of these microorganisms. Cyanobacteria have three major enzymes directly associated with H<sub>2</sub> metabolism: nitrogenase, uptake hydrogenase and bidirectional hydrogenase (Figure 2).

## **2.2 Cyanobacterial enzymes involved in hydrogen metabolism**

### **2.2.1 Nitrogenase**

The capacity to fix atmospheric N<sub>2</sub> is found in certain microorganisms within bacteria and archaea, but not in the eukaryote lineage (Burris, 1991; Zehr et al., 1998; Berman-Frank et al., 2003). The nitrogenase produces H<sub>2</sub>, obligatorily, as a byproduct of its main reaction during the assimilatory reduction of N<sub>2</sub>.



The nitrogenase complex consists of two protein components encoded by the *nif* (nitrogen fixation) genes: 1) the dinitrogenase (MoFe protein) and 2) the dinitrogenase reductase (Fe protein). The dinitrogenase reductase transfers electrons of very low redox potential to dinitrogenase. The reduction of N<sub>2</sub> is catalyzed by the dinitrogenase. The dinitrogenase reductase is a homodimer (60-80kDa) composed of two identical subunits (NifH) containing one central [4Fe4S]-cluster and two Mg-ATP binding sites, one on each subunit. Its function is to lower the redox potential of electrons derived from ferredoxin or flavodoxin, in an ATP dependent reaction, and to donate these electrons to dinitrogenase. The dinitrogenase is a heterotetramer (250 kDa) composed of NifD and NifK polypeptides. The P-cluster ([8Fe7S]) functions as a channel for electron transfer,

accepting electrons from the [4Fe4S]-cluster of the Fe protein and donating them to the MoFe cofactor, the site of substrate reduction (Burgess and Lowe, 1996; Berman-Frank et al., 2003; Igarashi and Seefeldt, 2003).

In addition to this “conventional” Mo-containing dinitrogenase, some diazotrophs (nitrogen fixers) synthesize alternative dinitrogenases that contain vanadium (V) or iron (Fe) (Eady, 1996). The three nitrogenase systems are encoded by distinct gene clusters: *nifHDK* coding for the Mo-, *vnfH/vnfDGK* for the V-, and *anfHDK* for the Fe-only nitrogenase (Bishop and Joerger, 1990; Eady, 1996). The V-nitrogenase is expressed in Mo deprived conditions whereas the Fe-nitrogenase (with a Fe-only cofactor) is expressed when both Mo and V are unavailable. The enzymes have somewhat different reaction kinetics (Burgess and Lowe, 1996; Eady, 1996). All the three nitrogenases are capable of H<sub>2</sub> production (Basak and Das, 2007).

Phylogenetic analyses of the nitrogenase gene sequence along with the physical and chemical characteristics of the nitrogenases, suggest that all the existing enzymes were derived from a single common ancestor that existed prior to the oxygenation of Earth (Broda and Peschek, 1983; Berman-Frank et al., 2003). The genes coding for nitrogenase are found in both archaea and bacteria, but are sporadically distributed within clades in most cases (Young, 1992). Perhaps the nitrogenase genetic information was lost in some strains in the course of adaptation to varied environmental conditions and/or the associated selective evolutionary pressures upon the microorganisms (Fay, 1992). All the cyanobacteria *nifH* genes cluster together, with the heterocyst-forming



cyanobacteria forming a tight cluster within the cyanobacterial group (Zehr et al., 1997; Zehr et al., 2003). Apart from the ‘conventional’ Mo-containing *nifH*, V-containing nitrogenases have also been reported in cyanobacteria (for instance in *Anabaena variabilis*) (Thiel, 1993; Zehr et al., 2003).

The dinitrogenase reductase and dinitrogenase components ([4Fe4S] and P-clusters, respectively) are inactivated by O<sub>2</sub>. However, the toxicity of this gas also involves other forms of reactive O<sub>2</sub> species (ROS), which affect the nitrogenase components (Fay, 1992; Gallon, 1992; Berman-Frank et al., 2003). Cyanobacteria are the only diazotrophs that actually produce O<sub>2</sub> as a by-product of the photosynthesis and, consequently, had to develop different strategies to protect their O<sub>2</sub>-sensitive nitrogenase. These strategies include spatial or temporal separation of the photosynthesis and N<sub>2</sub> fixation processes (Adams, 2000; Gallon, 2001; Berman-Frank et al., 2003). The spatial separation occurs by the formation of specialized cells, known as heterocysts where N<sub>2</sub> fixation occurs. The heterocyst cells are fundamentally different from vegetative cells and are characterized by the lack of PSII (no O<sub>2</sub> produced), a robust cell wall that is impermeable to O<sub>2</sub> and an ability to obtain energy from the adjacent vegetative cells.

As discussed earlier, the cyanobacterial nitrogenase-mediated H<sub>2</sub> production reported in *Cyanothece* sp. ATCC 51142 is a highly promising avenue (Bandyopadhyay et al., 2010). Using a physiological approach focused on production by N<sub>2</sub>-fixing strains, a study (Allahverdiyeva et al., 2010) surveyed a large number of existing isolates from culture collections (from Baltic Sea and Finnish lakes) for H<sub>2</sub> evolution. They found that about

50% of strains produced detectable amounts of H<sub>2</sub>, and about 2% displayed production rates similar to those obtained in standard strains optimized by genetic modification.

An advantage of nitrogenases over hydrogenase-mediated systems is that the H<sub>2</sub> production activity by the nitrogenase enzyme itself is irreversible. There is no evidence of H<sub>2</sub> uptake activity by any known nitrogenase. However, many nitrogen-fixing organisms contain uptake hydrogenases to recover the H<sub>2</sub> produced by the nitrogenase. Strategies to knock out the uptake hydrogenase have worked well in increasing the short-term H<sub>2</sub> evolution via nitrogenase (Lindberg et al., 2002; Yoshino et al., 2006; 2007).

For the production of significant amounts of biohydrogen via nitrogenase, the cells need to be flushed with argon, in order to promote proton reduction and prevent N<sub>2</sub> reduction (Masukawa et al., 2002a, Yoshino et al., 2006; 2007; Bandyopadhyay et al., 2010). Other challenges include the nitrogenase reaction being energetically expensive (ATP dependent H<sub>2</sub> production) and the O<sub>2</sub> sensitivity of the enzyme. Additionally, the turnover number of this enzyme is extremely low (6.4 s<sup>-1</sup>), necessitating the biosynthesis of enormous quantities of the enzyme for H<sub>2</sub> production at a reasonable rate (Hallenbeck and Benemann, 2002).

### **2.2.2 Hydrogenases**

Hydrogenases are metalloenzymes that catalyze the reversible interconversion of protons and H<sub>2</sub>.



The direction of the reaction is determined by the redox potential of the substance interacting with the enzyme. The hydrogenase evolves H<sub>2</sub> in the presence of an electron donor and consumes it in the presence of an electron acceptor and H<sub>2</sub> (Vignais and Billoud, 2007). A characteristic feature of all hydrogenases is that the iron atoms are ligated by small inorganic ligands (carbon monoxide and/or cyanide) (Happe et al., 1997; Pierik et al., 1998; Pierik et al., 1999).

There are three known types of hydrogenase enzymes, categorized by the metal cluster at the core of their active sites: 1. [Fe] hydrogenases (earlier referred to as metal free hydrogenases), known to occur in methanogenic archaea (synonymous with methylenetetrahydromethanopterin dehydrogenases or Hmd); 2. [Fe-Fe] hydrogenases, common in bacteria, algae, and some fungi; and 3. [Ni-Fe] hydrogenases, widespread through bacteria, cyanobacteria, and archaea (Vignais and Billoud, 2007; Heinekey, 2009).

*i. [Fe]-hydrogenases:*

Methylenetetrahydromethanopterin dehydrogenase (Hmd) also known as the [Fe]-hydrogenase is found in many methanogenic archaea growing on H<sub>2</sub> and CO<sub>2</sub>. This enzyme is also referred to as FeS-cluster-free hydrogenase because it lacks the FeS cubane centers, as opposed to the other two hydrogenases (Lyon et al., 2004). The active centre of this hydrogenase consists of a labile light-sensitive cofactor (Shima et al., 2004), with a mononuclear low-spin iron, most probably Fe(II) (Shima et al., 2005). In this structure,

the iron center takes a square pyramidal geometry in which the nitrogen atom of the pyridinol derivative binds apically to the iron and two carbon monoxide (CO) groups with a cystenyl thiolate and an unknown ligand occupying the basal positions (Figure 3) (Tard and Pickett, 2009). The reversible reduction of methenyltetrahydromethanopterin with H<sub>2</sub> to methylenetetrahydromethanopterin and a proton is catalyzed by the O<sub>2</sub>-sensitive [Fe]-hydrogenase (Zirngibl et al., 1990; 1992). This H<sub>2</sub>-based reduction of the methenyl substrate is an intermediary step in the biological conversion of CO to CH<sub>4</sub>. This enzyme is not universal in all methanogenic archaea and its absence in some methanogens is explained by the existence of two other enzymes, a F<sub>420</sub>-reducing [NiFe]-hydrogenase (F<sub>rh</sub>) and a F<sub>420</sub>-dependent methylene-H<sub>4</sub> methanopterin dehydrogenase (Tard and Pickett, 2009). Even though the [Fe]-hydrogenases themselves catalyze a bidirectional reaction, only H<sub>2</sub> consumption has been reported *in vivo* (Zirngibl et al., 1990; 1992; Afting et al., 2000).

*ii. [FeFe]-hydrogenases:*

The [Fe-Fe]-hydrogenases are common in bacteria, algae, and some fungi. The catalytic active site (also known as H cluster) of the [FeFe]-hydrogenases hosts only iron and sulfur atoms. The H cluster is arranged as a unique [2Fe] center connected by a conserved cysteine to a [4Fe4S] cluster (Figure 3) (Adams, 1990; Nicolet et al., 1999; Peters et al., 1998). In the [FeFe]-hydrogenases, reduced ferredoxin molecules deliver the electrons directly to the H cluster. All the eukaryotic [FeFe]-hydrogenase genes are nucleus-encoded, while the enzyme itself is localized either in the chloroplasts (green algae), the hydrogenosomes (trichomonads, anaerobic ciliates, and chytrid fungi), or in the cytosol

(few protozoan parasites, including *Entamoeba* and *Spironucleus*)(Horner et al., 2002;Ghirardi et al., 2007). In bacteria, the location of the [FeFe]-hydrogenases reflects the enzyme's function (Nicolet et al., 2000). The periplasmic [FeFe]-hydrogenase (DdH) in *Desulfovibrio desulfuricans* is involved in H<sub>2</sub>uptake and the cytoplasmic [FeFe]-hydrogenase I (CpI) in *Clostridium pasteurianum* is involved in H<sub>2</sub> production. The [FeFe]-hydrogenase is mainly monomeric (in the cytoplasm), but dimeric, trimeric, and tetrameric enzymes are also known (in the periplasm) (Nicolet et al., 2000). The [FeFe]-hydrogenases are rapidly and irreversibly inactivated by O<sub>2</sub>(Ghirardi et al., 2007;Stripp et al., 2009;Vincent et al., 2005). The turnover numbers of the [FeFe]-hydrogenases during H<sub>2</sub> evolution corresponds to 6000s<sup>-1</sup>for *C. pasteurianum* and 9000s<sup>-1</sup>for *Desulfovibrio* sp.(Hallenbeck and Benemann, 2002).

### **iii. [NiFe]-hydrogenases:**

The general fold of the hydrogenase enzyme with the [NiFe] bimetallic active site was described by crystallographic studies of the proteins isolated from *Desulfovibrio gigas* (Volbeda et al., 1995; Volbeda et al., 1996a), *Desulfovibrio vulgaris* Miyazaki F (Higuchi et al., 1997; Higuchi et al., 1999), *Desulfovibrio fructosovorans*(Rousset et al., 1998), *Desulfomicrobium norvegium* (formerly *Desulfomicrobium baculatum*) (Garcin et al., 1999), and *Desulfovibrio desulfuricans*(Matias et al., 2001). The NiFe active site is present in the large subunit of the enzyme (Figure 4). Four cysteine residues in the [NiFe]-hydrogenases (or three cysteine plus a selenocysteine residue in the [NiFeSe]-hydrogenase of *D. norvegium*) coordinate the nickel atom. Two of these cysteines also bind the iron atom. The iron possesses three diatomic ligands, namely one carbon

monoxide (CO) group and two cyanide (CN<sup>-</sup>) groups in *D. gigas* and *Allochromatium vinosum* (formerly *Chromatium vinosum*) (Volbeda et al., 1996b; Happe et al., 1997), or as sulfur monoxide, CO, and CN<sup>-</sup> in *D. vulgaris* (Higuchi et al., 1997). The small subunit of the hydrogenase hosts either three [4Fe4S]-clusters; or one [3Fe4S]-cluster and two [4Fe4S]-clusters (Frey, 2002; Shima and Thauer, 2007). The [NiFe] active site is at a distance of ~10 Å from the closest [FeS]-cluster in the small subunit, which is deemed essential for the H<sub>2</sub> activation. This proximity helps the cluster lead the electrons, one at a time, to or from the [NiFe]-centre and transfer them to the distal [4Fe4S]-cluster which is close to the surface of the molecule (Volbeda et al., 1995; Vignais and Colbeau, 2004). Certain hydrophobic channels link the active site to the surface of the molecule, and have been proposed to be involved in the access of gas molecules to the active site (Fontecilla-Camps et al., 1997; Montet et al., 1997; Volbeda et al., 2002). These enzymes are reversibly inactivated by O<sub>2</sub>. The turnover rates of the [NiFe]-hydrogenase in *Desulfovibrio fructosovorans* during H<sub>2</sub> evolution corresponds to 98 s<sup>-1</sup> (Hallenbeck and Benemann, 2002).

### **3. Cyanobacterial Hydrogenases**

All cyanobacterial strains examined so far have either an uptake [NiFe]-hydrogenase, a bidirectional [NiFe]-hydrogenase, or both enzymes (Lindblad et al., 2002; Tamagnini et al., 2007a). Since cyanobacteria are not known to host the [FeFe]-hydrogenases or the [Fe]-hydrogenases, only the [NiFe]-hydrogenases shall be discussed in further detail.

#### **3.1 Cyanobacterial uptake hydrogenase**

As the name suggests, this enzyme only performs the uptake of H<sub>2</sub> *in vivo*. The main physiological function attributed to this enzyme is to catalyze the consumption of the H<sub>2</sub> produced by the nitrogenase (Bothe et al., 1977; Howarth and Codd, 1985; Weisshaar and Boger, 1985). The occurrence of the uptake hydrogenase in cyanobacteria does not correlate with a specific habitat or morphology. It rather correlates with diazotrophy. As an exception, two *Synechococcus* strains (Ludwig et al., 2006; Steunou et al., 2008) and *Cyanothece* sp. PCC 7425 are known to harbor the nitrogenase genes but not the uptake hydrogenase genes.

The uptake hydrogenase is encoded by the *hup* (hydrogen uptake) genes, including *hupL*, coding for the large subunit (60 kDa) hosting the active site and *hupS*, coding for the small subunit (35 kDa) with a role in electron transfer. The uptake hydrogenase structural genes are generally contiguous with the gene encoding the smaller subunit (*hupS*) located upstream from the gene encoding the larger one (*hupL*). The *hupSL* always constitute a transcriptional unit (Happe et al., 2000b; Lindberg et al., 2000; Oliveira et al., 2004; Leitao et al., 2005). The amino acid sequences of uptake hydrogenases are highly conserved in

cyanobacteria, ranging from 93–99.7% similarity (Tamagnini et al., 2002). The position and orientation of the uptake hydrogenase specific endopeptidase coding *hupW* in the cyanobacterial chromosome is rather variable, however. In several cases they are located near the corresponding hydrogenase structural genes (Tamagnini et al., 2007a). In the unicellular N<sub>2</sub>-fixing *Gloeotheca* sp. ATCC 27152, *hupW* is the ORF immediately downstream of *hupL*, and is cotranscribed with *hupSL* (Oliveira et al., 2004). However, in the heterocystous strains *Nostoc* sp. PCC 7120 and *N. punctiforme*, *hupW* is not part of any known hydrogenase cluster, and it was shown to be transcribed under N<sub>2</sub>- and non-N<sub>2</sub>-fixing conditions in both organisms, contrasting with the *hupL* transcription (Wunschiers et al., 2003).

In cyanobacteria, a strong correlation exists between the N<sub>2</sub>-fixation and the uptake hydrogenase activity. In the cyanobacterium *Nostoc* sp. PCC 7120, the *hupL* is transcribed only after a photosynthetic vegetative cell differentiates into a N<sub>2</sub>-fixing heterocyst (Carrasco and Golden, 1995). The presence of combined N<sub>2</sub> in the growth medium has been shown to abolish or strongly repress the transcription and the uptake hydrogenase activity in several strains (Axelsson et al., 1999; Happe et al., 2000a; Hansel et al., 2001; Oliveira et al., 2004). In terms of the expression patterns of the uptake hydrogenase, there is an obvious light/dark regulation with the highest levels of N<sub>2</sub>-fixation and H<sub>2</sub> uptake activity occurring during the dark period in *Lyngbya majuscula* and *Gloeotheca* sp. ATCC 27152 (Reade et al., 1999; Lundgren et al., 2003; Oliveira et al., 2004; Leitao et al., 2005). The transcript levels of *hupSL* and uptake activity were positively influenced by the presence of nickel and H<sub>2</sub> (Houchins and Burris,



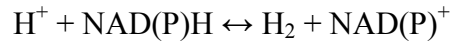
1981d;Oxelfelt et al., 1995;Axelsson and Lindblad, 2002) and negatively influenced by O<sub>2</sub>(Houchins and Burris, 1981d;Axelsson and Lindblad, 2002). On the transfer of *Nostoc muscorum*, from non-N<sub>2</sub>-fixing conditions to N<sub>2</sub>-fixing conditions *hupL* transcripts are induced, followed by an increase of the H<sub>2</sub>-uptake activity (Axelsson et al., 1999;Axelsson and Lindblad, 2002). The relationship between uptake activity and N<sub>2</sub>fixation is not very strong in certain strains such as *L. majuscula* (Leitao et al., 2005) and *A. variabilis*(Boison et al., 2000;Troshina et al., 1996) where residual H<sub>2</sub> uptake activity could be discerned even in presence of combined sources of nitrogen in growth media.

The uptake hydrogenase is not capable of H<sub>2</sub>production in physiological conditions; in fact, it has to be knocked out to attain considerable H<sub>2</sub>production via the nitrogenase (Yoshino et al., 2007;Lindberg et al., 2002;Lindblad et al., 2002;Masukawa et al., 2002a) and also via the fermentation pathway (Kim et al., 2006;Zhao et al., 2009).

### **3.2 Cyanobacterial bidirectional hydrogenase**

Thebidirectional [NiFe]-hydrogenase couples proton/hydrogen interconversion at the [NiFe] active site with the interconversion of its redox partner (NAD(P)<sup>+</sup>/ NAD(P)H). This enzyme is capable of catalyzing both H<sub>2</sub> uptake and evolution under physiological conditions (Tamagnini et al., 2000; 2002). The net reaction is represented below:





The reversible [Ni-Fe] hydrogenase in cyanobacterium *Synechocystis* sp. PCC 6803 can utilize NADPH or NADH along with protons and electrons as substrates to produce H<sub>2</sub>. Whereas most previously characterized bacterial [NiFe]-hydrogenases seem to be preferential H<sub>2</sub>oxidizing catalysts, the cyanobacterial enzyme works effectively in both directions and is biased towards proton reduction as opposed to H<sub>2</sub>oxidation (Carrieri, 2011).

### 3.2.1 Distribution

The bidirectional hydrogenase gene (*hox*) is present in N<sub>2</sub>-fixing and non-N<sub>2</sub>-fixing strains. The presence of a bidirectional hydrogenase has been reported in approximately half of the N<sub>2</sub>-fixing cyanobacteria and in almost all of the non-N<sub>2</sub>-fixing strains with no obvious connection to different habitats or strain morphology (Tamagnini et al., 2000;2002;Ludwig et al., 2006). Some authors suggested that bidirectional hydrogenases are common where anaerobic or micro-aerobic conditions are ecologically relevant and consequently *hox* genes are highly unlikely to be found at high frequency in open ocean waters (Ludwig et al., 2006). Another study revealed a clear increase in abundance of *hoxH* in freshwater lakes, ponds, marine coastal waters, and microbial mats in comparison to the open oceans (Barz et al., 2010). The authors link the increased abundance of *hoxH* to micro-oxic or anaerobic conditions in those environments, which favors H<sub>2</sub>production (Barz et al., 2010). Chapter II reports the presence of *hoxH* in

cyanobacterial strains belonging to both fresh water and marine microbial mats but not in those isolated from the terrestrial environments.

### **3.2.2 Phylogeny**

A *Chloroflexus*-like bacterium is hypothesized to be the closest ancestor of both the cyanobacterial (uptake and bidirectional) hydrogenase clusters. Vertical transmission of both the [NiFe]-hydrogenase genes from the *Chloroflexus*-like bacterium to the different cyanobacterial species was followed by loss of either none, one, or both of the enzymes (as per evolutionary pressure), thus producing the current distribution (Ludwig et al., 2006). Phylogenetic analysis of hydrogenases shows that the *hup* and *hox* hydrogenases form two well-defined clusters (Vignais et al., 2001).

### **3.2.3 Physiological role**

The exact physiological role of the bidirectional hydrogenase has not been successfully elucidated yet. It is proposed to function as an electron valve. This mechanism may occur either during photoautotrophic growth (briefly) to avoid the accumulation of electrons in the photosynthetic electron transport chain during dark-to-light transition states (Appel and Schulz, 1996b; Appel et al., 2000; Cournac et al., 2002; Cournac et al., 2004) or during fermentation to regenerate the NAD(P)<sup>+</sup> (Stal and Moezelaar, 1997; Troshina et al., 2002). Thus, the bidirectional hydrogenase in cyanobacteria primarily functions as a redox regulator for maintaining a proper oxidation/reduction state in the cell (Carrieri, 2011).

### 3.2.4 Cellular localization

The bidirectional hydrogenase enzyme appears in the soluble fraction after cell disruption and consequently has been considered to be soluble in *Nostoc* sp. PCC 7120 and *Anabaena cylindrica* (Houchins and Burris, 1981c). However, investigations in other cyanobacteria - *Anabaena variabilis*, *Synechocystis* sp. PCC 6803, and *Synechococcus elongatus* PCC 6301 (= *Anacystis nidulans*) suggest a weak association of the enzyme with the cell membranes (Kentemich et al., 1989; Appel et al., 2000).

### 3.2.5 Genes involved and enzyme structure

The Hox(Hydrogen oxidation) enzyme has five subunits namely HoxEFUYH. HoxUYH constitutes the hydrogenase moiety whereas HoxEF constitutes the diaphorase moiety. The hydrogenase interacts with protons, electrons, and hydrogen. The diaphorase primarily interacts with NAD(P)<sup>+</sup> and NAD(P)H (Schmitz et al., 1995; Appel and Schulz, 1996a; Boison et al., 1996; 1998; Schmitz et al., 2002; Sheremetieva et al., 2002). The genes coding for the bidirectional hydrogenase (*hoxEFUYH*) are often grouped together as in the strain *Microcoleus chthonoplastes* PCC 7420, but a few other ORFs are interspersed in the cluster of *Synechocystis* sp. PCC 6803 (Schmitz et al., 1995). In *Synechococcus elongatus* and in *Anabaena* sp. PCC 7120 (Boison et al., 1998; Kaneko et al., 2001) the two clusters, *hoxEF* and *hoxUYH*, are separated by several kb. In the *Lyngbya* strains CCAP 1446/4 and PCC 8106 the *hoxEF* and *hoxUYH* clusters are separated by a single gene coding for *hcp* (encoding a putative hybrid cluster protein) (Ferreira, 2009). All the completely sequenced cyanobacterial strains that harbor the bidirectional hydrogenase genes also harbor the gene of a pyruvate:flavodoxin/ferredoxin

oxidoreductase (PFOR), which in fact is in close proximity to the *hox* or *hyp* gene cluster in *Synechococcus* WH 5701, *Arthrospira maxima*, *Lyngbya aestuarii* PCC 8106 and *Lyngbya majuscula* 1446/4 suggesting that the bidirectional hydrogenase is used to dispose the electrons during fermentation via a PFOR-like enzyme (Barz et al., 2010).

### **3.2.6 Maturation proteins**

The bidirectional [NiFe]-hydrogenase needs action of six proteins encoded by the *hyp* genes (hydrogenases-pleiotropic genes) and a protease for post-translational processing to yield an active enzyme. The large number of genes (typically six for cyanobacteria) involved in the maturation of the structural subunit is probably indicative of the complexity of the hydrogenase molecular structure.

The role of Hyp proteins in the incorporation of ligands (CN<sup>-</sup> and CO) and metal ions (nickel and iron) in the active site of the bidirectional hydrogenase enzyme in *E. coli* has been studied extensively. *Hyp* gene homologs have been found in all organisms harboring [NiFe]-hydrogenases. Since the hydrogenase structural genes from various organisms are also homologous, it has been proposed that the maturation mechanisms (Figure 5) of hydrogenases are similar (Casalot and Rousset, 2001; Vignais and Colbeau, 2004). Likely, iron is the first metal to be incorporated into the active site. This iron is associated with one CO and two CN<sup>-</sup> groups as ligands. HypF and HypE are required for the synthesis of the CN<sup>-</sup> ligands wherein HypF catalyzes the transfer of the carbamoyl group of carbamoyl phosphate to the C terminal thiolate of HypE, forming an adenylated carbamoyl phosphate derivative with the concomitant generation of AMP and

pyrophosphate from ATP (Paschos et al., 2002;Reissmann et al., 2003). The HypE-thiocyanate gives the  $\text{CN}^-$  ligand to the HypC-HypD complex (Blokesch and Bock, 2002;Blokesch et al., 2004). HypD has been predicted to provide the electrons for the attachment of iron to all the three ligands (Blokesch and Bock, 2002;Blokesch et al., 2004). It is predicted that the ligands are attached at the HypC-HypD complex (Blokesch and Bock, 2002; Blokesch et al., 2004). The iron with the three attached ligands is delivered to pre-HycE, the precursor of the large subunit of the *E. coli* hydrogenase 3 by HypC (Forzi and Sawers, 2007). HypD gets liberated once the HypC and pre-HycE interact (Blokesch and Bock, 2002; 2006). The proteins HypA, a nickel binding protein (Mehta et al., 2003; Blokesch et al., 2004) and HypB, a GTPase (Maier et al., 1993;Maier et al., 1995) help insert the nickel into the active site (Jacobi et al., 1992;Olson et al., 2001). After the coordination of Ni and Fe, the C-terminal polypeptide is cleaved by a specific endopeptidase (Rossmann et al., 1994;Theodoratou et al., 2000). Nickel is employed as a recognition motif for the cleavage (Theodoratou et al., 2000;2005), liberating a short chain of aminoacids, the length and sequence of which varies greatly amongst different organisms (Wunschiers et al., 2003). This cleavage results in the internalization of the complete bimetallic active site into the hydrogenase enzyme (Magalon and Bock, 2000;Theodoratou et al., 2005;Bock et al., 2006). This endopeptidase-mediated proteolytic process has been reported to be highly specific. In *E. coli* three specific C-terminal endopeptidase were found, namely, HyaD, HybD and HycI, each one cleaving the C-terminal from the large subunit precursors of hydrogenases 1, 2 and 3, respectively (Menon et al., 1991; Menon et al., 1993;Rossmann et al., 1995).

The genes *hypFCDEAB* code for the maturation of bidirectional hydrogenase in cyanobacteria (Lutz et al., 1991; Jacobi et al., 1992). Deletion mutants of *hypA1*, *B1*, *C*, *D*, *E*, and *F* mutants in *Synechocystis* sp. PCC 6803, harboring only the bidirectional hydrogenase, proved positively that these genes encode proteins involved in the maturation of its native hydrogenase (Hoffmann et al., 2006). In *Synechocystis* sp. PCC 6803, additional homologs *hypA2* and *hypB2* exist but seem to have no effect on the bidirectional hydrogenase activity (Hoffmann et al., 2006). Although *hyp* genes are frequently clustered and in the vicinity of one of the hydrogenases structural genes (*hox*), they may also be scattered throughout the genome (Boison et al., 1996; Kaneko et al., 1996; Gubili and Borthakur, 1998; Hansel et al., 2001; Tamagnini et al., 2002; Wunschiers et al., 2003; Agervald et al., 2008). The presence of a single copy of most *hyp* genes in cyanobacterial genomes, regardless of possessing either one or both hydrogenases, suggests that the *hyp* genes might be responsible for the maturation of both the uptake and bidirectional hydrogenase (Tamagnini et al., 2007a).

The construction of a cyanobacterial C-terminal specific endopeptidase deficient mutant has demonstrated that *hoxW* is required for the bidirectional hydrogenase activity in *Synechocystis* sp. PCC 6803 (Hoffmann et al., 2006). The fact that both *hupW* and *hoxW* are present in strains containing the uptake and the bidirectional hydrogenase, suggests that each gene encodes the protease specific for one of the hydrogenases (Wunschiers et al., 2003). The position and orientation of *hoxW* in the cyanobacterial chromosome is rather variable although in several cases they are located near the corresponding hydrogenase structural genes (Tamagnini et al., 2007b).

### 3.2.7 Transcriptional studies

The transcription of the *hox* genes is in agreement with the patterns found in their physical organization. They may either be transcribed as a single unit together with the interspersed ORF(s), as in *Synechocystis* sp. PCC 6803 (Boison et al., 2000), or as two different transcripts: *hoxEF* and *hoxUYH*, as seen in *S. elongatus* PCC 6301 (Boison et al., 2000). Upon limiting cells of nitrate and ammonium, an increase in the *hox* transcript level was observed in *Synechocystis* sp. PCC 6803 (Antal et al., 2006) whereas no significant change was detected in *Gloeocapsa alpicola* CALU 743 under similar conditions (Sheremetieva et al., 2002). Depriving cells of sulfur resulted in increased transcriptional activity in both *G. alpicola* CALU 743 and *Synechocystis* sp. PCC 6803 (Antal and Lindblad, 2005). In several heterocystous cyanobacteria microaerobic/anaerobic conditions increase the transcriptional levels (and enzyme activity) of the bidirectional hydrogenase (Houchins and Burris, 1981b; Houchins, 1984; Serebriakova et al., 1994; Schmitz and Bothe, 1996; Axelsson and Lindblad, 2002; Sheremetieva et al., 2002; Sjöholm et al., 2007). Addition of nickel to the growth medium increases the transcript level (and enzyme activity) of *hoxH* in *Nostoc muscorum* (Axelsson and Lindblad, 2002). The transcription of bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 is regulated by transcriptional activators, LexA (Gutekunst et al., 2005) and *abrB1* (Oliveira and Lindblad, 2008), along with the transcriptional repressor, *abrB2* (Ishii and Hihara, 2008; Dutheil et al., 2012). The maturase protein *hoxW* is known to be transcribed under both N<sub>2</sub>-fixing conditions and non-N<sub>2</sub>-fixing conditions in *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803

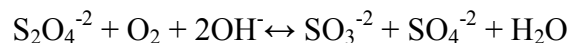


(Wunschiers et al., 2003). In *S. elongatus* PCC 6301 and *S. elongatus* PCC 7942, *hoxW* transcribed as *hoxUYHWhypAB* (Boison et al., 2000). In *S. elongatus* PCC 7942, although *hoxW* constitutes a transcriptional unit together with *hoxUYH*, it is mainly expressed from its own promoter (Schmitz et al., 2001).

### 3.2.8 Hydrogen production via the bidirectional hydrogenase

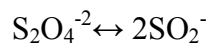
#### 3.2.8.1. Assaying the potential capacity (standard assay)

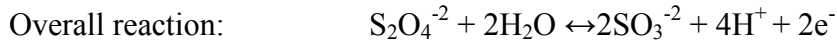
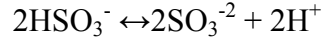
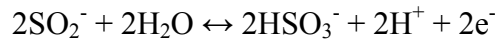
The potential capacities of cyanobacterial strains to produce H<sub>2</sub> via the bidirectional hydrogenase can be assayed by providing excess reductants under dark anaerobic conditions (Peck and Gest, 1956). Sodium dithionite reduced methyl viologen is most widely used procedure to elicit H<sub>2</sub> production from cyanobacteria (Houchins and Burris, 1981; Houchins, 1984; Appel et al., 2000, Appel et al., 2006; Baebprasert et al., 2010; Gutekunst et al., 2006; Gutthann et al., 2007; Schutz et al., 2004). Sodium dithionite (represented as Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> or simply S<sub>2</sub>O<sub>4</sub><sup>-2</sup>) reacts with O<sub>2</sub> to make the assay solution anaerobic (Jhaveri and Sharma, 1968).



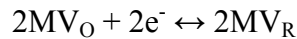
Sodium dithionite also reduces methyl viologen. Initially, the S<sub>2</sub>O<sub>4</sub><sup>-2</sup> dissociates to the radical SO<sub>2</sub><sup>-</sup> followed by 1-electron oxidation to HSO<sub>3</sub><sup>-</sup> (Mayhew, 1978, Dijk et al., 1979).

The electrons generated reduce methyl viologen as per the equations below.





Reaction with methyl viologen:



( $\text{MV}_\text{O}$  = oxidized methyl viologen and  $\text{MV}_\text{R}$  = reduced methyl viologen)

The reduced methyl viologen acts as an electron carrier, supplying reducing equivalents to the bidirectional hydrogenase eliciting  $\text{H}_2$  production (Houchins and Burris, 1981; Houchins, 1984). Thus, the specific hydrogenase activity assay is a measure of the potential capacity of the hydrogenase to make  $\text{H}_2$  when not limited by reducing equivalents. Analysis of the various *hox* subunit and subcomplex mutants in the *Synechocystis* sp. PCC 6803 reveals that 1) the full complex (*hoxEFUYH*) is necessary for  $\text{H}_2$  production by the enzyme in presence of NAD(P)H and that 2) *hoxYH* is the minimal hydrogenase required for  $\text{H}_2$  production in presence of reduced methyl viologen. The specific hydrogenase activity of the *hoxEFU* mutant was 30% of the wild type activity (Eckert et al., 2012).

### **3.2.8.2. Assaying the natural capacity**

As discussed earlier, cyanobacteria have the capability to produce H<sub>2</sub> under certain physiological conditions. H<sub>2</sub> can be produced directly via photosynthesis or indirectly involving a carbohydrate-storage intermediate as described below.

#### **3.2.8.2.1 Direct photobiological hydrogen production**

Direct photobiological H<sub>2</sub> production involves the production of H<sub>2</sub> directly from the activity of the hydrogenase enzyme and photosynthetic processes without the intermediate storage of carbohydrate. Certain strains of cyanobacteria adapted to dark anaerobic conditions display a brief period (few seconds) of H<sub>2</sub> production when illuminated. This bidirectional-hydrogenase-mediated generation of H<sub>2</sub> is referred to as photohydrogen. The H<sub>2</sub> production is followed by H<sub>2</sub> uptake until the inactivation of hydrogenase by the accumulation of photosynthetically generated O<sub>2</sub> (Gutthann et al., 2007). Photohydrogen has been reported in *Oscillatoria chalybea* (Abdel-Basset and Bader, 1997; 1998) and *Synechocystis* sp. PCC 6803 (Abdel-Basset and Bader, 1998).

#### **3.2.8.2.2 Indirect photobiological hydrogen production**

NAD(P)H can be directed towards carbon fixation and regenerated later through glycolytic and fermentative metabolic pathways to power the bidirectional hydrogenase leading to indirect photobiological H<sub>2</sub> production. In cyanobacteria, the intermediate storage carbohydrate, glycogen (Troshina et al., 2002), or osmolytes, trehalose (Heyer et al., 1989) and glucosylglycerol (Moezelaar et al., 1996), are known to be fermented. Since the reductant is made available through the dark fermentative breakdown of stored

organic carbon(in the night) after energy had been directed toward biomass accumulation(earlier in the day), a temporal separation occurs between the O<sub>2</sub> and H<sub>2</sub> production processes.

Cyanobacteria are not known to respire external electron acceptors other than O<sub>2</sub>, and thus, when subjected to nighttime anoxia must resort to fermentation in order to maintain ATP production and regenerate reduction equivalents. Depending on the strain, they have been shown to carry out a variety of fermentative metabolisms including the homolactate, homoacetate, heterolactate and mixed acid pathways (Stal and Moezelaar, 1997). The homolactate pathway primarily produces lactate (Oren and Shilo, 1979), whereas the heterolactate pathway evolves lactate along with ethanol and acetate (Heyer et al., 1989). The homoacetate pathway produces mostly acetate along with minor quantities of lactate, CO<sub>2</sub> and H<sub>2</sub> (Heyer et al., 1989; De Philippis, 1996). The mixed acid fermentation pathway is known to produce acetate, lactate, ethanol, formate and/or CO<sub>2</sub> and H<sub>2</sub> (Vanderoost et al., 1989; Moezelaar et al., 1996b; Aoyama et al., 1997; Troshina et al., 2002). Thus, the mixed acid and, to a certain extent, the homoacetate pathways result in H<sub>2</sub> production. Amongst cyanobacteria, the theoretical maximum of four moles of H<sub>2</sub> per mole of glucose has been observed only in *G. alpicola* CALU 743, albeit two moles arise via the fermentation pathway and the remaining two moles from glycolysis (Troshina et al., 2002). *Microcystis* sp. PCC 7806 yields 0.51 (Moezelaar and Stal, 1994) and *Cyanothece* sp. PCC 7822 yields 0.76 moles H<sub>2</sub>/mol glucose (Oost et al., 1989).

### 3.2.9 Oxygen sensitivity

Despite being expressed under aerobic conditions, the bidirectional hydrogenase is O<sub>2</sub>sensitive and gets reversibly inactivated in presence of O<sub>2</sub>. During photosynthesis, O<sub>2</sub> is produced at PSII, usually at a faster rate than consumed by aerobic respiration, thus, arresting the activity of the hydrogenase. From an evolutionary standpoint, this adaptation helps prevent the loss of biochemical energy. The O<sub>2</sub> toxicity of the [NiFe]-hydrogenases results from the reversible reaction of the enzyme with O<sub>2</sub>, giving rise to a mixture of inactive oxidized states (Lacey et al., 2007; Vincent et al., 2007). In *Synechocystis* sp. PCC 6803, the proton reduction continues at 25-50% of the maximal rate in the presence of 1% O<sub>2</sub>. Aerobic inactivation of the bidirectional hydrogenase in the same strain is total and nearly instantaneous, producing two inactive states. Both of these states are quickly (<90 s) reactivated by removal of O<sub>2</sub> and exposure to reducing conditions (McIntosh et al., 2011).

The mechanism of O<sub>2</sub> tolerance is not clearly understood, although it is likely related to the number and placement of ligands coordinating the bi-metallic active site (Ghirardi et al., 2007) and the gas channel which presumably controls access of both H<sub>2</sub> and O<sub>2</sub> to the active site (Montet et al., 1997; Kleihues et al., 2000). None of the reversible hydrogenases from oxygenic phototrophs examined so far are truly tolerant of atmospheric O<sub>2</sub> levels (Maness et al., 2002; Bleijlevens et al., 2004). In order to use the bidirectional hydrogenase to produce photohydrogen coupled with the oxygenic photosynthesis, the O<sub>2</sub> tolerance of the enzyme needs to be improved. The proposed

avenues for increased O<sub>2</sub> tolerance in cyanobacteria are alteration of the native enzyme or transformation with other O<sub>2</sub> tolerant hydrogenases (Ghirardi et al., 2007).

### ***3.2.10 Attempts at optimization of cyanobacterial hydrogen production***

#### *3.2.10.1. Genetic modifications*

Metabolic engineering has great potential as a tool to bring major breakthroughs in biohydrogen process by eliminating bottlenecks, increasing the carbon flow to hydrogen-producing pathway(s) and/or increasing substrate utilization and enzyme efficiencies.

Attempts have been made to increase the carbon flow to H<sub>2</sub> producing pathways by eliminating alternate sinks for the reducing equivalents. In *Synechocystis* sp. PCC 6803, a mutant was constructed which lacked the type I NADPH-dehydrogenase complex (NDH-1), a major sink for NADPH. This mutant exhibited an increase in the rate (5 X increase) and amount (20 X increase) of photohydrogen produced (Gutthann et al., 2007). The deletion of NDH-1 leads to low photosynthetic O<sub>2</sub> production (3 X decrease) implying a poor capacity to fix CO<sub>2</sub>. This mutant relied on glucose for sustained H<sub>2</sub> production (Gutthann et al., 2007) making it unsuitable for long term H<sub>2</sub> production. Additionally, deletion of the three terminal respiratory oxidases resulted in an increase in the specific hydrogenase activity (2 X increase), fermentative H<sub>2</sub> production (1.2 X increase) and the amount of photohydrogen production (4 X increase) in *Synechocystis* sp. PCC 6803.

Given the impaired respiration capabilities, this strain is also not suitable for long term H<sub>2</sub> production. The enzymes nitrate reductase and nitrite reductase involved in conversion of nitrate to ammonia are also sinks for reducing equivalents (Flores and Herrero, 2004). The

deletion of the genes coding for these enzymes lead to increase in the specific hydrogenase activity (2 X increase) and the amount of H<sub>2</sub> produced (6 X increase) in presence of combined ammonium in the media (Baebprasert et al., 2011).

In principle, overexpression of enzymes directly related to H<sub>2</sub> production (bidirectional hydrogenase and the accessory maturase proteins, pyruvate ferredoxin oxidoreductase, ferredoxin NADP oxidoreductase and/or pyruvate formate lyase and hydrogen formate lyase) should lead to increased H<sub>2</sub> evolution. Of these, the only study attempted in cyanobacteria was overexpression of the native bidirectional hydrogenase and the accessory maturase proteins from *Anabaena* sp. PCC 7120 in *Synechocystis* sp. PCC 6803 (Germer et al., 2009). This study reports higher specific hydrogenase activities (3.3 X increase) when compared to the wild-type strain.

Another approach is the introduction of highly active [FeFe]-hydrogenase in cyanobacteria. The heterologous expression of the *Clostridium* [FeFe]-hydrogenase in the cyanobacterium *Synechococcus* PCC 7942 led to higher specific hydrogenase activities (4.5 X increase) (Asada et al., 2000). Similarly, expression of *Shewanella oneidensis* MR-1 [FeFe]-hydrogenase genes in *Anabaena* sp. PCC 7120 uptake hydrogenase mutant resulted in higher specific hydrogenase activities (10 X increase) than the wild-type (Gartner et al., 2012).

The ultimate breakthrough in biohydrogen production will most likely be via successful coupling of an oxygen-tolerant hydrogenase directly to photosystem I, thus getting rid of

all the competing sinks for electrons (Ihara et al., 2006; Lubner et al., 2009; Krassen et al., 2009). Although, the initial attempts in this area have met with some success, the rates are rather low (Lubner et al., 2009).

Even though certain results from genetic engineering appear promising, none of these strategies have proved useful for long-term H<sub>2</sub> production in cyanobacteria.

#### 3.2.8.10.2. *Changes in physiological parameters*

Exploring the effects of optimizing the physiological assay and/or growth parameters can lead to enhanced H<sub>2</sub> production in cyanobacteria. The important parameters studied with respect to cyanobacterial bidirectional hydrogenases are as follows.

##### a) pH:

In principle, H<sub>2</sub> production processes should be dependent on the internal pH of the cells since the pH determines the concentration of protons (a substrate for the H<sub>2</sub> producing enzymes) (Supplementary Information). *In vitro*, the bidirectional hydrogenase from *Synechocystis* sp. PCC 6803 displayed higher activity (2.4 X increase) at pH 5 than at pH 7.5 (McIntosh et al., 2011). The specific hydrogenase activity in *Synechocystis* sp. PCC 6803 increased with an increase in external pH from 6.5 to 7.5; and a decrease in activity was observed on increasing the pH thereafter (quantification unavailable) (Baebprasert et al., 2010).



b) Light intensity:

Light is an important factor in the biohydrogen production process determining the reductant production via photosynthesis and synthesis of carbon storage molecules. Different strains require different light intensities for optimized H<sub>2</sub> production. For instance, in *Aphanothece halophytica* increase of light intensity from 15 to 30  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  only slightly increased the rate of fermentative H<sub>2</sub> production (1.1 X increase). Further increase of light intensity from 30 to 150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  led to a decline in the rate of fermentative H<sub>2</sub> production (1.1 X decrease) (Taikhao et al., 2013).

c) Nickel:

Nickel ions are essential cofactors of the cyanobacterial bidirectional hydrogenases (Dismukes et al., 2008). Addition of nickel to media increased the specific hydrogenase activity (18 X increase) and the rate of fermentative H<sub>2</sub> production (6 X increase) in *Arthrospira maxima* by enhancing the activity of the bidirectional hydrogenase (Dismukes et al., 2008). In *Aphanothece halophytica* supplementing the cells with 1  $\mu\text{M}$  nickel increased the rate of fermentative H<sub>2</sub> production (3 X increase). Addition of more than 1  $\mu\text{M}$  nickel led to a decrease in the rates of fermentative H<sub>2</sub> produced (Taikhao et al., 2013). Similarly, an increase in rates of fermentative H<sub>2</sub> production (15 X increase) is observed in the strain *Lyngbya aestuarii* BL J when the nickel concentration is increased from zero to 0.5  $\mu\text{M}$  (Chapter III).

d) Combined nitrogen:

In cyanobacteria, reduction of nitrate to ammonia requires reducing equivalents (Flores and Herrero, 2004). Therefore, elimination of nitrate from the growth media increases the reductant flow to the hydrogenase (Gutthann et al., 2007). When limited of nitrate (9 X decrease in final concentration of nitrate), the non-N<sub>2</sub>fixing cyanobacterium *G. alpicola* displayed an increase in glycogen accumulation (4 X increase), rate of fermentative H<sub>2</sub> production (4 X increase) and specific hydrogenase activity (4 X increase)(Troshina et al., 2002). In absence of combined nitrate and ammonium in the medium, the non-N<sub>2</sub>fixing *Synechocystis* sp. PCC 6803 exhibited higher specific hydrogenase activity (1.7 X increase)(Baebprasert et al., 2010) than that observed in the presence of combined nitrate in the medium. Similarly, *Aphanothece halophytica* grown in absence of nitrate and ammonium displayed an increase in the rates of fermentative H<sub>2</sub> production (4 X increase) along with inhibition of cell growth (Taikhao et al., 2013). The non-N<sub>2</sub> fixing strains are not known to survive for long in absence of combined nitrate or ammonium in the medium.

Ammonium is the preferred source of nitrogen in all examined cyanobacteria so far (Bhaya et al., 2000). As expected, addition of ammonium instead of nitrate leads to increase in the amounts of fermentative H<sub>2</sub>(quantification unavailable), in *Synechocystis* sp. PCC 6803, since electrons are not directed towards reduction of nitrate to ammonium (Ely et al., 2008).

e) Sulfur starvation:

As discussed earlier, sulfur deprivation is a commonly used method for promoting anaerobic conditions and thereby increasing H<sub>2</sub> production in the green algae *Chlamydomonas reinhardtii* (Melis et al., 2000; Zhang et al., 2002). Similarly, depriving cells of sulfur lead to increased specific hydrogenase activity in *G. alpicola* (4 X increase) and *Synechocystis* sp. PCC 6803 (4 X increase) (Antal and Lindblad, 2005). Interestingly, halotolerant cyanobacterium *Aphanothece halophytica* exhibited a decrease in cell growth (quantification unavailable), H<sub>2</sub> production rate (7 X decrease), and bidirectional specific hydrogenase activity (15 X decrease) when deprived of sulfur (Taikhao et al., 2013).

f) Carbon dioxide:

It is expected that in presence of higher levels of CO<sub>2</sub>, carbon fixation would increase resulting in fewer electrons being directed to the bidirectional hydrogenase for photohydrogen production. However, for fermentative H<sub>2</sub> production, it is expected that increased carbon-dioxide concentrations would increase the carbon storage compounds leading to increased H<sub>2</sub> production. In *Synechocystis* sp. PCC 6803, increase in the concentration of bicarbonate in the media (hydrated form of CO<sub>2</sub>) from zero to 120 mM HCO<sub>3</sub><sup>-</sup>, led to the increased amount of fermentative H<sub>2</sub> production (2 X increase) (Ely et al., 2008).

g) Temperature:

It is unclear how temperature directly affects the bidirectional-hydrogenase-mediated H<sub>2</sub> production and it most likely has an indirect effect. In *Synechocystis* sp. PCC 6803, the bidirectional specific hydrogenase activity increased with an increase in temperature from 30 °C to 60 °C with the highest activity at 70 °C (quantification unavailable). The authors note that it is unclear whether the increased activities observed at higher temperatures are due to cell lysis (Baebprasert et al., 2010). Increasing the temperature from 25 °C to 35 °C in *Aphanothece halophytica* led to an increase in rates of fermentative H<sub>2</sub> production (6 X increase) (Taikhao et al., 2013).

h) Salinity:

In the freshwater *Synechocystis* sp. PCC 6803, an increase in the specific hydrogenase activity (1.5 X increase) was seen on increasing the concentration of NaCl from zero to 0.01M. Further increase in salinity resulted in decline of the specific hydrogenase activity possibly due to diversion of cell's energy and reductants for the maintenance of osmotic balance (Baebprasert et al., 2010). In the unicellular halotolerant (optimal growth at 0.5-1.0 M NaCl) cyanobacterium, *Aphanothece halophytica*, on increasing the concentration of NaCl from zero to 0.75M NaCl, an increase in the rate of fermentative H<sub>2</sub> production (15 X increase) was observed (Taikhao et al., 2013). Further increase in the NaCl concentrations upto 3 M led to a decline in the rate of fermentative H<sub>2</sub> production (4 X decrease).

In short, different strains have different requirements for optimum H<sub>2</sub> production. Therefore individual approaches at optimizing the physiological parameters for improved H<sub>2</sub> production are required for each promising H<sub>2</sub>-producing strain. Optimizing the nickel concentrations had maximum impact on H<sub>2</sub> production via the bidirectional hydrogenase. Other important parameters are sulfur and nitrogen starvation. A study on optimizing H<sub>2</sub> production in *Synechocystis* sp. PCC 6803 reported nearly 150-fold increase in the rate of fermentative H<sub>2</sub> production by optimizing concentrations of key nutrients such as ammonium, carbonate, phosphate and sulfate in the media. The optimized rate of fermentative H<sub>2</sub> production reported was 0.8 nmol (μg chl. *a*)<sup>-1</sup> h<sup>-1</sup> (Ely et al., 2008).

Instead of genetically modifying or optimizing parameters for the standard strains such as *Synechocystis* sp. PCC 6803, with average capacities of H<sub>2</sub> production, it would be desirable to genetically modify (if possible) and/or optimize physiological parameters for a strain with high H<sub>2</sub> production capacities. Hence this dissertation focuses on bioprospecting natural communities to seek cyanobacterial strains with strong H<sub>2</sub> production capacities.

#### **4. Approach Used in this Study**

In theory, H<sub>2</sub> production via the cyanobacterial bidirectional hydrogenases is extremely promising because this process uses the inexhaustible sunlight and water for generation of H<sub>2</sub>. Also, the bidirectional [NiFe]-hydrogenase enzyme is less sensitive to O<sub>2</sub> than the nitrogenases and [FeFe]-hydrogenases. In comparison to the nitrogenases, the bidirectional hydrogenases have the advantage of having higher turnover number and being ATP independent. In spite of the bidirectional-hydrogenase-mediated H<sub>2</sub> production being a highly desirable approach, the currently known cyanobacterial strains are not very suitable for long-term H<sub>2</sub> production given the O<sub>2</sub> sensitivity, low amounts and rates of H<sub>2</sub> production and the reversibility of the enzymatic system (Tamagnini et al., 2007b). As discussed earlier, genetic modifications and optimization methods have been employed for improved H<sub>2</sub> production via the bidirectional hydrogenase in certain cyanobacterial strains. Although some results look promising, none of them, in their current capacity, can be successfully used in large-scale processes.

Alternatively, one can also try a bio-prospecting approach, to probe existing biological diversity in the search for natural variants of the enzyme or its regulatory networks that constitute better production platforms. Perhaps with this in mind, extensive genomic surveys have been conducted on aquatic environments in an attempt to understand the distribution and diversity of the *hoxH* gene, coding for the large subunit of the bidirectional hydrogenase (Barz et al., 2010). Another approach studied eleven strains

from various labs/culture collections and probed for the presence/absence of *hoxH* gene and activity (Ludwig et al., 2006).

This study is novel in carrying out a blend of both of the aforementioned approaches, by isolating novel strains from diverse environments and surveying them for the presence of the bidirectional hydrogenase gene and a concurrent quantitative comparison of their hydrogenase activities under non-N<sub>2</sub>-fixing conditions (Chapter II). Since public databases have not reported bidirectional hydrogenase genes from terrestrial ecosystems, cyanobacteria from these understudied environments were targeted. Cyanobacterial strains from marine microbial intertidal mats were of special interest since a high flux of H<sub>2</sub> has been reported from certain mats (Skyring et al., 1989; Hoehler et al., 2001). To this a survey of freshwater plankton was added, a habitat well known to harbor cyanobacteria with bidirectional hydrogenases (Schmitz et al., 1995; Appel et al., 2000; Schutz et al., 2004; Allahverdiyeva et al., 2010). It was deemed important to carry out these determinations in newly isolated strains, given the tendency for phenotype loss that may ensue during continued cultivation in the laboratory. Thus, this research exploits the cyanobacterial diversity to unearth novel strains with strong H<sub>2</sub> producing capacity.

These studies were extended to probe the innate H<sub>2</sub> evolving capacity, of certain promising strains, under fermentative conditions. The strain *Lyngbya aestuarii* BL J exhibited the highest rates and steady state H<sub>2</sub> concentrations in optimized fermentation assays. Therefore, using metabolomic and genomic approaches, the fermentation pathway of *L. aestuarii* BL J was analysed (Chapter III).

And finally, the sequence and 3D structure of the bidirectional hydrogenase in *L. aestuarii* BL J and the standard strain *Synechocystis* sp. PCC 6803 was comparatively analysed to ascertain any potential genetic or structural basis for the powerful H<sub>2</sub> production in the former strain (Chapter IV).



## Tables/Figures

| Avenue                                    | Organism                   | Advantages                                                                                                    | Disadvantages                                                                                                                                                                                    | External Organic ? | Examples                                                                                                                                                                                                                                                                                               |
|-------------------------------------------|----------------------------|---------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Microbial Electrolysis Cells              | Heterotrophic Bacteria     | - High H <sub>2</sub> yield (moles of H <sub>2</sub> produced per mole of glucose or other organic substrate) | - Low rates of H <sub>2</sub> production<br>- External energy supply requirement                                                                                                                 | +                  | <i>Geobacter</i> , <i>Shewanella</i> , <i>Klebsiella</i> , <i>Pseudomonas</i> , <i>Clostridium</i> , <i>Desulfuromonas</i> , <i>Escherichia</i>                                                                                                                                                        |
| Nitrogenase-Mediated                      | Cyanobacteria              | - High rates reported in the strain <i>Cyanothece</i> sp. ATCC 51142                                          | - Low turnover number<br>- High ATP requirement<br>- Additional energy costs involved in heterocyst differentiation and maintenance<br>- Uptake hydrogenase consumes the H <sub>2</sub> produced | -                  | <i>Anabaena</i> sp. PCC 7120, <i>Cyanothece</i> sp. ATCC 51142                                                                                                                                                                                                                                         |
| Nitrogenase-Mediated                      | Purple non-sulfur bacteria | - O <sub>2</sub> sensitivity is not an issue                                                                  | - Low turnover number<br>- High ATP requirement<br>- Uptake hydrogenase consumes the H <sub>2</sub> produced                                                                                     | +                  | <i>Rhodospirillum rubrum</i> , <i>Rhodobacter capsulatus</i> , <i>Rhodobacter sulidophilus</i> , <i>Rhodopseudomonas capsulata</i> , <i>Rhodobacter sphaeroides</i> , <i>Rhodopseudomonas palustris</i> , <i>Chlorobium limicola</i> , <i>Thiocapsa roseopersicina</i> , <i>Halobacterium halobium</i> |
| Dark Fermentation                         | Heterotrophic Bacteria     | - High rates of H <sub>2</sub> production<br>- Simple bioreactor construction with low energy input demands   | - Low H <sub>2</sub> yield                                                                                                                                                                       | +                  | <i>Enterobacter cloacae</i> DM 11, <i>Clostridium</i> sp. strain No. 2                                                                                                                                                                                                                                 |
| Direct Biophotolysis (Photohydrogen)      | Cyanobacteria Algae        | - Direct coupling of light to the splitting of water                                                          | - Transitory H <sub>2</sub> production<br>- Low rates of H <sub>2</sub> production<br>- Low concentrations of steady state H <sub>2</sub><br>- O <sub>2</sub> sensitivity of enzyme              | -                  | <i>Chlamydomonas reinhardtii</i> , <i>Synechocystis</i> sp. PCC 6803                                                                                                                                                                                                                                   |
| Direct Biophotolysis (Sulfur deprivation) | Algae                      | - High rates of H <sub>2</sub> production<br>- High concentrations of steady state H <sub>2</sub> production  | - Low photon to H <sub>2</sub> conversion efficiencies given that algae have large light-harvesting antenna complexes (LHC)<br>- Supplementation of acetate in media                             | +                  | <i>Chlamydomonas reinhardtii</i> , <i>Scenedesmus obliquus</i> , <i>Scenedesmus vacuolatus</i>                                                                                                                                                                                                         |
| Indirect Biophotolysis                    | Cyanobacteria Algae        | - Temporal separation to deal with enzyme's O <sub>2</sub> sensitivity                                        | - Low H <sub>2</sub> yield                                                                                                                                                                       | -                  | <i>Chlamydomonas reinhardtii</i> , <i>Lyngbya aestuarii</i> PCC 8106                                                                                                                                                                                                                                   |

Table 1: Comparison of the various avenues of biological hydrogen production.

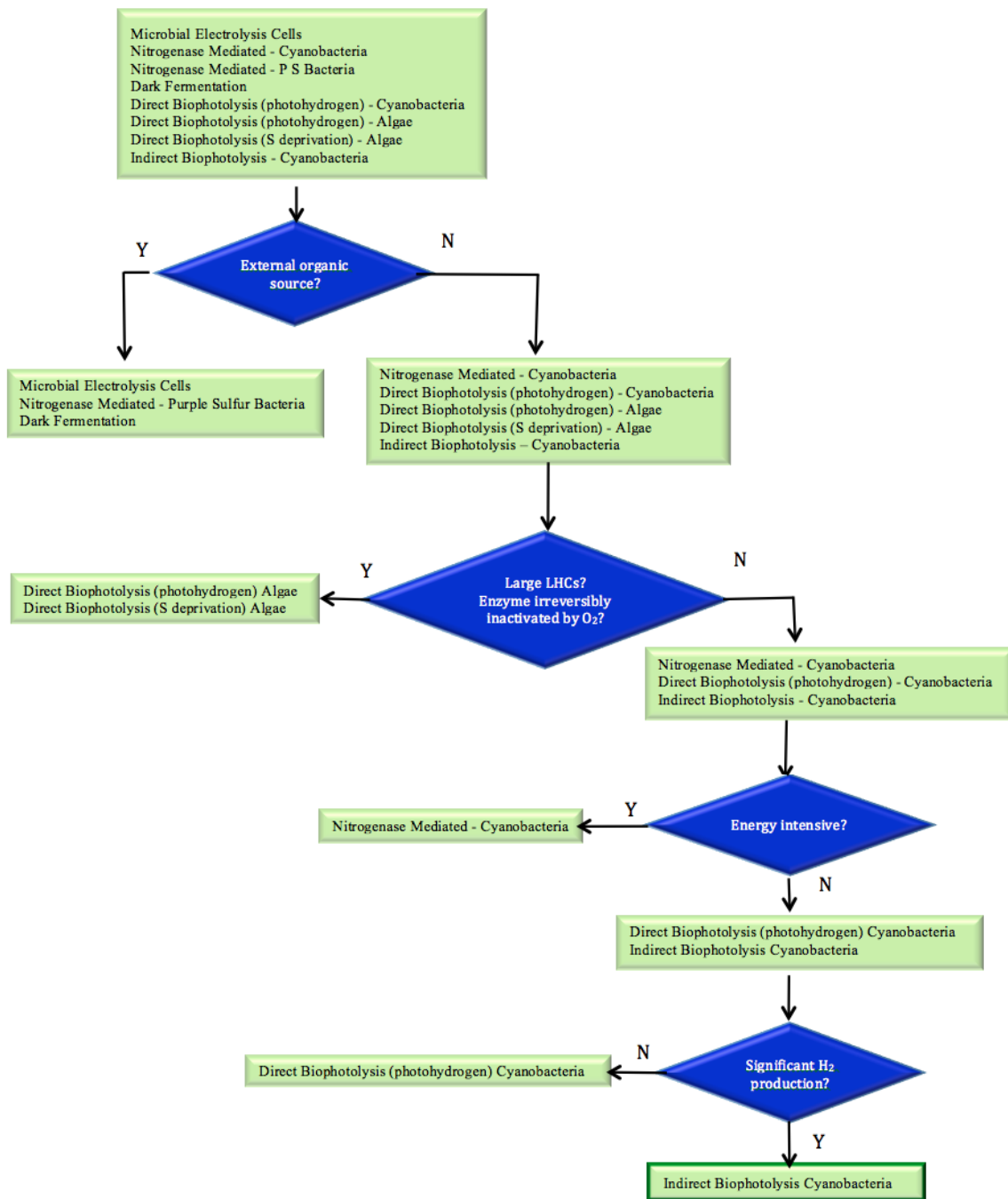


Figure 1. Biological methods of hydrogen production and the rationale for focusing on indirect photolysis via cyanobacteria.

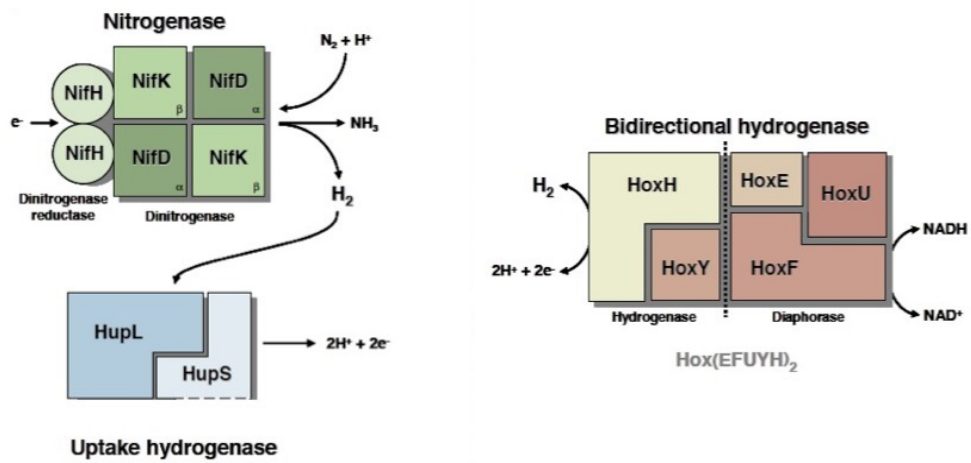
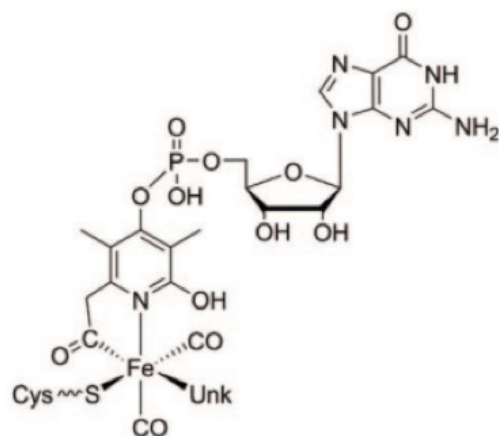


Figure 2. Cyanobacterial enzymes involved in H<sub>2</sub> metabolism, namely, the nitrogenase, uptake hydrogenase and the bidirectional hydrogenase. Figure adapted from Ferreira (2009).

a)



b)

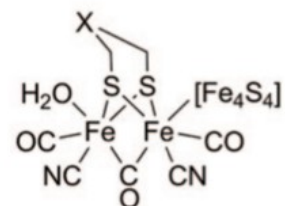


Figure 3. X-ray crystal structures and schematic representations of the active site a) [Fe]-hydrogenase, (Unk= unknown ligand; this site appears to bind cyanide). b) [FeFe]-hydrogenase (X = CH<sub>2</sub>, NH, or O). Figure adapted from Tard and Pickett(2009).

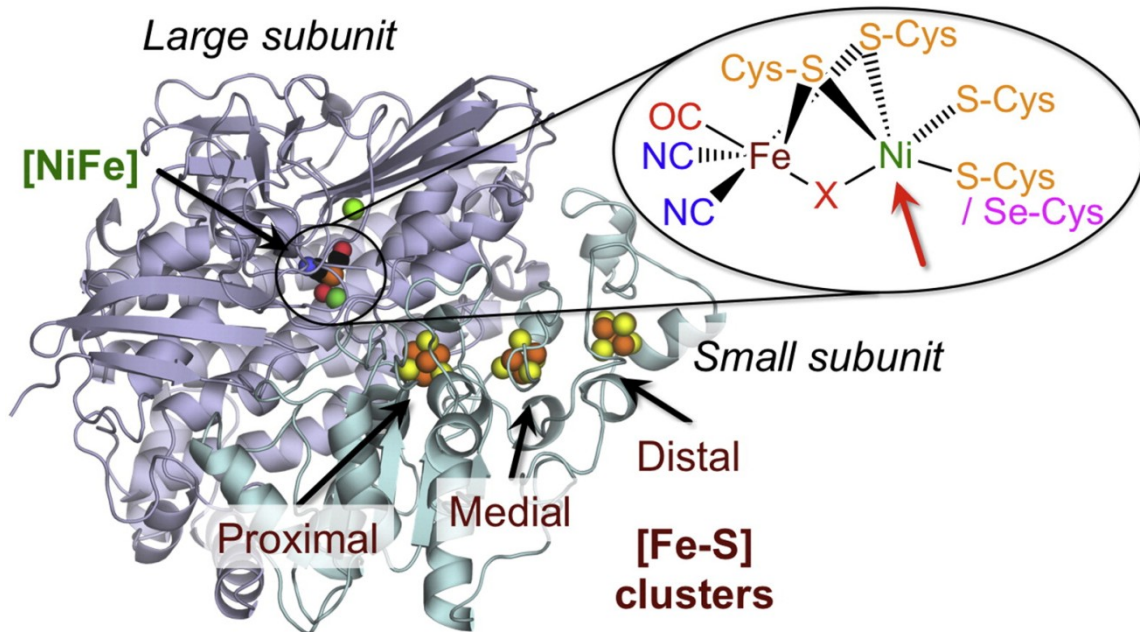


Figure 4. Three-dimensional protein structure of the [NiFe]-hydrogenase from *Desulfovibrio vulgaris miyazaki* (PDB: 1H2R) displaying the [NiFe] active site, [4Fe-4S] proximal and distal clusters, and [3Fe-4S] medial cluster indicated. (*Inset*) The molecular structure of the active site (Higuchi et al., 1999). The red arrow marks the proposed H<sub>2</sub>-binding site; the terminal cysteine that is replaced by selenocysteine in the [NiFeSe]-hydrogenase is displayed. Figure from Shafaat et al.(2013).

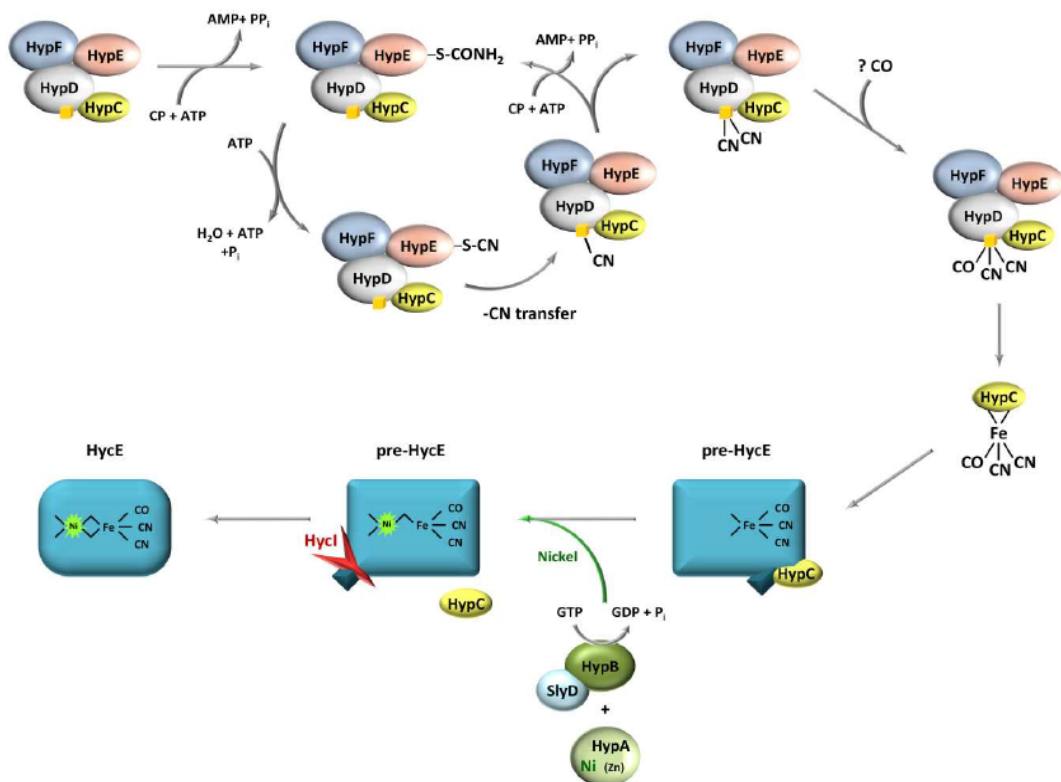


Figure 5. Hypothesized maturation pathway of the hydrogenase3 large subunit from *E. coli* (HycE) elucidating the formation of the [NiFe] active centre. The small yellow cube in HypD denotes a [4Fe4S]-cluster. The question mark on CO suggests its uncertain metabolic origin and whether CO it is added prior or subsequent to CN<sup>-</sup> incorporation. Abbreviations: Pi, inorganic phosphate; PPi, pyrophosphate; CO, carbonyl ligand; CP, carbamoyl phosphate; CN, cyano ligand (Blokesch et al., 2002; Forzi and Sawers, 2007). Figure from Ferreira (2009).

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**II. DIVERSITY IN HYDROGEN EVOLUTION FROM BIDIRECTIONAL  
HYDROGENASES IN CYANOBACTERIA FROM TERRESTRIAL,  
FRESHWATER AND MARINE INTERTIDAL ENVIRONMENTS.**

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*(Published: International Journal of Biotechnology)*

## **Abstract**

We characterized a set of 36 strains of cyanobacteria isolated from terrestrial, freshwater and marine intertidal settings to probe their potential to produce hydrogen from excess reductant, in the hope of finding novel strains with improved traits for biohydrogen production. The set was diverse with respect to origin, morphology, taxonomy and phylogeny. We found that about one half of the strains could produce H<sub>2</sub> from hydrogenases in standard assays, a trait that corresponded invariably with the presence of homologues of the gene *hoxH*, coding for subunit H in the bidirectional Ni-Fe hydrogenase. Strains from freshwater and intertidal settings had a high incidence of H<sub>2</sub>producing, *hoxH*-containing strains, but all terrestrial isolates were negative for both. While specific rates of H<sub>2</sub>production varied among strains, some novel strains displayed rates several fold higher than those previously reported. Two different patterns in H<sub>2</sub>production were detected. Pattern 1, corresponding to that previously known in *Synechocystis* PCC 6803, encompassed strains whose hydrogenase system produced H<sub>2</sub>only temporarily to revert to H<sub>2</sub>consumption within a short time and after reaching moderate H<sub>2</sub>concentrations. Cyanobacteria displaying Pattern 2, in the genera *Lyngbya* and *Microcoleus*, tended to have higher rates, did not reverse the direction of the reaction, and reached much higher concentrations of H<sub>2</sub>at steady state, making them of interest as potential platforms for biohydrogen production.

## **1. Introduction**

Interest in the generation of renewable fuels has gained momentum in the last decades in the face of global warming associated with the continued use of fossil fuels and because

of the finite nature of their reserves. Biohydrogen production from photosynthetic organisms constitutes a conceptually promising avenue in renewable bioenergy, because it would couple directly solar radiant energy, essentially inexhaustible, to the generation of clean, carbon-neutral biofuels, particularly if water-splitting (oxygenic) phototrophs were used (Weaver et al., 1980; Akkerman et al., 2002; Prince and Khesghi, 2005). In spite of the efforts geared towards developing biological platforms for hydrogen production, none has yet reached large-scale production stages. Cyanobacteria, the only group of oxygenic phototrophs among the bacteria, have been regarded as good models for research and eventual application in this area for several reasons: they are capable of growth with minimal nutritional requirements, they are demonstrable producers of hydrogen ( $H_2$ ) under certain physiological conditions, and some can be genetically modified with ease. Among cyanobacteria, three different enzymes participate in  $H_2$  metabolism (Ghirardi et al., 2007; Tamagnini et al., 2007): nitrogenase, and two types of Ni-Fe hydrogenases (uptake and bidirectional). The nitrogenase produces  $H_2$  as a byproduct of its main reaction, the assimilatory reduction of  $N_2$ . In principle, production of biohydrogen-based on nitrogenase systems requires significant modifications of the enzyme or cumbersome growth conditions in order to promote proton reduction and prevent  $N_2$  reduction. In spite of this, significant improvements have recently been achieved by extending the panoply of cyanobacterial species investigated in order to take advantage of pre-existing physiological diversity (Bandyopadhyay et al., 2010). The  $H_2$  produced by nitrogenase is often recycled back into metabolic reducing equivalents by means of the uptake hydrogenase (Peterson and Burris, 1978; Eisbrenner and Evans, 1983). Under physiological conditions, and as the name suggests, the latter enzyme can

only consume, rather than produce H<sub>2</sub>, and so does not constitute a viable platform for biohydrogen production; in fact, it needs to be inactivated to improve yields of nitrogenase-based H<sub>2</sub> production yields (Happe et al., 2000; Lindblad et al., 2002; Yoshino et al., 2007).

Certain cyanobacteria, however, host a bidirectional hydrogenase that can catalyze both the production and the uptake of H<sub>2</sub> under physiological conditions (Fujita and Myers, 1965). This enzyme is attractive because it is naturally less sensitive to long-term inactivation by O<sub>2</sub> than other hydrogenases (Vignais et al., 2001; Cournac et al., 2004; Vignais and Colbeau, 2004) and because it does not depend on nitrogen metabolism. Much of the research on biohydrogen production in cyanobacteria has focused on this enzyme as platform. Physiological studies of the bidirectional hydrogenase revealed that it can play multiple roles in the cell: it can be a part of fermentative metabolism (Oost et al., 1989; Troshina et al., 2002), act as an emergency electron valve to release excess electrons during photosynthesis (Appel et al., 2000), and perhaps also as a general regulator for maintaining optimal redox state in the cell (Carrieri *et al.*, 2011). Direct production of H<sub>2</sub> by the bidirectional hydrogenase with electrons coming from the photolysis of water has been demonstrated during the transition from (anaerobic) dark to light conditions in *Synechocystis* PCC 6803, but this production is slow and transient (Houchins 1984, Apple and Schulz, 1998). In fact, one of the major disadvantages for sustained H<sub>2</sub> production via the bidirectional hydrogenase is the easy reversal of the reaction direction (Tamagnini et al., 2007). Not only is the H<sub>2</sub> production transient, but also the enzyme soon reverts to H<sub>2</sub> oxidation. Genetic modification of standard strains has

been an approach used in trying to address the shortcomings of the bidirectional hydrogenase platform (Cournac et al., 2004; Gutthann et al., 2007; Marques et al., 2011; Masukawa et al., 2002).

Alternatively, one can also try a bio-prospecting approach, to probe existing biological diversity in the search for natural variants of the enzyme or its regulatory networks that constitute better production platforms. Perhaps with this in mind, extensive genomic surveys have been conducted on aquatic environments in an attempt to understand the distribution and diversity of the *hoxH* gene, coding for the large subunit of the bidirectional hydrogenase. Some authors suggested that bidirectional hydrogenases are common where anaerobic or micro-aerobic conditions may be relevant ecologically and hence, *hox* genes are highly unlikely to be found at high frequency in open ocean waters (Ludwig et al., 2006). Barz and colleagues could show a clear increase in abundance of *hoxH* in freshwater and marine coastal environments, in comparison to the open oceans (Barz et al., 2010). Using a more physiological approach, focused on production by N-fixing strains, Allahverdiyeva and co-authors surveyed a large number of existing isolates from culture collections for H<sub>2</sub> evolution (Allahverdiyeva et al., 2010). They found that about 50% of strains produced detectable amounts of H<sub>2</sub>, and about 2% displayed production rates similar to those obtained in standard strains optimized by genetic modification.

In this study a blend of both of the aforementioned bio-prospecting approaches was carried out by surveying newly isolated cultures from diverse environments for the



presence of the bidirectional hydrogenase genes and for their H<sub>2</sub> production capacity under non nitrogen-fixing conditions. Cyanobacteria from terrestrial environments were targeted, since no bidirectional hydrogenase genes originating in these environments were known from public databases, suggesting that they had been differentially under-sampled. Marine microbial intertidal mats were also of special interest since a high flux of H<sub>2</sub> had been reported from these cyanobacterial mats (Skyring et al., 1989; Hoehler et al., 2001). To this, a survey of freshwater plankton, a habitat well known to harbor cyanobacteria with bidirectional hydrogenases and H<sub>2</sub> producing capabilities (Schmitz et al., 1995; Appel et al., 2000; Schutz et al., 2004; Allahverdiyeva et al., 2010), was added. It was deemed important to carry out these determinations in newly isolated strains, given the tendency for phenotype loss that may ensue during continued cultivation in the laboratory.

## **2. Material and Methods**

### **2.1 Sampling and isolation of strains.**

Cyanobacterial strains were isolated from terrestrial, fresh water and marine benthos. The terrestrial cyanobacterial strains were isolated from different layers of a gypsum outcrop (34°19'12''N; 106°42'39.1''S) protruding from the biological soil crust in the Chihuahuan Desert at the Sevilleta LTER, Palo Duro Canyon, New Mexico, U.S.A. Pulverized sample from each layer was used to inoculate liquid BG11 media, with 0.1 g/L cyclohexamide to prevent fungal growth. Strains were isolated from these enrichments by repeated streaking on Petri dishes (Rippka, 1988). The freshwater cyanobacterial strains were isolated from the epilimnetic waters of Saguaro Lake

(33°33'56"N, 111°32'10"W), Lake Roosevelt (33°40'18"N 111°09'40"W) and from the benthos of a coastal hot spring in Puertecitos, Baja California, Mexico (30°20'46.6''N; 114°38'11.2''S). Water was filtered onto GF/F filters, and the biomass on the filter used to inoculate liquid BG11 media. Repeated streaking as above led to isolation of clonal strains of cyanobacteria. Marine microbial intertidal mats were sampled from Guerrero Negro, Baja California Sur, Mexico, with the low- and mid-tide mats being collected at 27°45'32.2''N; 113°59'51.3''W and the high-tide mat at 27°44'34.6''N; 113°57'2.5''W (Rothrock and Garcia-Pichel, 2005). Small pieces of low, mid and high tide mats were inoculated directly on plates of IMR media (Eppley et al., 1968) solidified with 1% Noble agar. Filaments of cyanobacteria moved or grew out of the inoculum and into the agar from where they could be sequentially plated. As observed previously (Garcia-Pichel et al., 1996), placing the field sample on the media, selected for fast growing opportunistic cyanobacterial species instead of the major mat-forming cyanobacteria. Therefore pieces of these mats were also viewed under a dissecting microscope and filaments that morphologically resembled *Microcoleus* (Garcia-Pichel et al., 1996), *Lyngbya* (Castenholz, 2001) and *Calothrix* (Rippka et al., 1979) were pulled out by micromanipulation using watchmaker's forceps, dragged through the agar for cleaning (Prufert-Bebout and Garcia-Pichel, 1994) and allowed to grow on fresh media. Thereafter, a single motile filament (hormogonium) was picked and allowed to grow into a culture, three consecutive times, to establish a clonal culture. In addition, other strains available in the laboratory or obtained from public culture collections were used to complement the survey or to serve as positive or negative controls. All strains used in this study were clonal and mono-cyanobacterial, but many contained

contaminant heterotrophic bacteria. For experimentation, the level of contamination was monitored by microscopic observation under phase contrast optics, and did not exceed 0.01% of the cyanobacterial biomass (assessed as bio-volume).

## **2.2 Cultivation and maintenance**

All non-marine isolates were grown on BG11 medium (Rippka et al., 1979) except for those that could fix nitrogen, which were grown on a nitrate and ammonium free version, BG11° medium. A 1:1 mixture of medium IMR and ASN III medium (Eppley et al., 1968) set at 3% salinity worked best for all *Microcoleus* strains. For the rest of marine strains IMR media (3%) was used. For strains *Chlorogloeopsis* O-89-Cgs., *Scytonema hyalinum* FGP-7A, *Microcoleus vaginatus* 9802, *Chroococciopsis* sp. CCMEE 029, *Calothrix* BECD30, *Mastigocoleus* BC008, *Euhalotheca* MPIN303 and *Microcoleus cthonoplastes* NDN-I media was used as described for each (Garcia-Pichel and Castenholz, 1991; Yeager et al., 2007; Billi et al., 2000; Chacon et al., 2006; Garcia-Pichel et al., 1998; Garcia-Pichel et al., 2010; Garcia-Pichel et al., 1996). For PCC strains, media used were as recommended by the culture collection. All cultures were supplemented with 0.5  $\mu\text{M}$   $\text{NiSO}_4$ , not always present in all formulations, and necessary to ensure a supply of nickel for appropriate function of [Ni-Fe]-hydrogenases. Cultures were maintained in appropriate standard media plates with 1% agar at 28°C and at a light intensity of 20  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ .

### **2.3 Molecular analyses**

Genomic DNA preparations were obtained from all strains after PCI (phenol; chloroform; isoamyl alcohol) extraction (Countway et al., 2005). DNA in the extracts was quantified using fluorometry of ethidium bromide-stained 1% agarose electrophoresis gels. To verify genetic homogeneity and to have an idea of the phylogenetic placement, the 16S rRNA genes fragments were amplified from each newly isolated strain. For the 16S rRNA genes amplification, the cyanobacteria-specific primers (CYA106F and CYA781R) and amplification conditions of Nübel et al. (1997) were used. The presence of bidirectional-hydrogenase-coding genes was assessed by PCR amplification using the *hoxH* specific primers and amplification protocols described by Barz et al. (2010). A Bio-Rad iCycler Thermal Cycler was used for all PCR reactions. All reactions included a positive and a negative control. Any reactions yielding no amplification were repeated independently three times, before reporting results as negative. Amplification products were documented on 1% agarose gels for quantity and expected size. Excised bands were purified using the QIAquick Gel Extraction Kit (Qiagen Sample and Assay Technologies) and used as template for commercial Sanger sequencing. In most cases both forward and reverse primers were used for independent sequencing reactions of the same template, and the consensus sequence used for further analysis. NCBI nucleotide BLAST searches were performed to assign the phylogenetically closest known/named strains to those newly isolated and sequenced here.

In addition, this tentative placement based on 16S rRNA gene sequence was confirmed by phylogenetic reconstructions using MEGA 5 (Tamura et al., 2011). About 600bp of

16S rRNA sequences were aligned using ClustalW. The alignment was manually curated, and Kimura 2 parameter model was used to construct Maximum Likelihood trees with 1000 bootstrap replicates. Since the sequences obtained from *Chroococciopsis* RP110, 114, 116, 118, *Tolypothrix* RP 102 and *Leptolyngbya* RP108 were less than 400bp, they were not used in the analysis. For phylogenetic analysis of the bidirectional hydrogenase, the DNA sequences were translated into amino acid sequence and aligned by ClustalW. The alignment was manually checked and all the HoxH sequences were cropped to a common length of 300 amino acids. MEGA 5 determined WAG to be the best model, and a maximum likelihood tree with 1000 bootstrap replicates was constructed using it. Since the sequence obtained from *Anabaena* 901 was less than 100 amino acids, it was not used in the phylogenetic analysis.

#### **2.4 Standard assay for hydrogen production**

Most cultures did not grow as homogenous suspensions, but rather as biofilms or clumps. Batch cultures of 100 ml volume were grown in cotton-plugged 250 ml Erlenmeyer flasks at 28°C and 20  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ , without additional bubbling. Small clumps of biomass from log phase cultures were used for the assay. They were suspended in fresh medium and placed in a custom made, airtight chamber with continuous stirring, so as to achieve biomass concentrations between 3 and 10  $\mu\text{g (chl } a) \text{ ml}^{-1}$ . Chl *a* was measured *a posteriori*. The chamber was endowed with a miniature Clark-type electrode to monitor H<sub>2</sub> partial pressure. The electrode was connected to a pico-ammeter set at a voltage of 0.8V. An A/D converter allowed the current signal data to be read on a computer using Sensor Trace Basic software. All electrodes and peripherals were from

Unisense, Aarhus, Denmark. Before each measurement, the electrode was subject to a 2-point calibration in culture medium bubbled with either air (0% H<sub>2</sub>) or bubbled with a custom gas mixture of 10% H<sub>2</sub> in N<sub>2</sub>. Media without cyanobacteria constituted a negative control. The specific hydrogenase activity assay was carried out in the dark by the addition of methyl viologen (5 mM, final concentration) and sodium dithionite (10 mM, final concentration) (Appel et al., 2000). Sodium dithionite makes the assay solution anaerobic (Jhaveri and Sharma, 1968) and reduces methyl viologen (Mayhew, 1978). The reduced methyl viologen acts as an electron carrier, supplying the reducing equivalents to the bidirectional hydrogenase eliciting H<sub>2</sub> production (Houchins and Burris, 1981; Houchins, 1984). Chlorophyll content was measured sacrificially in the spent suspension after extraction with 100% methanol (MacKinney, 1941).

### **3. Results**

#### **3.1 Diversity of the set of strains surveyed**

The survey involved a total of 36 strains from diverse environments. Table 1 gathers information on origin and taxonomic assignments. Strains are arranged there by ecological origin. In all, 11 strains were of terrestrial origin, seven from freshwater systems and 18 originated in the marine intertidal benthos. In certain cases multiple isolates from a single genus/species were obtained and studied, which allowed us to probe internal variability. In terms of taxonomic diversity, all major groups were represented, but not equally. Most strains (19) belong to Subsection III, (i.e., order Oscillatoriales), 8 are in Section IV (order Nostocales), and 5 are in section II (Pleurocapsales). Sections I (Chroococcales), and VI (Stigonematales) are only

represented each by two strains. The phylogenetic placement of the strains according to 16S rRNA gene trees indicate that the survey encompasses also a wide representation of cyanobacterial biodiversity (Supplementary Information. 1), but that it is not exhaustive in its coverage as several important phylogenetic clusters are not encompassed. Light microscopic images depicting the morphology of strains surveyed can be seen in Figure 1. Morphological diversity in this set of strains spans most of that known for all cyanobacteria, from simple unicells (such as *Synechocystis* sp.) to large, complex, multicellular, branching filamentous strains (such as *Mastigocoleus* sp.).

### **3.2 Identity of the isolates and phylogenetic placements.**

In most cases, PCR amplification of 16S rRNA genes yielded clean products of about 600 bp in length that resulted in good-quality Sanger sequences with no background and few hard to call bases (less than 1% undefined bases), indicating the absence of mixed or multiple alleles in the amplificate and corroborating that each strain was likely monocyanobacterial, as also indicated by microscopic observation. In a few cases, however, multiple sequences were obtained. Such strains were submitted to further rounds of purification until a complete separation was achieved; those that did not result in a complete separation were not used in this study. BLAST analyses against public databases of 16S rRNA sequences were used to confirm the validity of the generic assignments based on morphology. In general the assignments obtained by BLAST corresponded to the morphogenus assignment based on Bergey's Manual, Phylum BX Cyanobacteria section (see Table 1). In a few cases, when the closest match had low similarity to any cultivated strains (7Y, 7C and RP114), morphogenus and phylogenetic

assignment differed. This is likely due to the fact that the isolates likely represent novel cyanobacterial taxa in need of description. Other mismatches were between thin filamentous strains (*Oscillatoria/ Leptolyngbya/ Geitlerinema*) where the microbiological and botanical nomenclatural traditions may differ.

### **3.3 Patterns of *hoxH* detectability**

We sought to detect the presence the *hoxH* gene that codes for the H subunit of the bidirectional NiFe hydrogenase using specific PCR amplification. Four strains were included as controls. *Synechocystis* sp. PCC6803 (Yang and McFadden, 1994) and *M. chthonoplastes* PCC 7420 were known to possess *hox* operons containing a *hoxH* gene in their genome and were used as gene positive control, whereas *N. punctiforme* PCC 73102 (Meeks et al., 2001) and *M. vaginatus* PCC 9802 (Starkenburg et al., 2011) lacked *hoxH* gene homologues in their completely sequenced genomes and were used as negative controls. All controls behaved as predicted (Table 1). Of the strains tested, approximately half were positive for *hoxH*. Certain clear patterns of distribution of *hoxH* emerged based on this analysis. While negatives for *hoxH* were seen in all environments (Table 1), none of the cyanobacterial strains from terrestrial environments possessed amplifiable homologues. The chances of this being a random result given an overall *hoxH* incidence of about 50% are clearly negligible. In contrast, 87% percent of the strains isolated from fresh water contained the gene. Marine benthic strains displayed 67% *hoxH* gene positives. The patterns of distribution of *hoxH* sequence obtained from multiple strain isolates of a single genus/species displayed a high level of consistency (i.e., all *Microcoleus chthonoplastes* were positive, while all *Chroococciopsis* sp. were negative).



In addition all strains in the same genus that were isolated from the same environment had 100% *hoxH* sequence similarity. At higher taxonomic level there were no discernible patterns. For example Group IV (Nostocales) cyanobacteria contained both positive strains and negative strains, as did Group III. Figure 2 depicts a Maximum Likelihood HoxH tree based on 300 amino acid long sequences from 36 strains, of which 14 were novel. Well-supported clades of *Arthospira*, *Limnothrix*, *Lyngbya* and *Microcoleus* HoxH sequences could be resolved.

### **3.4 Physiology of hydrogen production**

A total of 32 strains were tested for H<sub>2</sub> production via the hydrogenase assay.

Approximately half of the strains tested produced detectable H<sub>2</sub> (Table 2). A direct correlation between the presence of *hoxH* (Table 1) and the ability to evolve H<sub>2</sub> in the assay was evident from all the strains tested. No evolution was detected in any *hoxH*-negative strains either. For quantitative comparisons,  $R_H$  – the maximal (typically also the initial) rate of H<sub>2</sub> production per unit biomass, was determined. The range in  $R_H$  (gathered in Table 2; between 14 and 156 nmol ( $\mu\text{g chl } a$ )<sup>-1</sup> h<sup>-1</sup>) was wider than the range of values already reported in the literature, typically from 30 to 80 nmol ( $\mu\text{g chl } a$ )<sup>-1</sup> h<sup>-1</sup> (Appel et al., 2006; Gutekunst et al., 2006; Gutthann et al., 2007; Schutz et al., 2004). The highest values were consistently seen in marine intertidal *Lyngbya* isolates, with strain BL J displaying average rates as high as five fold faster than those seen in *Synechocystis* sp. PCC 6803 under identical assay conditions, but also with considerable variability among *Lyngbya* strains. In all, however, this is only a moderate improvement with respect to  $R_H$ .

Certain other aspects of the dynamics of H<sub>2</sub> production that have not usually been considered or reported in detail in the literature were also quantified. As advanced in the introduction, the production of H<sub>2</sub> is reported to be only transient, and, after H<sub>2</sub> reaches a certain maximum concentration,  $[H_2]_M$  at time  $T_R$ , H<sub>2</sub> production stops and reverts to consumption, bringing concentrations to very low levels or even consuming all available hydrogen. These dynamics are exemplified in *Synechocystis* sp. PCC 6803, shown in Figure 2a. However, these parameters varied widely among the different strains (Table 2). Amongst the H<sub>2</sub> producing strains, 2 distinct dynamic Patterns of H<sub>2</sub> production could be distinguished. Pattern 1 was the most common and involved an initial H<sub>2</sub> production up to relatively low  $[H_2]_M$ , between 9 and 62 μM. This was followed by reversal of the enzyme's activity into consumption of all the H<sub>2</sub> that had been produced. Strains *Synechocystis* sp. PCC 6803, *Limnothrix* HS, *Anabaena* sp. 901, *Limnothrix* 005, *Eubhalotheca* MPI N303 and *Geitlerinema* G066 conformed to Pattern 1. Pattern 2 was observed in some filamentous isolates, and was characterized by relatively high  $[H_2]_M$  values, between 68 and 486 μM. The concentration of H<sub>2</sub> was sustained thereafter, implying that enzyme activity stopped, but the system never reverted to significant H<sub>2</sub> consumption, at least during the duration of the assay. This pattern is exemplified in Figure 2b. Strains in the clade of *Lyngbya aestuarii* (BL G, BL E, BL J, BL AA, PCC 7419), in that of *Microcoleus chthonoplastes* (BM 001, BM 002, BM 003, NDN-1, PCC 7420), all isolated from benthic microbial mats, conformed to this pattern of production. Values for  $R_H$ ,  $[H_2]_M$ , and  $T_R$  for each strain have been gathered in Table 2.

The highest values of  $[H_2]_M$  were attained in marine intertidal *Lyngbya* isolates, with strain BL J reaching high as sixteen fold more than that seen in *Synechocystis* sp. PCC 6803 under identical assay conditions, but also with considerable variability among *Lyngbya* strains. This coupled with the lack of reversal of enzyme under the assay conditions in *Microcoleus* and *Lyngbya* strains is a significant improvement with respect to  $[H_2]_M$  and  $T_R$ .

#### **4. Discussion**

Ours is the first survey to encompass concurrent assessment of the *hoxH* gene presence and  $H_2$  evolution capacity from excess reductant, and it is significant that the correspondence between gene presence and activity was absolute (Table 2). Since whenever *hoxH* was absent, no  $H_2$  could be detected, it is likely that this enzyme is responsible for the activity in a vast majority of cyanobacteria. Phylogenetic analyses of HoxH confirmed the degree of overall conservation and allowed us to increase the resolution of the evolutionary history of the enzyme within oxygenic phototrophs from those that have been presented elsewhere (see Vignais et al., 2001). Even though the tree was not well resolved with respect to higher-order relationships among clades, all sequences fit clearly and within the “cyanobacterial” lineage, and well-populated clusters of strains seemed to contain consistently closely related versions of the gene (Figure 3). This seems to speak for an evolutionary path devoid of events of lateral transfer. This is possibly a consequence of the rather complex composition of the *hox* operon itself and to the presence of necessary “maturation” systems that help obtain functional enzymes (Hoffmann et al., 2006). Among the database sequences in Figure 2., there does seem to

be a couple of cases of clear “misplacement”: a *Mastigocladus* sequence, which should be among the heterocystous clade, falls among a group of unicellulars (*Synechocystis*, *Microcystis*, *Cyanothece*...), and a single *Arthrospira* strain falls away from its main clade. It is hard to interpret this data as being due to lateral transfer events, rather than just being the result of database entry or other analytical errors, and it would be advisable to re-sequence those two strains before attempting to read too much into it.

Interestingly, this Ni-Fe hydrogenase is clearly not universal among cyanobacteria. Our results and those of others tend to converge in a figure around 50% incidence. Clearly the bidirectional hydrogenase system is subject to facile evolutionary loss from cyanobacterial genomes. We could show, as others have also hinted (Barz et al., 2010), that the prevalence of these hydrogenase genes was clearly not independent of habitat. Currently, no satisfactory explanation is available as to why cyanobacteria from terrestrial environments are virtually devoid of bidirectional hydrogenases (we hesitate to write *completely*, given that *Nostoc* PCC 7422, a symbiont with cycads and probably having a terrestrial part of its life cycle is known to possess a known bidirectional hydrogenase gene (Yoshino et al., 2007)). It is possible that terrestrial environments, which tend to be oxic, may select against enzymes that are involved in anaerobic pathways or are very sensitive to oxygen, but then again, the epilimnion of freshwater lakes is also a rather oxygenated environment, and yet the incidence of *hoxH*-positive strains there was quite large.

It is of interest to uncover the reason for the strong potential H<sub>2</sub> production in *L. aestuarii*BL J (exemplary of Pattern 2) in comparison to that of *Synechocystis*sp. PCC 6803 (exemplary of Pattern 1). In comparison to *Synechocystis*sp. PCC 6803, the initial rates of H<sub>2</sub> production were five-fold higher in *L. aestuarii*BL J in presence of excess external reductants. This could result from slightly higher amounts of hydrogenase given other factors like pH and substrate concentrations are unaltered. The steady-state H<sub>2</sub> concentrations are determined by the concentration of NAD(P)H/NAD(P)<sup>+</sup> (electron source) and protons. Therefore, it is expected that in presence of excess reductants (electrons and protons), the steady-state H<sub>2</sub> concentrations would be similar in both *Synechocystis*sp. PCC 6803 and *L. aestuarii*BL J. However, the steady-state H<sub>2</sub> concentration in *Synechocystis*sp. PCC 6803 was 15-fold lower than the strain BL J. Currently, no satisfactory explanation is available for this behavior in presence of excess reductants other than differential regulation of the enzyme in the strain PCC 6803. Unlike Pattern 2, the Pattern 1 cyanobacteria exhibited a decline in the concentrations of H<sub>2</sub> leading to the consumption of almost all the H<sub>2</sub> produced. The observed decline also has little to do with the loss of enzyme activity, since the bidirectional hydrogenase works in the direction of hydrogen consumption (enzyme still active). It perhaps, again has something to do with the regulation of the bidirectional hydrogenase enzyme in Pattern 1 strains.

With respect to the discovery of novel strains of high biotechnological potential our findings had two clearly different aspects. We did not detect maximal specific activities that were much faster than what had previously reported in the literature. *L. aestuarii* BL

J was five-fold more potent than *Synechocystis* sp. PCC 6803. However, in the literature, a range of specific hydrogenase activities have been reported for *Synechocystis* sp. PCC 6803 itself (Appel et al., 2006; Baebprasert et al., 2010; Gutekunst et al., 2006; Gutthann et al., 2007; Schutz et al., 2004). While most are in the range of several tens of nmol (H<sub>2</sub>) per µg Chl per hour, Schutz et al reported as much as 235. If one takes the typical variability into account, the improvement may be just a mere two-fold; including with the high-level outlier no improvement at all. However, the fact that we discovered the presence of “Pattern 2” cyanobacteria and having bidirectional hydrogenases that run unidirectionally under excess reductant may be quite relevant for potential biotechnological applications. Cyanobacteria in this class can accumulate high concentrations of H<sub>2</sub> in the medium, thus facilitating an eventual H<sub>2</sub> harvesting in large-scale processes. Unfortunately, all strains conforming to Pattern 2 belong to the Oscillatoriales (filamentous, non-heterocystous cyanobacteria), which are notoriously difficult to grow in large scale and are not yet amenable to even the simplest forms of genetic modification. Therefore future attempts to use these cyanobacteria as platform for H<sub>2</sub> production will have to rely on optimization through variations of basic cultivation and physiological parameters. As an immediate goal, it will be imperative to carry out a detailed study of the enzymology, physiology and the molecular basis of H<sub>2</sub> generation in model strain of Pattern 2. Differences in genomic sequence and architecture, enzyme maturation, and regulation of the enzyme must be understood. A simple comparison of the 300 amino acids in our HoxH alignment did not reveal any obvious difference in sequence between the Pattern 1 and 2 cyanobacteria. Heterologous expression of Pattern

2 H<sub>2</sub>generating systems under controlled conditions in model strains such as *Synechocystis* sp. PCC 6803 may eventually constitute the best platform.

## Tables/Figures

Table 1. Cyanobacterial strains used in this survey, with their origin, morphological and phylogenetic assignments and the presence of *hoxH* in their genome (as determined by PCR amplification).

| Origin                | Strain     | Morphogenus <sup>a</sup>     | Closest 16S rRNA-based cultivated relative <sup>b</sup> | Similarity (%) <sup>c</sup> | <i>hoxH</i> presence | Closest <i>hoxH</i> allele <sup>b</sup> | Similarity (%) <sup>c</sup> | Reference                            |
|-----------------------|------------|------------------------------|---------------------------------------------------------|-----------------------------|----------------------|-----------------------------------------|-----------------------------|--------------------------------------|
| <b>Terrestrial</b>    |            |                              |                                                         |                             |                      |                                         |                             |                                      |
| Desert                | RP110      | <i>Chroococci diopsis</i>    | <i>Chroococcidiopsis</i> sp. CC1                        | 95                          | -                    | -                                       | -                           | This study                           |
|                       | RP114      | <i>Chroococci diopsis</i>    | <i>Pleurocapsa concharum</i>                            | 89                          | -                    | -                                       | -                           | This study                           |
|                       | RP116      | <i>Chroococci diopsis</i>    | <i>Chroococcidiopsis</i> sp. CC1                        | 93                          | -                    | -                                       | -                           | This study<br>This study             |
|                       | RP118      | <i>Chroococci diopsis</i>    | <i>Chroococcidiopsis</i> sp. CC1                        | 95                          | -                    | -                                       | -                           | This study                           |
|                       | RP102      | <i>Tolypothrix</i>           | <i>Tolypothrix</i> sp. LQ-10                            | 98                          | -                    | -                                       | -                           | This study                           |
|                       | RP108      | <i>Leptolyngbya</i>          | <i>Leptolyngbya</i> sp. CCALA 094                       | 98                          | -                    | -                                       | -                           | This study                           |
|                       | FGP-7A     | <i>Scytonema</i>             | NA <sup>d</sup>                                         | NA                          | -                    | -                                       | -                           | (Yeager et al., 2007)                |
|                       | PCC 9802   | <i>Microcoleus vaginatus</i> | NA                                                      | NA                          | -                    | -                                       | -                           | (Garcia-Pichel et al., 1996)         |
| Concrete sidewalk     | CCME E 029 | <i>Chroococci diopsis</i>    | NA                                                      | NA                          | -                    | -                                       | -                           | (Bili et al., 2000)                  |
|                       | O-89-Cgs   | <i>Chlorogloeopsis</i>       | NA                                                      | NA                          | -                    | -                                       | -                           | (Garcia-Pichel and Castenholz, 1991) |
| Soil / Cycad symbiont | PCC 73102  | <i>Nostoc</i>                | NA                                                      | NA                          | -                    | -                                       | -                           | (Rippka et al., 1979)                |



| Origin                    | Strain   | Morphogenus <sup>a</sup> | Closest 16S rRNA-based cultivated relative <sup>b</sup> | Similarity (%) <sup>c</sup> | <i>hoxH</i> presence | Closest <i>hoxH</i> allele <sup>b</sup> | Similarity (%) <sup>c</sup> | Reference  |
|---------------------------|----------|--------------------------|---------------------------------------------------------|-----------------------------|----------------------|-----------------------------------------|-----------------------------|------------|
| <b>Freshwater</b>         |          |                          |                                                         |                             |                      |                                         |                             |            |
| Pond<br>Lake-<br>Plankton | PCC 6803 | <i>Synechocystis</i>     | NA                                                      | NA                          | +                    | <i>Synechocystis</i> sp. PCC6803        | 100                         | -          |
|                           | 901      | <i>Anabaena/Nostoc</i>   | <i>Anabaena oscillarioides</i><br>BO HINDAK 1984/43     | 97                          | +                    | <i>Lyngbya majuscula</i><br>CCAP 1446/4 | 83                          | This study |
|                           | 081      | <i>Oscillatoria</i>      | <i>Leptolyngbya</i> sp. LEGE                            | 92                          | +                    | <i>Lyngbya majuscula</i><br>CCAP 1446/4 | 69                          | This study |
|                           | 005      | <i>Oscillatoria</i>      | <i>Limnothrix redekei</i><br>2LT25S01                   | 100                         | +                    | <i>Spirulina subsalsa</i><br>FACHB351   | 77                          | This study |
|                           | NH       | <i>Anabaena/Nostoc</i>   | <i>Nostoc</i> sp. 'Mollenhauer 1:1-067'                 | 98                          | -                    | -                                       | -                           | This study |
| Hot<br>Spring             | 011      | <i>Oscillatoria</i>      | <i>Limnothrix redekei</i><br>2LT25S01                   | 100                         | +                    | <i>Spirulina subsalsa</i><br>FACHB351   | 77                          | This study |
|                           | HS       | <i>Oscillatoria</i>      | <i>Limnothrix redekei</i><br>2LT25S01                   | 99                          | +                    | <i>Lyngbya majuscula</i><br>CCAP1446/4  | 70                          | This study |

| Origin                | Strain      | Morpho<br>genus <sup>a</sup> | Closest 16S<br>rRNA-<br>based<br>cultivated<br>relative <sup>b</sup> | Simila<br>rity<br>(%) <sup>c</sup> | <i>hoxH</i><br>prese<br>nce | Closest <i>hoxH</i><br>allele <sup>b</sup>         | Similar<br>ity(%) <sup>c</sup> | Reference                           |
|-----------------------|-------------|------------------------------|----------------------------------------------------------------------|------------------------------------|-----------------------------|----------------------------------------------------|--------------------------------|-------------------------------------|
| <b>Marine benthos</b> |             |                              |                                                                      |                                    |                             |                                                    |                                |                                     |
| Microbial<br>mat      | BL G        | <i>Lyngbya</i>               | <i>Lyngbya<br/>aestuarii</i><br>PCC7419                              | 99                                 | +                           | <i>Lyngbya<br/>majuscula</i><br>CCAP1446/4         | 89                             | This study                          |
|                       | BL E        | <i>Lyngbya</i>               | <i>Lyngbya<br/>aestuarii</i><br>PCC7419                              | 99                                 | +                           | <i>Lyngbya<br/>majuscula</i><br>CCAP1446/4         | 89                             | This study                          |
|                       | BL J        | <i>Lyngbya</i>               | <i>Lyngbya<br/>aestuarii</i><br>PCC7419                              | 99                                 | +                           | <i>Lyngbya<br/>majuscula</i><br>CCAP1446/4         | 89                             | This study                          |
|                       | BL AA       | <i>Lyngbya</i>               | <i>Lyngbya<br/>aestuarii</i><br>PCC7419                              | 99                                 | +                           | <i>Lyngbya<br/>majuscula</i><br>CCAP1446/4         | 89                             | This study                          |
|                       | BM<br>001   | <i>Microco<br/>leus</i>      | <i>Microcoleus<br/>chthonoplast<br/>es</i> CCY9602                   | 100                                | +                           | <i>Spirulina<br/>subsalsa</i><br>FACHB351          | 72                             | This study                          |
|                       | BM<br>002   | <i>Microco<br/>leus</i>      | <i>Microcoleus<br/>chthonoplast<br/>es</i> CCY9602                   | 100                                | +                           | <i>Spirulina<br/>subsalsa</i><br>FACHB351          | 72                             | This study                          |
|                       | BM<br>003   | <i>Microco<br/>leus</i>      | <i>Microcoleus<br/>chthonoplast<br/>es</i> CCY9602                   | 100                                | +                           | <i>Spirulina<br/>subsalsa</i><br>FACHB351          | 72                             | This study                          |
|                       | BC 2        | <i>Calothri<br/>x</i>        | <i>Calothrix</i><br>sp. BC001                                        | 97                                 | -                           | -                                                  | -                              | This study                          |
|                       | BC 4        | <i>Calothri<br/>x</i>        | <i>Calothrix</i><br>sp. BC001                                        | 97                                 | -                           | -                                                  | -                              | This study                          |
|                       | 7Y          | <i>Oscillat<br/>oria</i>     | <i>Spirulina</i> sp.<br>SCyano57                                     | 93                                 | -                           | -                                                  | -                              | This study                          |
|                       | 7C          | <i>Oscillat<br/>oria</i>     | <i>Spirulina</i> sp.<br>SCyano57                                     | 93                                 | -                           | -                                                  | -                              | This study                          |
|                       | G066        | <i>Oscillat<br/>oria</i>     | <i>Geitlerinem<br/>a</i> sp. kopara-<br>FE                           | 99                                 | +                           | ND                                                 | -                              | This study                          |
|                       | PCC<br>7419 | <i>Lyngbya</i>               | NA                                                                   | NA                                 | +                           | <i>Lyngbya<br/>majuscula</i><br>CAP1446/4          | 92                             | -                                   |
|                       | NDN-I       | <i>Microco<br/>leus</i>      | NA                                                                   | NA                                 | +                           | <i>Spirulina<br/>subsalsa</i><br>FACHB351          | 76                             | (Garcia-<br>Pichel et<br>al., 1996) |
|                       | PCC<br>7420 | <i>Microco<br/>leus</i>      | NA                                                                   | NA                                 | +                           | <i>Microcoleus<br/>chthonoplaste<br/>s</i> PCC7420 | 100                            | (Garcia-<br>Pichel et<br>al., 1996) |
|                       | MPI<br>N303 | <i>Euhalot<br/>hece</i>      | NA                                                                   | NA                                 | +                           | <i>Euhalothece<br/>halophytica</i><br>ATCC43922    | 82                             | (Garcia-<br>Pichelet al.,<br>1998)  |

| Origin                | Strain     | Morpho<br>genus <sup>a</sup>                       | Closest 16S<br>rRNA-<br>based<br>cultivated<br>relative <sup>b</sup> | Simila<br>rity<br>(%) <sup>c</sup> | <i>hoxH</i><br>prese<br>nce | Closest <i>hoxH</i><br>allele <sup>b</sup> | Similar<br>ity(%) <sup>c</sup> | Reference                          |
|-----------------------|------------|----------------------------------------------------|----------------------------------------------------------------------|------------------------------------|-----------------------------|--------------------------------------------|--------------------------------|------------------------------------|
| Carbonate<br>surfaces | BECD3<br>0 | <i>Calothri</i><br><i>x</i> sp.                    | NA                                                                   | NA                                 | -                           | -                                          | -                              | (Chaconet<br>al., 2006)            |
|                       | BC008      | <i>Mastigo</i><br><i>coleus</i><br><i>testarum</i> | NA                                                                   | NA                                 | -                           | -                                          | -                              | (Garcia-<br>Pichelet al.,<br>2010) |

<sup>a</sup>: Assigned according to Bergey's manual (Castenholz, 2001).

<sup>b</sup>: According to NCBI BLAST search

<sup>c</sup>: Similarity of the isolate's sequence to that of the closest *cultivated* strain in database

NA stands for not applicable, ND for not determined

Table 2. Parameters in the dynamics of anaerobic H<sub>2</sub> production with excess exogenous reductant in cyanobacterial strains during 24 h long standard specific activity assays.

| Strain | H <sub>2</sub> production | $R_H$ (nmol (µg chl.a) <sup>-1</sup> h <sup>-1</sup> ) | [H <sub>2</sub> ] <sub>M</sub> (µM) | $T_R$ (h)         |
|--------|---------------------------|--------------------------------------------------------|-------------------------------------|-------------------|
| RP110  | -                         |                                                        |                                     |                   |
| RP114  | -                         |                                                        |                                     |                   |
| RP116  | -                         |                                                        |                                     |                   |
| RP118  | -                         |                                                        |                                     |                   |
| RP102  | -                         |                                                        |                                     |                   |
| RP 108 | -                         |                                                        |                                     |                   |
| FGP-7A | -                         |                                                        |                                     |                   |
| 9802   | -                         |                                                        |                                     |                   |
| O-89-  | -                         |                                                        |                                     |                   |
| Cgs    | -                         |                                                        |                                     |                   |
| 73102  | -                         |                                                        |                                     |                   |
| 6803   | +                         | 31 ±7                                                  | 31 ±6                               | 0.06 ±0.02        |
| HS     | +                         | 18 ±11                                                 | 41 ±15                              | 1.66 ±0.08        |
| 901    | +                         | 40 ±6                                                  | 9 ±5                                | 8.66 ±1.55        |
| 005    | +                         | 35 ±8                                                  | 17 ±5                               | 3.66 ±2.50        |
| NH     | -                         |                                                        |                                     |                   |
| BL G   | +                         | 123 ±22                                                | >24 <sup>a,b</sup>                  |                   |
| BL E   | +                         | 75 ±10                                                 | 320 <sup>a</sup>                    | ND                |
| BL J   | +                         | 156 ±77                                                | 487 ±12                             | ND                |
| BL AA  | +                         | 57 ±25                                                 | 389 ±10                             | ND                |
| BM 001 | +                         | 17 ±5                                                  | >14 <sup>a,b</sup>                  |                   |
| BM 002 | +                         | 14 ±3                                                  | >80 <sup>a,b</sup>                  | ND                |
| BC 2   | -                         |                                                        |                                     |                   |
| BC 4   | -                         |                                                        |                                     |                   |
| 7Y     | -                         |                                                        |                                     |                   |
| 7C     | -                         |                                                        |                                     |                   |
| G066   | +                         | 15 ±3                                                  | 62 <sup>a</sup>                     | 9.12 <sup>a</sup> |
| 7419   | +                         | 115 ±28                                                | 200 <sup>a</sup>                    | ND                |
| NDN-I  | +                         | 44 ±18                                                 | 315 <sup>a</sup>                    | ND                |
| 7420   | +                         | 18 ±10                                                 | 68 ±24                              | ND                |
| N303   | +                         | 14 ±13                                                 | 21 ±6                               | 0.81 ±0.34        |
| BECD30 | -                         |                                                        |                                     |                   |
| BC008  | -                         |                                                        |                                     |                   |

a: maximal value is given if less than 3 replicates available. Lack of replication was due to incidental termination of an assay before 24 h of monitoring

b: concentration of H<sub>2</sub> still increasing at assay termination

ND: none detected within 24 h

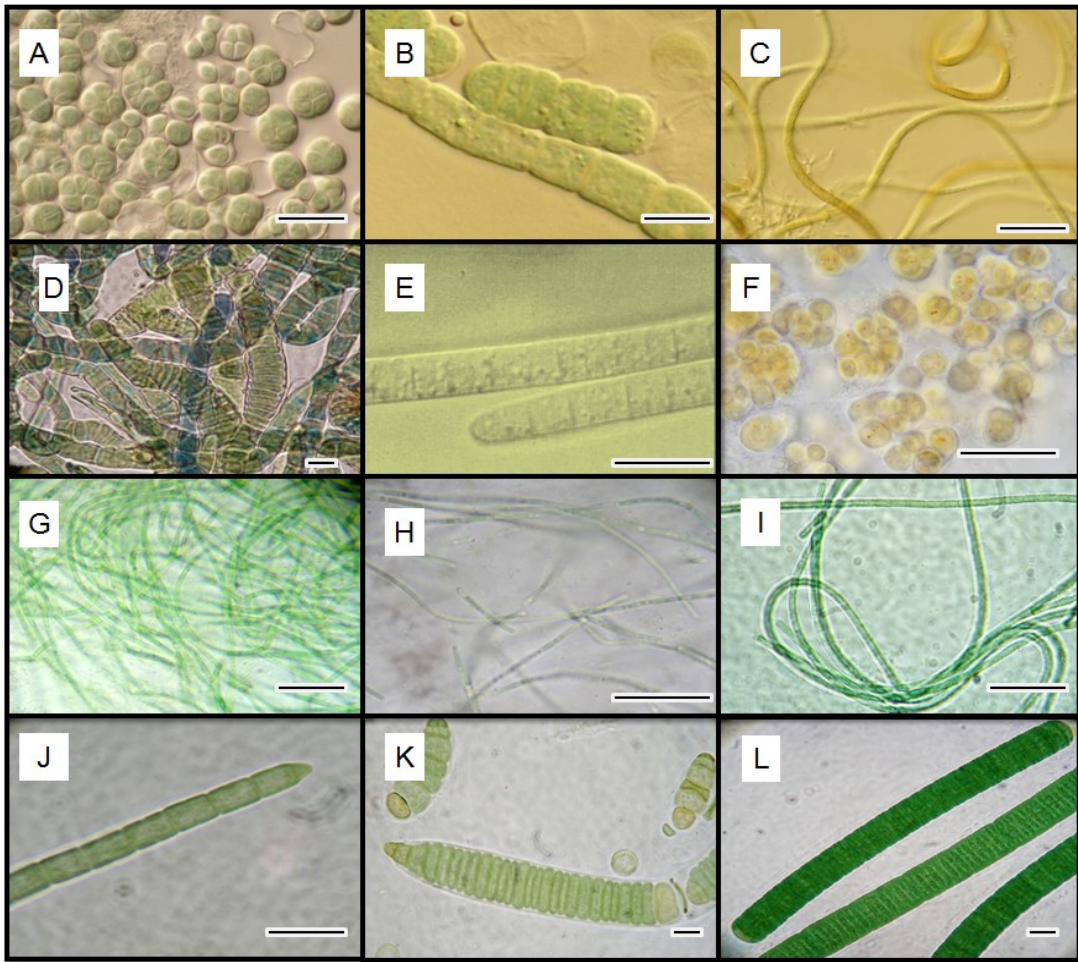


Figure 1. Morphological diversity of the strains surveyed. Scale bars represent 10  $\mu\text{m}$ . A: *Chroococidiopsis* RP114. B: *Tolypothrix* RP102, detail of filament, C: *Leptolyngbya* RP108, a thin filamentous oscillatorian. D: *Mastigocoleus testarum* BC003. E: *Microcoleus vaginatus* PCC 9802. F: *Euhalothece* sp. MPI N303. G: *Limnothrix* sp. 005. H: *Limnothrix* sp. 081. I: *Oscillatoria* sp. 7C. J: *Microcoleus chthonoplastes* BM 001. K: *Calothrix* sp. BC2, with terminal heterocysts. L: *Lyngbya* sp. BL J.

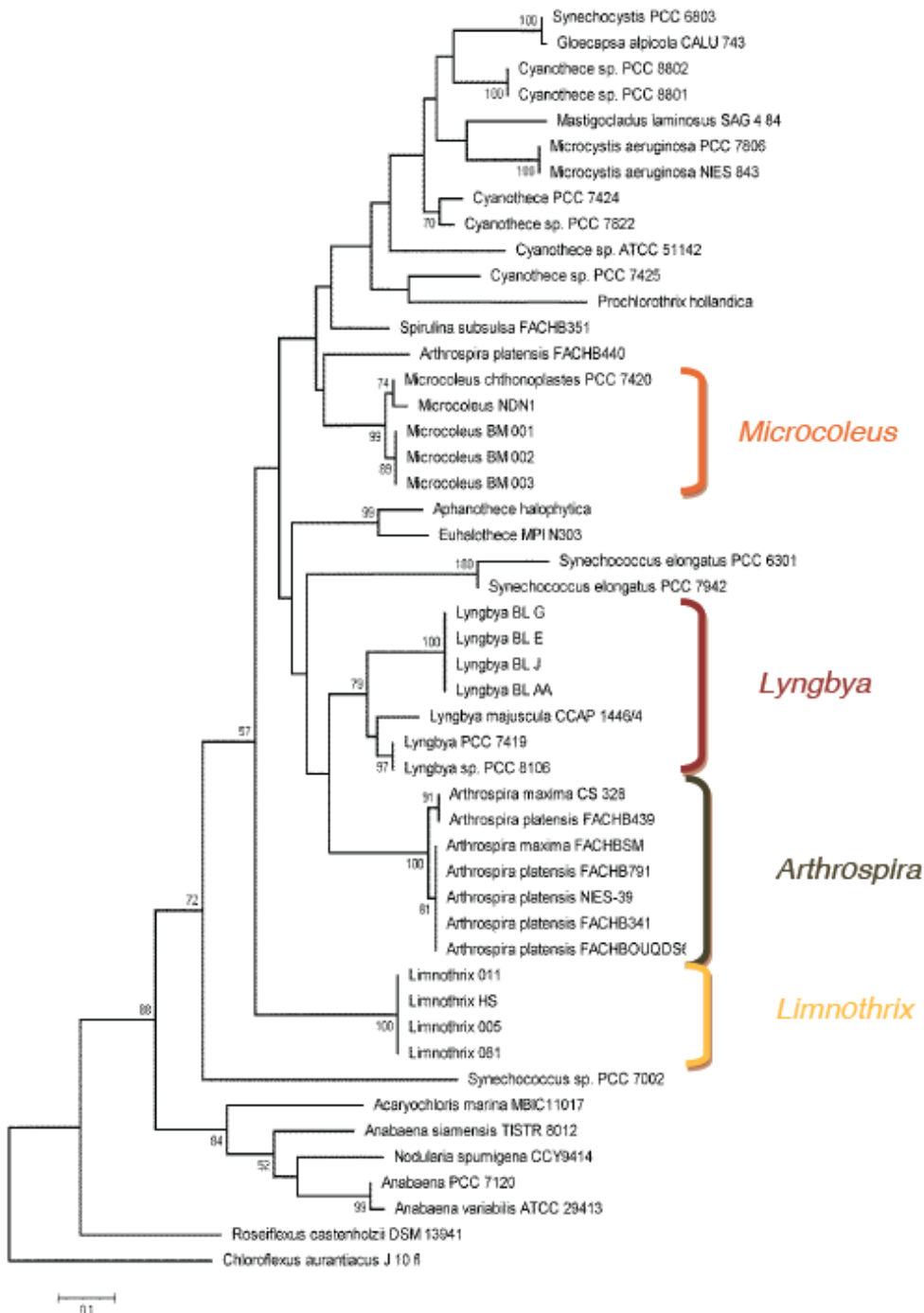


Figure 2. Maximum likelihood tree of the HoxH cyanobacterial sequences (based on 300 amino acids). Bootstrap values (%) for well-supported nodes (>50) are indicated, obtained from analyses of 1000 replicate trees. The sequence from *Chloroflexus aurantiacus* J-10-fl was used as outgroup. Coherent clades are indicated to the right.

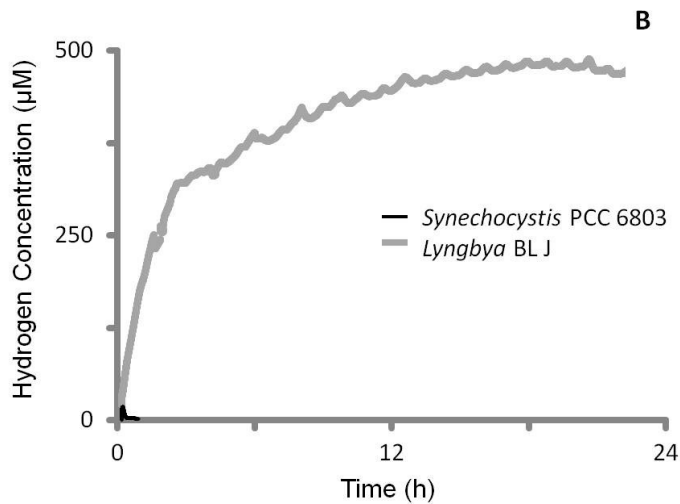
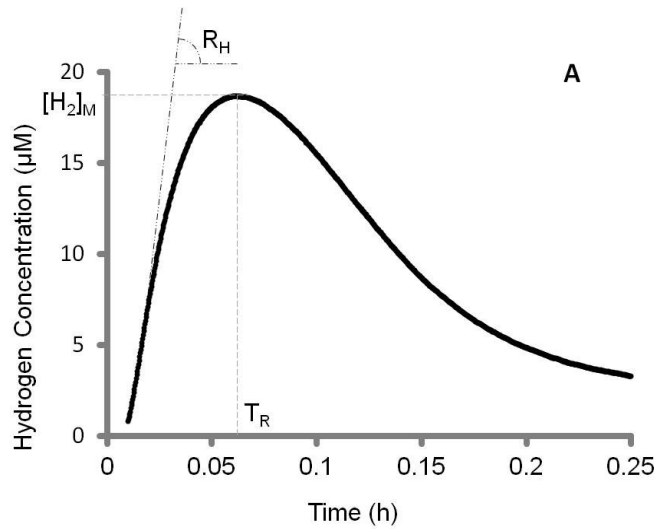
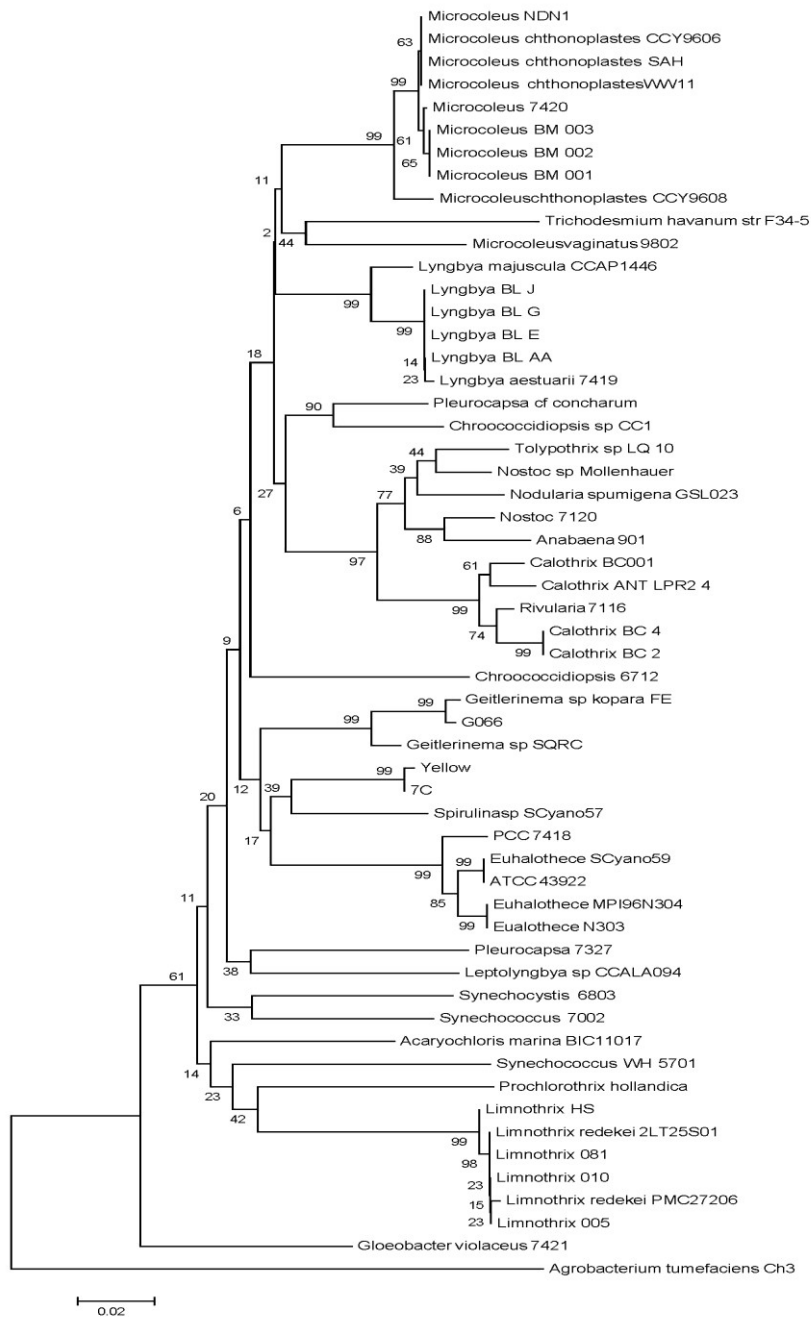


Figure 3. Patterns in  $H_2$  concentration dynamics during specific activity assays. A. *Synechocystis* sp. PCC 6803 (Pattern 1)  $H_2$  evolution with a maximal initial rate of  $H_2$  production,  $R_H$ , decreasing fast and reaching a relatively low maximum concentration of  $H_2$   $[H_2]_M$  at time  $T_R$ , after which hydrogenase activity reverses, bringing  $[H_2]$  to zero. B. Pattern 2, exemplified by *Lyngbya aestuarii* BL J (curve in gray), also with an initial maximal rate, slowly decreasing to reach a high value of  $[H_2]_M$  and without significant reversal into  $H_2$  consumption. Curve in black shows the  $H_2$  production dynamics of *Synechocystis* sp. PCC 6803 (shown previously in A) for scale comparison.

**Supplementary Information**



Supplementary Information. 16S rRNA Phylogenetic tree based on 600bp sequence. *Agrobacterium tumefaciens*Ch3 was used as an outgroup. Evolutionary distance was determined by Kimura 2 parameter model and the tree was constructed by maximum likelihood algorithm using MEGA 5.



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**III. POWERFUL FERMENTATIVE HYDROGEN EVOLUTION OF  
PHOTOSYNTHATE IN THE CYANOBACTERIUM *LYNGBYA AESTUARII* BL J  
MEDIATED BY A BIDIRECTIONAL HYDROGENASE.**

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*(In Review: Environmental Microbiology)*



## **Abstract**

Cyanobacteria are considered good models for biohydrogen production because they are relatively simple organisms with a demonstrable ability to generate H<sub>2</sub> under certain physiological conditions. However, they present low amounts of H<sub>2</sub> production, a facile reaction reversal towards H<sub>2</sub> consumption, and enzyme sensitivity to O<sub>2</sub>. Previously, it was found that strains of the cyanobacteria *Lyngbya aestuarii* and *Microcoleus chthonoplastes* obtained from marine intertidal cyanobacterial mats displayed stronger potential for H<sub>2</sub> production than many other cyanobacteria. Because of their ecological origin in environments that become quickly anoxic in the dark, we hypothesized that this capacity may have evolved to serve a role in the fermentation of the photosynthate. Here we show that, when forced to ferment, these cyanobacteria display similarly desirable characteristics of physiological H<sub>2</sub> production. Among them, the strain *L. aestuarii* BL J had the fastest specific rates and attained the highest H<sub>2</sub> concentrations during fermentation of photosynthate, which proceeded via a mixed-acid fermentation pathway to yield acetate, ethanol, lactate, H<sub>2</sub>, CO<sub>2</sub> and pyruvate. Contrary to expectations, the H<sub>2</sub> yield per mole of glucose was only average compared to that of other cyanobacteria. Nevertheless, the high specific rates and H<sub>2</sub> concentrations coupled with the lack of reversibility of the enzyme, at the expense of internal, photosynthetically generated reductants, makes *L. aestuarii* BL J and/or its enzymes, a potentially feasible platform for large-scale H<sub>2</sub> production.

## **1. Introduction**

Cyanobacteria have great potential to act as cell factories, because they have the ability to use light to split water, potentially generating H<sub>2</sub> (Weaver et al., 1980; Akkerman et al., 2002; Prince and Kheshgi, 2005). They do in fact evolve H<sub>2</sub> naturally, but as a by-product of N<sub>2</sub> fixation or as an end-product of fermentation. Very transitorily, a burst in H<sub>2</sub> production is sometimes seen when the light is switched on suddenly during dark fermentative metabolism. The latter is the only known form of direct “photohydrogen” production in cyanobacteria. The enzyme responsible for N<sub>2</sub> fixation, nitrogenase, does also reduce protons and releases H<sub>2</sub> as an unavoidable side reaction (Peterson and Burris, 1978; Eisbrenner and Evans, 1983). This process requires significant cellular energy inputs and most often does not result in any net H<sub>2</sub> production, because it is reoxidized via an uptake hydrogenase (Peterson and Burris, 1978). It has been proposed that the enzyme bidirectional hydrogenase is involved in fermentative H<sub>2</sub> production (Stal and Moezelaar, 1997; Troshina et al., 2002) and photohydrogen generation (Appel et al., 2000). As the name implies this enzyme has the ability to both produce and oxidize H<sub>2</sub> (Fujita and Myers, 1965). Direct photohydrogen production in cyanobacteria is extremely short-lived (a few seconds) with rather negligible H<sub>2</sub> yields (Appel et al., 2000). Fermentative H<sub>2</sub> production represents an indirect hydrophotolytic route that proceeds through an organic intermediary (glycogen). It is relatively long-lived (hours) with somewhat better H<sub>2</sub> yields than the photohydrogen production (Cournac et al., 2002; Troshina et al., 2002). Fermentative H<sub>2</sub> production is in fact the natural mode by which cyanobacteria release H<sub>2</sub> for extended periods of time in nature, making it of potential biotechnological interest.

Cyanobacteria have the intrinsic ability to ferment in order to survive dark anaerobic conditions (Gottschalk, 1979). Depending on strain, they have been shown to carry out a variety of fermentative metabolisms including the homolactate, homoacetate, heterolactate, and mixed acid pathways (Stal and Moezelaar, 1997). The homolactate pathway primarily produces lactate (Oren and Shilo, 1979), whereas the heterolactate pathway evolves lactate along with ethanol and acetate (Heyer et al., 1989). The homoacetate pathway produces mostly acetate along with minor quantities of lactate, CO<sub>2</sub>, and H<sub>2</sub> (Heyer et al., 1989; De Philippis, 1996). The mixed acid fermentation pathway is known to produce acetate, lactate, ethanol, formate and/or CO<sub>2</sub> and H<sub>2</sub> (Van der Oost et al., 1989; Moezelaar et al., 1996; Aoyama et al., 1997; Troshina et al., 2002). Thus, the mixed acid and, to a certain extent, the homoacetate pathways result in H<sub>2</sub> production.

Cyanobacteria are not known to respire external electron acceptors other than O<sub>2</sub>, and thus, when subjected to nighttime anoxia must resort to fermentation in order to maintain ATP production and regenerate excess reduction equivalents. A classic example of an environment conducive to this are cyanobacterial benthic mats (Walter, 1976; Bauld, 1981; Javor and Castenholz, 1981). In these mats, oxygenic photosynthetic activity causes the top mat layers to become supersaturated with O<sub>2</sub> during the daytime, but strong respiration rates overwhelm diffusive O<sub>2</sub> import in the dark, establishing strong anoxia (Revsbech et al., 1983) and forcing the constituent cyanobacteria to ferment its daytime photosynthate. Fermentation products have been directly detected in hot spring

microbial mats (Anderson et al., 1987; Nold and Ward, 1996). Amongst the mat inhabiting cyanobacteria, fermentation has been reported in *Oscillatoria terebriformis* (Richardson and Castenholz, 1987) and *Synechococcus* sp. strains OS-A and OS-B' (Steunou et al., 2006) from hot springs. Fermentation has also been studied in marine microbial mat-building *Lyngbya aestuarii* CCY 9616 (= PCC 8106, also known as *Oscillatoria limosa* in the early literature) and *Oscillatoria* sp. SAG 3192 (Garcia-Pichel et al., 1996) (also referred to as *M. cthonoplastes* 11 or *M. cthonoplastes* SAG 3192 before (Stal and Krumbein, 1985)). *L. aestuarii* CCY 9616 follows a homoacetate-heterolactate pathway (Heyer et al., 1989) whereas *Oscillatoria* sp. SAG 3192 ferments via a mixed acid fermentation pathway (Moezelaar et al., 1996).

Owing to the presence, multiplicity and avidity of potential H<sub>2</sub> consumers in the complex microbial communities where H<sub>2</sub> is being produced, steady state concentrations of H<sub>2</sub> tend to remain very low, usually undetectable in natural systems (Ebert and Brune, 1997; Schink, 1997). This general rule finds a clear exception in some intertidal microbial mats, where intense net H<sub>2</sub> accumulation and export has been reported (Skyring et al., 1989; Hoehler et al., 2001). Similarly, it was observed that intertidal microbial mats from Baja California, maintained in a greenhouse setting for more than three years under an artificial intertidal regime, continue to produce H<sub>2</sub> at night, exporting significant amounts to the overlying waters (D. Hoffmann, J. Maldonado, personal communication). The organisms fermenting under such conditions must thus be able to produce H<sub>2</sub> even against high partial pressures in the mat. In an earlier report surveying a set of cyanobacterial strains for H<sub>2</sub> production in presence of excess reductants, two different

Patterns were observed wherein Pattern 1 (as was known from fresh water strains such as *Synechocystis* sp. PCC 6803) exhibited lower rates and steady state H<sub>2</sub> concentrations followed by uptake of most of the produced H<sub>2</sub> and Pattern 2 (as was known from *Lyngbya aestuarii* and *Microcoleus chthonoplastes* strains from the marine intertidal mats) exhibited much higher rates, steady state H<sub>2</sub> concentrations and a lack of H<sub>2</sub> uptake throughout the assay (Kothari et al., 2012). Indeed, the cyanobacterial strains isolated from these mats displayed an extraordinary potential to produce/sustain H<sub>2</sub> under the unusually high concentrations of H<sub>2</sub> prevailing in their micro-environment in standard assays (Kothari et al., 2012). Now these studies are extended to include their innate H<sub>2</sub> evolving capacity under fermentative conditions, using microbiological and genomic evidence.

## **2. Material and Methods**

### **2.1 Strains, media and growth conditions.**

Five strains of cyanobacteria were used for this work. *Lyngbya aestuarii* BL J, *Lyngbya aestuarii* BL AA and *Microcoleus chthonoplastes* BM003 were isolated from marine intertidal microbial mats in Baja California (Kothari et al., 2012). *Microcoleus chthonoplastes* PCC 7420 was originally isolated from a microbial mat in a salt marsh, Woods Hole, Massachusetts. *Synechocystis* sp. PCC 6803, originally a freshwater isolate, has been used in this study since it is a popular model cyanobacterium for biohydrogen research. The latter two strains were obtained from the Pasteur Culture Collection (<http://www.pasteur.fr/ip/easysite/pasteur/en/institut-pasteur>). *L. aestuarii* strains were grown in IMR medium with 3% salinity (Eppley et al., 1968), modified to incorporate

commercially available Instant Ocean salt mixture instead of natural seawater. *M. chthonoplastes* strains were grown in a 1:1 mixture of IMR and ASN III media (Rippka et al., 1979) with 3% salinity. *Synechocystis* sp. PCC 6803 was grown in BG11 medium (Rippka et al., 1979). All media were supplemented with 0.5 mM (final concentration) NiSO<sub>4</sub> to ensure adequate supply of nickel for the working of Ni-Fe hydrogenases.

The strains *L. aestuarii* BL J, *L. aestuarii* BL AA and *M. chthonoplastes* BM003 were clonal and monocyanobacterial, but not always axenic. Therefore phase contrast microscopy was used to confirm that the level of contaminating bacteria was less than 0.01% of the cyanobacterial biomass (assessed as bio-volume) for the physiological experiments. *M. chthonoplastes* PCC 7420 and *Synechocystis* sp. PCC 6803 were always used in axenic form.

For the purpose of whole genome sequencing, an axenic culture of *L. aestuarii* BL J was established by picking up the motile hormogonia developing on IMR medium – 1% nobel agar plates (Rippka, 1988). These hormogonia were allowed to grow on IMR–PGY medium 1% nobel agar plates (0.25% peptone, 0.25% yeast extract, 0.25% glucose, 1.5% agar) and axenicity was determined by lack of heterotrophic bacterial growth, and through direct microscopic observation. All strains were maintained in 250 ml Erlenmeyer flasks, with 100 ml medium, starting with similar amounts of inoculum in presence of light at an intensity of 100  $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ .

## **2.2 Fermentative H<sub>2</sub> production assay.**

All strains were subjected to two different sets of growth conditions for the fermentation assays. In the first set, filaments were grown in continuous light without any bubbling (CL). In the second set, the strains were grown in 12-hour light and 12-hour dark cycle. The cultures were bubbled with air in the light period and with N<sub>2</sub> in the dark period to establish anoxia, forcing cells to ferment. These conditions are referred to as “Light Oxidic Dark Anoxic” (L<sub>O</sub>D<sub>A</sub>) conditions. All cultures were incubated for a minimum of two weeks before making any measurements. The assay itself was carried out in the dark using whole cells (*in vivo*) without the addition of any external reductants. Particularly, for the cultures growing in L<sub>O</sub>D<sub>A</sub> conditions, the assay was commenced at the beginning of the dark period. Small pea size pellets of biomass from log phase cultures were placed in a custom-made, 2.5 ml volume chamber with continuous stirring. Fresh medium was added to completely fill the chamber, which was sealed with no headspace. The chamber was endowed with two miniature Clark-type electrodes to monitor H<sub>2</sub> and O<sub>2</sub> partial pressure. The electrodes were connected to a pico-ammeter set at a voltage of 0.8 V for H<sub>2</sub> and -0.8V for O<sub>2</sub>. An A/D converter allowed the current signal data to be read on a computer using Sensor Trace Basic software. All electrodes and peripherals were from Unisense, Aarhus, Denmark. Before each measurement, the H<sub>2</sub> electrode was subject to a 2-point calibration in culture medium bubbled with either air (0% H<sub>2</sub>) or with a custom gas mixture (10% H<sub>2</sub> in N<sub>2</sub>). The O<sub>2</sub> electrode was also subjected to a 2-point calibration system wherein culture medium was bubbled with either air (21% O<sub>2</sub>) or with 100% N<sub>2</sub> (0% O<sub>2</sub>). During calibration the sealed chamber showed negligible leakage over a period of 2-3 hours.

Each strain was measured in independent triplicate experiments. From the electrode traces, the following parameters were derived: the initial specific rate of fermentative H<sub>2</sub> production,  $R_H$ , the maximum steady state H<sub>2</sub> concentration reached,  $[H_2]_M$  and the time after which H<sub>2</sub> production stopped and reverted to consumption,  $T_R$ . The measurements lasted for 24 h. At the end of the assay, chlorophyll was extracted from the biomass with 100% methanol and measured spectrophotometrically (MacKinney, 1941). This was done to ensure that all assays had roughly comparable biomass and to obtain specific rates of initial H<sub>2</sub> production (i.e, per unit biomass).

### **2.3 Analysis of fermentation metabolism in *L. aestuarii* BL J.**

*L. aestuarii* BL J grown in L<sub>0</sub>D<sub>A</sub> conditions was used. A couple of hours before the onset of the dark anaerobic period, biomass was harvested by centrifugation and acclimatized in fresh medium, and washed twice with fresh medium to get rid of any existing fermentation products. The filaments can form tight clumps, and hence attempts were made to break the clumps using forceps and mild sonication at the lowest speed setting for 4 s to get a homogeneous cell suspension. It was required to split the biomass into two aliquots with approximately equal amounts of biomass to conduct the initial and final analyses quantifying the fermentation substrates and products. Since a non-destructive procedure that does not impart any kind of stress to the cells was necessary for quantifying the biomass, wet weights were used. Optical density cannot be employed for biomass estimation given the filamentous and clumpy nature of this strain. As described



below, since only the wet weights from the two halves of the same filter were compared to each other, the errors in biomass estimation were minimized.

For obtaining two aliquots with approximately equal amounts of biomass using wet weights, the following procedure was adopted. The biomass was vacuum filtered onto a 0.4  $\mu\text{m}$  polycarbonate filter to establish a homogenous layer on it. The filter was cut into half, biomass scrapped off and the wet weight of the cells on each half was measured. The biomass from each half of the filter was then introduced into a 10 ml serum bottle (one for initial and one for final analyses). To each bottle 5 ml of fresh medium was added and the bottles were sealed. To confirm that no fermentation products were present at the onset of the assay, 1 ml of medium was drawn out from the “initial” serum bottle for High Pressure Liquid Chromatography (HPLC) analysis. The rest was immediately frozen in liquid  $\text{N}_2$  and stored at  $-80\text{ }^\circ\text{C}$  to be used eventually to measure the initial fermentable glycogen content in the cells. The “final” serum bottle was bubbled with nitrogen for 30 min to establish anoxia. Gas Chromatography (GC) confirmed the absence of  $\text{O}_2$  and the serum bottle was incubated in the dark on a rocking bench for 24 h at  $25\text{ }^\circ\text{C}$ . After incubation, 1 ml of medium was withdrawn for HPLC analyses of organic acids and ethanol. Hydrochloric acid was added to the serum bottle to lower the pH of the solution and ensure that all the inorganic carbon was present as  $\text{CO}_2$ . The gases in the headspace ( $\text{CO}_2$ ,  $\text{H}_2$ , and/or  $\text{O}_2$ ) were sampled by syringe and quantified by GC equipped with a thermal conductivity detector. GC was performed with Helium as the carrier gas and the concentrations of  $\text{H}_2$  and  $\text{CO}_2$  in the headspace were estimated as described (Parameswaran et al., 2009). Total amounts of gas in the bottle were back-calculated according to volumetric partitioning. Subsequently, the biomass in the serum bottle was

frozen in liquid N<sub>2</sub> and kept at -80 °C for eventual glycogen content quantification. Glycogen was extracted as per Ernst et al.(1984) and quantified using a BioAssay Systems glycogen assay kit. To determine organic acids and ethanol, HPLC was employed. All the liquid samples for HPLC were filtered through a 0.2 mm PVDF filter and the filtrate used. The HPLC was performed with Aminex HPX - 87H column at 50 °C with 2.5 mM sulfuric acid as eluent at a flow rate of 0.6 mL/min using a photodiode array and refractive index indicator (Parameswaran et al., 2009). Most of the common products of bacterial fermentation can be detected under these settings.

#### **2.4 Whole genome sequencing.**

Genomic DNA preparations of *L. aestuarii* BL J were obtained by PCI (phenol; chloroform; isoamyl alcohol) extraction (Countway et al., 2005). DNA was quantified using fluorometry of ethidium bromide-stained 1% agarose electrophoresis gels and sent for commercial MiSeq 250 Illumina sequencing at the University of Maryland School of Medicine, Institute for Genome Sciences, Genome Resource Center. Genomic DNA libraries were constructed for sequencing on the Illumina platform using the NEBNext® DNA Sample Prep Master Mix Set 1 (New England Biolabs, Ipswich, MA). First, the DNA was fragmented with the ultrasonicator, Covaris E210. Libraries were prepared using the manufacturer's protocol. The DNA was purified between enzymatic reactions and the size selection of the library was performed with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA). Libraries were sequenced using the 250 bp paired-end protocol on an Illumina MiSeq sequencer. Raw data from the sequencer was processed using Illumina's RTA and CASAVA pipeline software, which includes image analysis,

base calling, sequence quality scoring, and index demultiplexing. Data were then processed through in-house pipelines for sequence assessment and quality control and FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). These pipelines report numerous quality metrics and perform a megablast-based contamination screen. By default, the quality control pipeline assesses basecall quality and truncates reads where the median Phred-like quality score falls below Q20 (implying more than 99% accuracy in base calling). Data were randomly sampled to create datasets at 100 X, 150 X and 200 X coverage. The different datasets were assembled with MaSuRCA (Zimin et al., 2013). The genome assembled by MaSuRCA was subjected to the Institute of Genomic Science prokaryotic annotation pipeline forms the core of the IGS Annotation Engine. The total contig base pair size was estimated to be 6.87 Mb. The draft genome contained 439 contigs of which 124 were large contigs (>10,000bp size). About 6.44 Mb of the entire genome was present in large contigs. The genome was also annotated using RAST -Rapid Annotation using Subsystem Technology (Aziz et al., 2008). The genomic sequence of *L. aestuarii* BL J was checked for the presence of orthologs of genes potentially coding for key enzymes involved in fermentation. Protein sequences coding for cyanobacterial fermentation enzymes from NCBI database were used as query and Psi BLAST was performed against the entire *L. aestuarii* BL J genome. Given that the genome is not closed, the absence of any one gene does not necessarily imply its absence from the genome, as there is a small probability that it is found in unsequenced regions. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AUZM00000000. The version described in this paper is version AUZM01000000.

### **3. Results**

#### **3.1 Fermentative H<sub>2</sub> production.**

The strains *L. aestuarii* BL J, *L. aestuarii* BL AA, *M. cthonoplastes* BM 003, *M. cthonoplastes* PCC 7420 and *Synechocystis* sp. PCC 6803 were all capable of fermentative H<sub>2</sub> production. All strains reached anoxic conditions solely by dark respiration without the addition of any external reductants or anoxia-inducing compounds. No fermentable substrates were externally provided. As soon as anoxia was established, H<sub>2</sub> production commenced without any measurable lag time in all strains.

Some variation in the parameters of fermentative H<sub>2</sub> production could be detected. These main parameters are initial specific rate,  $R_H$ , the maximum H<sub>2</sub> steady state concentration,  $[H_2]_M$ , and the time after which the enzyme reverts in direction,  $T_R$  (see Figure 1). Table 1 gathers information on these parameters for all the 5 tested strains. In general, *Lyngbya* and *Microcoleus* strains from microbial mats produced H<sub>2</sub> faster and could reach higher equilibrium concentrations of H<sub>2</sub> than the standard strain *Synechocystis* sp. PCC 6803. *Lyngbya* and *Microcoleus* strains did not consume the H<sub>2</sub> produced during the assay (for up to 24 h) unlike *Synechocystis* sp. PCC 6803. The highest specific rate of H<sub>2</sub> production was seen in *L. aestuarii* BL AA and the highest steady state H<sub>2</sub> concentration was seen in *M. cthonoplastes* BM 003.

### 3.2 Optimization of fermentative H<sub>2</sub> production.

Attempts were made to optimize the H<sub>2</sub> produced by acclimatizing the cells to 12h Light/Dark cycles wherein the cells were made anaerobic in the dark (L<sub>0</sub>D<sub>A</sub>). *Synechocystis* sp. PCC 6803 showed no significant improvements by this preconditioning in any of the parameters.

The specific rates and steady state concentrations of fermentative H<sub>2</sub> production attained in *Lyngbya* strains, but not those of *Microcoleus* strains, could be enhanced when cultures were pre-acclimated to recurrent nighttime anaerobiosis during growth. On subjecting the strains to L<sub>0</sub>D<sub>A</sub> preconditioning all strains retained their characteristic feature of reversibility of reaction direction (or the lack of it). *L. aestuarii* BL J was characterized by the highest specific rates and steady state concentrations of H<sub>2</sub>. The  $R_H$  of *L. aestuarii* BL J grown in L<sub>0</sub>D<sub>A</sub> condition doubled compared to that of cells grown in continuous light conditions; its  $[H_2]_M$  increased twenty eight fold (Figure 2). In L<sub>0</sub>D<sub>A</sub> conditions, the strain BL J performed exceptionally better than the standard *Synechocystis* sp. PCC 6803, (its  $R_H$  was twenty-fold faster and  $[H_2]_M$  forty-five fold higher (Figure 2)). While calculating the average  $R_H$  of *L. aestuarii* BL J grown in L<sub>0</sub>D<sub>A</sub> one abnormally high specific rate of 44.2 nmol (μg chl. *a*)<sup>-1</sup>h<sup>-1</sup> was removed from the tally. Had this been incorporated, the  $R_H$  value would have been 13.1 ± 17.5 nmol (μg chl. *a*)<sup>-1</sup>h<sup>-1</sup>.

Attempts made to further optimize the fermentative H<sub>2</sub> production from *L. aestuarii* BL J in L<sub>0</sub>D<sub>A</sub> conditions by varying the salinity, nickel and nitrate content in the medium did not lead to any significant increase in the specific rates or steady state H<sub>2</sub> concentrations

(data not shown). On starving cells of nickel, however, a fifteen-fold decrease in the specific rates of fermentative H<sub>2</sub> production was observed, indicating the nickel dependency of the enzyme system involved in the process.

### **3.3 Fermentation in *L. aestuarii* BL J.**

Along with H<sub>2</sub>, the fermentative production of lactate, ethanol, acetate and CO<sub>2</sub> was observed. The ratio of the products of fermentation remained similar for the three independent replicate experiments. Small amounts of pyruvate were also excreted. Other common bacterial fermentation products such as formate, succinate, propionate and butyrate were never detected. Table 2 depicts a quantitative balance analysis of the fermentation process in *L. aestuarii* BL J. Ethanol and acetate were produced in equimolar amounts. Lactate, ethanol and acetate were seen in 1:2:2 molar ratios. One mol of H<sub>2</sub> was produced for every two moles of CO<sub>2</sub>. The stoichiometry of carbon recovery and the recovery of H available, was 100.07% and 100.58%, respectively.

### **3.4 Genomic evidence.**

Orthologs of the following genes involved in glycogen metabolism in *L. aestuarii* BL J were detected: Glucose-1-phosphate adenylyltransferase, glycogen synthase, ADP-glucose transglucosylase, glycogen branching enzyme (GH-57-type, archaeal), 1,4-alpha-glucan (glycogen) branching enzyme (GH-13-type), glycogen debranching enzyme, glycogen phosphorylase, and 4-alpha-glucanotransferase (amylomaltase). Orthologs of genes coding for all the enzymes involved in the pentose phosphate pathway and glycolysis, potential routes for the breakdown of glucose into pyruvate, were detected.

Genomic evidence for the presence of mixed acid fermentation pathway was clear. *L. aestuarii* BL Jhas orthologs coding for the enzymes pyruvate:ferredoxin oxidoreductase, ferredoxin:NADP oxidoreductase, bidirectional hydrogenase, lactate dehydrogenase, phosphotransacetylase, acetaldehyde dehydrogenase, alcohol dehydrogenase, and acetate kinase. Based on the fermentation products obtained experimentally and the presence of these orthologs, the pathway proposed for fermentative degradation of glycogen is depicted in Figure 3. Notably, the gene for pyruvate formate lyase, involved in the reversible conversion of pyruvate and coenzyme-A into formate and acetyl-CoA, was not detected. This was consistent with a lack of formate amongst the fermentation products. Also worth noting is that no genomic evidence could be found for formate hydrogen lyase, involved in splitting of formate into H<sub>2</sub> and CO<sub>2</sub>.

#### **4. Discussion**

We had previously demonstrated that in standard H<sub>2</sub> production assays, strains of *Lyngbya* and *Microcoleus* displayed optimal H<sub>2</sub> evolution characteristics compared to a large number of other strains from diverse environments (Kothari et al., 2012). Since excess reductants were externally provided during that assay, the results likely are maximal potential specific rates and do not actually reflect physiologically realistic conditions. This naturally prompted us to study the actual H<sub>2</sub> production capacity of these strains. Here we demonstrate that all the 4 strains studied had the capacity to produce fermentative H<sub>2</sub> naturally, at the expense of photosynthetically fixed carbon, as did the standard strain *Synechocystis* sp. PCC 6803, which is included for reference. As reported earlier (Cournac et al., 2002), the fermentative H<sub>2</sub> evolution in *Synechocystis* sp.

PCC 6803 commenced without any lag time, as was the case in *Microcoleus* and *Lyngbya* strains, and unlike what was observed in *Microcystis aeruginosa* M-176 (Asada and Kawamura, 1984). In general, the specific H<sub>2</sub> production rates and the steady state concentrations under fermentative conditions were about an order of magnitude lower than the potential seen in standard assays in presence of excess reductant (Kothari et al., 2012). The *Microcoleus* and *Lyngbya* strains from the marine intertidal mats are capable of sustained fermentative H<sub>2</sub> production for at least 24 h. This was in accordance to the Pattern 2 hydrogen production earlier reported in these strains via the hydrogenase activity assay wherein sustained H<sub>2</sub> production was also measured for up to 24 h.

In comparison, the H<sub>2</sub> production phase did not last more than about 3 h in the standard strain *Synechocystis* sp. PCC 6803. This is consistent with the notion that cyanobacteria isolated from environments experiencing recurring nighttime anoxia (marine microbial mats) may be innately better H<sub>2</sub> producers, thus validating a general approach of bio-prospecting in Nature for biotechnologically useful properties of extant but little known microbes.

That H<sub>2</sub> production metabolism in intertidal mat harboring *Lyngbya* strains was enhanced by prior exposure to recurrent dark anaerobic growth conditions was expected under the premise that this type of fermentative metabolism would be regulated and thus subject to induction. This was clearly not the case in *Microcoleus* strains, where the capacity for fermentative H<sub>2</sub> generation, while high, seemed to be constitutive. *Lyngbya* typically colonizes microbial mats that desiccate frequently and may not be exposed to nighttime



anoxia as frequently as *Microcoleus*, which tends to dominate mats lower in the tidal gradient, with more recurrent flooding or always flooded (Javor and Castenholz, 1981; Rothrock and Garcia-Pichel, 2005). Perhaps the different responsiveness of the fermentative H<sub>2</sub> physiology has to do with this differential ecology. *Synechocystis* sp. PCC 6803, which has been in culture since 1968 does presumably not see many periods of dark anoxia during cultivation, and displayed a low-yield, non-inducible H<sub>2</sub> physiology.

When forced to ferment on a diel cycle, the highest specific rate and steady state H<sub>2</sub> concentration was exhibited by *L. aestuarii* BL J. In fact, the optimized steady state H<sub>2</sub> concentrations in fermentative assays in this strain were only three-fold lower in magnitude than those seen in standard assays in the presence of excess reductant (Kothari et al., 2012). This is suggestive of the presence of a strong H<sub>2</sub> producing system, which, might be of particular fitness value in the uniquely H<sub>2</sub> accumulating intertidal mats. Since the  $p\text{H}_2$  in the mat pore-waters of the cyanobacterial layers of these mats in the nighttime is high (6-10 $\mu\text{M}$  H<sub>2</sub>, D. Hoffmann, J. Maldonado, personal communication), the resident cyanobacteria must be able to ferment even in the presence of high H<sub>2</sub>, a scenario that surely would benefit from unusually powerful H<sub>2</sub> producing physiology. Recently, it has been proposed that *Microcoleus* sp. are the dominant hydrogenogens in the Elkhorn Slough mats based on the bidirectional hydrogenase transcript levels (Burow et al., 2012). However, based on the H<sub>2</sub>producing physiologies of the strains isolated from the intertidal mats of Baja California, we propose that *L. aestuarii* strains can also play a major role as hydrogenogens in these systems.

In comparison to *Synechocystis* sp. PCC 6803, the initial rates of H<sub>2</sub> production were 17-fold higher in *L. aestuarii* BL J in the optimized fermentation assays. Most likely, this is due to increased amounts of bidirectional hydrogenase in the strain BL J or because *Synechocystis* sp. PCC 6803 employed alternative strategies in dark anaerobic conditions to regenerate NAD(P)<sup>+</sup>. In optimized fermentation assays, the steady-state H<sub>2</sub> concentration in *Synechocystis* sp. PCC 6803 was 45-fold lower than the strain BL J. The high steady-state H<sub>2</sub> concentration could result from a low intracellular pH or high ratios of NAD(P)H/NAD(P)<sup>+</sup> in the strain BL J. The high H<sub>2</sub> concentrations could also result from higher amounts of glycogen or more efficient means of metabolizing glycogen in the strain BL J. Presence of an alternate electron donor with a more negative redox potential than NAD(P)H could also make the H<sub>2</sub> production reaction more feasible in the strain BL J. It is also possible that *Synechocystis* sp. PCC 6803 employs alternative strategies in fermentative conditions to regenerate NAD(P)<sup>+</sup>, thus not producing much of H<sub>2</sub>. It is most likely that the decline in H<sub>2</sub> concentration observed in the strain PCC 6803 is due to some sort of enzymatic regulation.

In certain cyanobacteria, the specific rates of H<sub>2</sub> evolution could be increased by increasing the medium's nickel content (*Arthrospira maxima* CS-328 (Dismukes et al., 2008)), its salinity (*Synechocystis* sp. PCC 6803 (Baebprasert et al., 2010)), or by imposing N limitation (*Gloeocapsa alpicola* CALU 743 (Troshina et al., 2002) and *Oscillatoria* sp. Miami BG7 (Kumazawa and Mitsui, 1981)). Our preliminary experiments attempting to further boost the fermentative H<sub>2</sub> production in *L. aestuarii* BL

J by varying the salinity, nickel content, or limiting cells of nitrate did not yield any favorable results (data not shown).

It may be of interest to discuss the fermentative metabolism of strain BL J in comparison to the few other closely related cyanobacterial species whose fermentation has been studied in any detail. *L. aestuarii* CCY 9616, a strain phylogenetically close to *L. aestuarii* BL J (99% identity based on 16S rRNA), behaves quite differently: it can ferment trehalose, its osmoprotectant, via the homoacetate pathway into mostly acetate with small amounts of H<sub>2</sub> and CO<sub>2</sub> (Heyer et al., 1989). It can also ferment glycogen by a heterolactic fermentation pathway producing equimolar amounts of ethanol, lactate and CO<sub>2</sub> (Heyer et al., 1989). It is unknown if *L. aestuarii* BL J has the same osmoprotectant, or if it can be metabolized via a similar fermentation. This is however unlikely, in that neither the enzyme trehalase, involved in trehalose breakdown, nor the carbon monoxide dehydrogenase, a key enzyme for homoacetic fermentation, could be detected in its genome. Notably, the genome had orthologs of the enzymes trehalose synthase and trehalose-6-phosphate synthetase involved in trehalose synthesis. This may be worth a direct assessment. Another closely related strain, the thermophilic *Oscillatoria terebriformis*, was also fermentatively distinct; it produced lactate by anaerobic degradation of photosynthetically accumulated glycogen, without producing acetate, butyrate, isobutyrate or n-butyrate (Richardson and Castenholz, 1987). In comparison, *L. aestuarii* BL J produces equimolar amounts of ethanol and acetate, a characteristic feature also seen in more distantly related *Oscillatoria* sp. SAG 3192, which also uses the

mixed acid fermentation pathway (Moezelaar et al., 1996). Phylogeny seems thus to be a poor predictor of fermentative pathways.

*L. aestuarii* BL J has all the products and the genes coding for all enzymes of the mixed acid fermentation pathway. In view of the stoichiometric ratios of the products of fermentation (Table 2), our strain does not follow any one ideal fermentation pathway or even a combination of pathways. This is also the case for *Cyanothece* sp. PCC 7822 (Van der Oost et al., 1989), *Microcystis* sp. PCC 7806 (Moezelaar and Stal, 1994), and *Oscillatoria* sp. SAG 3192 (Moezelaar et al., 1996). The fermentation pathway is likely to be similar to that observed in *Microcystis* sp. PCC 7806 (Moezelaar and Stal, 1994) and *Gloeocapsa alpicola* CALU 743 (Troshina et al., 2002). The high recovery percentages in terms of recovery of C (100.07%) and the recovery of H (100.58%) indicates that no major fermentation product or substrate was missing.

It may also be of interest to positively identify the enzyme responsible for the intense H<sub>2</sub> evolution observed in this study, as this may be the target of future studies. H<sub>2</sub> production in fermentative pathways can be either of *Enteric* type, with H<sub>2</sub> evolving from formate breakdown by formate hydrogen lyase, or of *Clostridial* type, wherein H<sub>2</sub> is evolved by pyruvate:ferredoxin oxidoreductase along with a hydrogenase (Hallenbeck, 2009). Based on its genome, *L. aestuarii* BL J lacks both enzymes for the *Enteric* pathway, but contains a pyruvate:ferredoxin oxidoreductase and two [Ni-Fe] hydrogenases, an uptake-type hydrogenase and a bidirectional-type hydrogenase. One of these two must be involved, given that fermentative H<sub>2</sub> production in *L. aestuarii* BL J was found to be Ni-dependent.

Because uptake hydrogenases are not known to produce H<sub>2</sub> under physiological conditions (Houchins and Burris, 1981; Houchins, 1984) it is proposed that the Ni-Fe bidirectional hydrogenase is the source of the fermentative H<sub>2</sub> produced in *L. aestuarii* BL J.

Since *L. aestuarii* BL J accumulates very high concentrations of H<sub>2</sub>, it was of significance to characterize the molar conversion ratio of glucose to H<sub>2</sub>. Theoretically 1 mole of glucose can give rise to a maximum of 4 moles of H<sub>2</sub> via fermentation (Thauer, 1977). Amongst cyanobacteria, this theoretical maximum has been observed only in *Gloeocapsa alpicola* CALU 743 (Troshina et al., 2002). *Microcystis* sp. PCC 7806 yields 0.51 (Moezelaar and Stal, 1994) and *Cyanothece* sp. PCC 7822 yields 0.76 moles /mol (Van der Oost et al., 1989). *L. aestuarii* BL J, at 0.6 moles/mol, was certainly not the best. The strong H<sub>2</sub> production characteristics of *L. aestuarii* BL J cannot be attributed to a high glucose to H<sub>2</sub> molar conversion ratio, but likely reside in the bidirectional hydrogenase enzyme system. A cursory comparison of the bidirectional hydrogenase locus in strain BL J does not reveal any conspicuous differences with those of other cyanobacteria. Heterologous expression of the hydrogenase of *L. aestuarii* BL J in other cyanobacteria may shed some light in the prediction.

H<sub>2</sub> production in *L. aestuarii* BL J is unique in that it seems impervious to end product inhibition, making it desirable for long-term applications. High steady state concentrations of H<sub>2</sub> are also desirable in that they simplify harvesting. The prospect of genome modifications to boost H<sub>2</sub> production, however, is not very likely in this strain,

which belongs to a large group of cyanobacteria for which genetic manipulation tools are still lacking.

## Tables/Figures

Table 1. Parameters characterizing the dynamics of fermentative H<sub>2</sub> production in various cyanobacterial strains along with the effect of prior exposure to nighttime anoxia. Averages  $\pm$  standard deviation with n=3 independent determinations shown for each strain and condition.

| Strain                               | Growth Conditions             | $R_H(\text{nmol } (\mu\text{g chl.a})^{-1}\text{h}^{-1})$ | $[H_2]_M(\mu\text{M})$ | $T_R(\text{h})$  |
|--------------------------------------|-------------------------------|-----------------------------------------------------------|------------------------|------------------|
| <i>L. aestuarii</i> BL J             | L <sub>0</sub> D <sub>A</sub> | 5.3 $\pm$ 2.7                                             | 159.8 $\pm$ 30.4       | $\geq$ 24        |
|                                      | CL                            | 2.8 $\pm$ 1.7                                             | 5.6 $\pm$ 4.0          | >24              |
| <i>L. aestuarii</i> BL AA            | L <sub>0</sub> D <sub>A</sub> | 4.0 $\pm$ 2.9                                             | 87.8 $\pm$ 43.0        | $\geq$ 24        |
|                                      | CL                            | 9.4 $\pm$ 0.1                                             | 4.70 $\pm$ 2.9         | >24              |
| <i>M. chthonoplastes</i><br>PCC 7420 | L <sub>0</sub> D <sub>A</sub> | 0.4 $\pm$ 0.4                                             | 2.3 $\pm$ 0.6          | $\geq$ 24        |
|                                      | CL                            | 0.4 $\pm$ 0.2                                             | 2.6 $\pm$ 1.2          | >24              |
| <i>M. chthonoplastes</i><br>BM 003   | L <sub>0</sub> D <sub>A</sub> | 0.6 $\pm$ 0.1                                             | 2.7 $\pm$ 1.5          | $\geq$ 24        |
|                                      | CL                            | 0.8 $\pm$ 0.1                                             | 35.5 $\pm$ 17.4        | >24              |
| <i>Synechocystis</i> sp.<br>PCC 6803 | L <sub>0</sub> D <sub>A</sub> | 0.3 $\pm$ 0.2                                             | 3.5 $\pm$ 2.8          | 5 $\pm$ 6.6      |
|                                      | CL                            | 0.2 $\pm$ 0.1                                             | 2.7 $\pm$ 2.0          | 3.3<br>$\pm$ 3.2 |

CL: Continuous Light; L<sub>0</sub>D<sub>A</sub>: light oxic and dark anoxic 12 h cycles

Table 2. Stoichiometry of fermentation of endogenous polyglucose and the fermentation mass balance of *L. aestuarii* strain BL J, after 24 h of dark incubation.

|                     | mol   | mol/100 mol<br>glucose | mol C/ 100<br>mol glucose | H<br>available | H available<br>mol/100 mol<br>glucose |
|---------------------|-------|------------------------|---------------------------|----------------|---------------------------------------|
| <i>Products</i>     |       |                        |                           |                |                                       |
| Glucose             | 13.27 | 100.00                 | 600.00                    | 24.00          | 2400.00                               |
| Pyruvate            | 0.46  | 3.48                   | 10.44                     | 10.00          | 34.81                                 |
| Lactate             | 5.91  | 44.54                  | 133.61                    | 12.00          | 534.44                                |
| Acetate             | 11.44 | 86.21                  | 172.41                    | 8.00           | 689.66                                |
| Ethanol             | 11.34 | 85.48                  | 170.95                    | 12.00          | 1025.72                               |
| H <sub>2</sub>      | 8.37  | 63.07                  | 0.00                      | 2.00           | 126.15                                |
| CO <sub>2</sub>     | 15.00 | 113.04                 | 113.04                    | 0.00           | 0.00                                  |
| <i>Recovery (%)</i> |       |                        | <i>100.07</i>             |                | <i>100.58</i>                         |

H available is determined by oxidizing a compound to carbon dioxide with water.

For example,  $C_6H_{12}O_6 + 6H_2O \rightarrow 24H + 6CO_2$

Thus, the H available value for glucose is 24(Gottschalk, 1979).



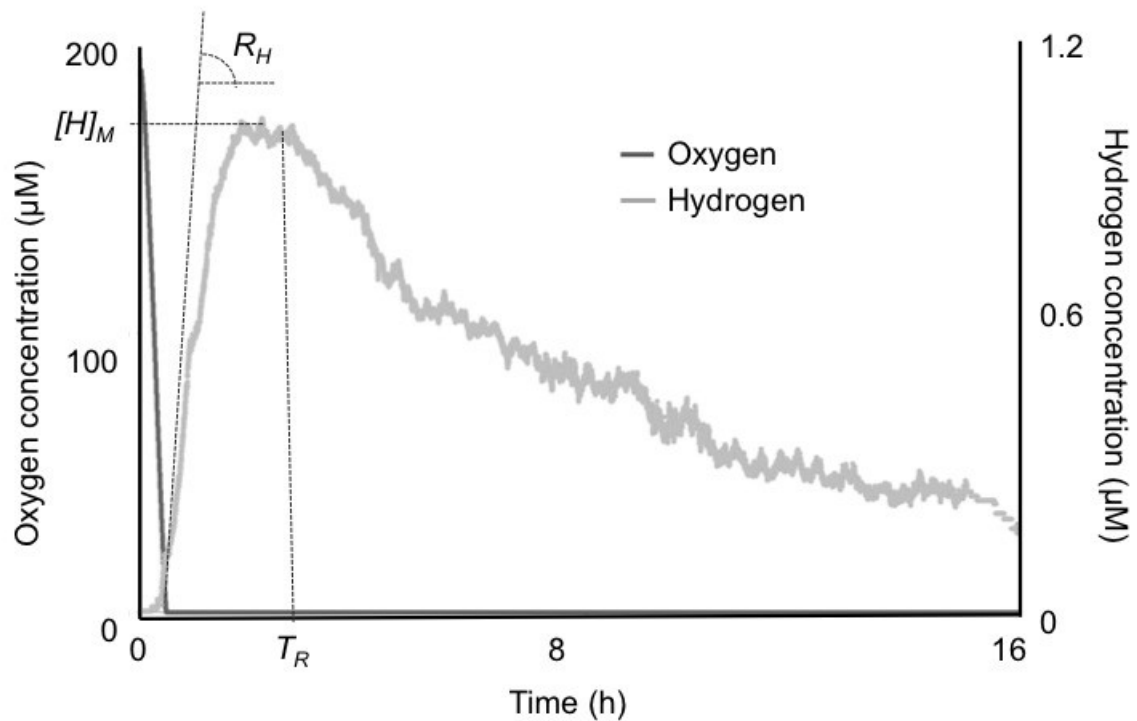


Figure 1. Oxygen and hydrogen concentrations during a fermentative  $H_2$  production assay in *Synechocystis* sp. PCC 6803. Anoxia is established in a few minutes by respiration in dark followed by onset of fermentative  $H_2$  production. The parameters of the  $H_2$  production studied are the maximal initial rate of  $H_2$  production,  $R_H$ , the maximum steady state  $H_2$  concentration  $[H_2]_M$  and the time, after which hydrogenase reverses in direction,  $T_R$ .

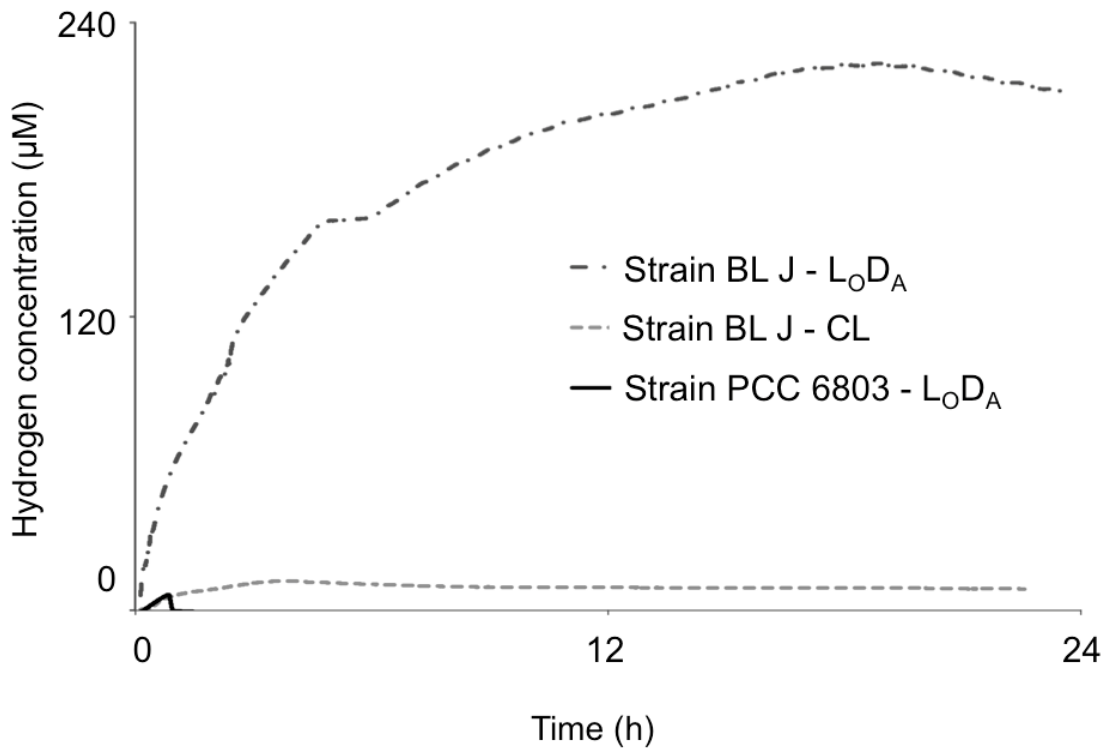


Figure 2. Comparison of the dynamics of fermentative hydrogen production in continuous light (CL) grown *L. aestuarii* BL J, along with Light oxic Dark anoxic (L<sub>0</sub>D<sub>A</sub>) cycle grown *L. aestuarii* BL J and *Synechocystis* sp. PCC 6803.

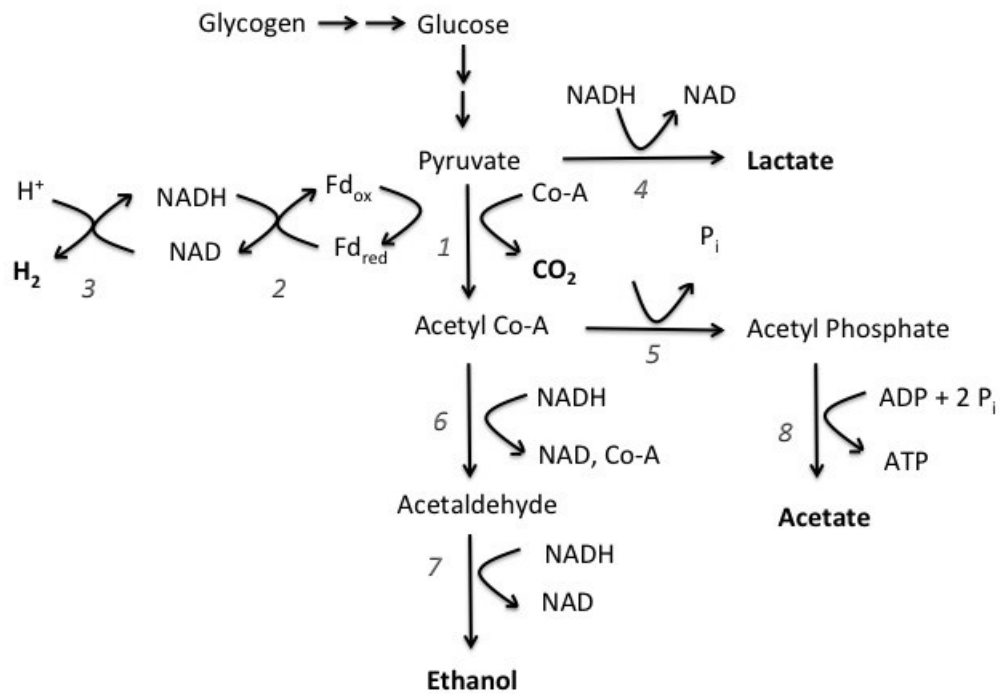


Figure 3. Proposed pathway for glycogen (polyglucose) fermentation in *L. aestuarii* BL J. Compounds in bold are fermentation end products. The numbers refer to the enzymes involved: 1, pyruvate ferredoxin oxidoreductase; 2, ferredoxin NADP oxidoreductase; 3, bidirectional hydrogenase; 4, lactate dehydrogenase; 5, phosphotransacetylase; 6, acetaldehyde dehydrogenase; 7, alcohol dehydrogenase; 8, acetate kinase.

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**IV. COMPARATIVE GENOMIC ANALYSES OF THE CYANOBACTERIUM  
*LYNGBYA AESTUARI* BL J, A POWERFUL HYDROGEN PRODUCER.**

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*(Accepted: Frontiers in Microbiology)*

## **Abstract**

The filamentous, non-heterocystous cyanobacterium *Lyngbya aestuarii* is an important contributor to marine intertidal microbial mats system worldwide. The recent isolate *L. aestuarii* BL J is an unusually powerful H<sub>2</sub>producer. Here we report a morphological, ultrastructural and genomic characterization of this strain to set the basis for future systems studies and applications of this organism. The filaments contain *circa* 17 µm wide trichomes, composed of stacked disk-like short cells (2 µm long), encased in a prominent, laminated exopolysaccharide sheath. Cellular division occurs by transversal centripetal growth of cross-walls, where several rounds of division proceed simultaneously. Filament division occurs by cell self-immolation of one or groups of cells (necridial cells) at the breakage point. Short, sheath-less, motile filaments (hormogonia) are also formed. Morphologically and phylogenetically, *L. aestuarii* belongs to a clade of important cyanobacteria that include members of the marine *Trichodesmium* and *Hydrocoleum* genera, as well as terrestrial *Microcoleus vaginatus* strains, and alkalyphilic strains of *Arthrospira*. A draft-genome of strain BL J was compared to those of other cyanobacteria in order to ascertain some of its ecological constraints and biotechnological potential. The genome had an average GC content of 41.1%. Of the 6.87 Mb sequenced, 6.44 Mb was present as large contigs (>10,000 bp). The genome contained 6515 putative protein-encoding genes, of which 43% encode proteins of known functional role, 26% corresponded to proteins with domain or family assignments, 19.6% encode conserved hypothetical proteins, and 11.3% encode apparently unique hypothetical proteins. The strain's genome reveals its adaptations to a life of exposure to intense solar radiation and desiccation. It likely employs the storage

compounds glycogen and cyanophycin but no polyhydroxyalkanoates, and can produce the osmolytes trehalose and glycine betaine. According to its genome, BL J strain also has the potential to produce a plethora of products of biotechnological interest such as Curacin A, Barbamide, Hemolysin-type calcium-binding toxin, the sunscreens scytonemin and mycosporines, as well as heptadecane and pentadecane alkanes. With respect to H<sub>2</sub> production, initial comparisons of the genetic architecture and sequence of relevant genes and loci, along with a comparative model of protein structure of the bidirectional [NiFe]-hydrogenase, did not reveal conspicuous differences that could explain its unusual H<sub>2</sub>-producing capacity.

## **1. Introduction**

Cyanobacteria are deemed ecologically important for their contributions to global nitrogen fixation and carbon flux (Paul, 1978; Capone et al., 1997) and their global biomass in the order of 10<sup>14</sup> g C (Garcia-Pichel et al., 2003) is a relevant component of both terrestrial and marine biomes. Biotechnologically, they possess a great potential to act as cell factories by virtue of their relatively simple structure, minimal nutritional requirements and an ability to synthesize a wide variety of metabolites. In this study, we focus on the cyanobacterium *L. aestuarii* BL J, a representative of an ecologically important species in marine intertidal mats endowed with an extraordinary capacity to produce H<sub>2</sub> (Kothari et al., 2012) and thus of potential biotechnological interest.

In nature, *L. aestuarii* forms extensive microbial mats in many marshes and intertidal mud flats (Horodyski and Bloeser, 1977; Mir et al., 1991; Paerl et al., 1991; Lopez-Cortes

et al., 2001). Microbial mats are dense laminated benthic communities of micro-organisms (Stal and Caumette, 1993). They present an environment that is extreme in many respects, with repeated cycles of desiccation and wetting, intense exposure to ultraviolet (UV) radiation, and changing regimes of salinity (as cell may be exposed to hypersaline marine waters to very dilute meteoric precipitation). The intertidal mats that are exposed to desiccation are restricted in their anaerobic components (Rothrock and Garcia-Pichel, 2005). Although, as in most microbial communities, H<sub>2</sub> is a key metabolite in interspecies metabolic linking, it rarely accumulates to concentrations high enough to be exported in significant amounts. This has been linked to the diverse populations of potential H<sub>2</sub> consumers that inhabit these communities (Ebert and Brune, 1997; Schink, 1997). However, certain intertidal microbial mats, where intense net H<sub>2</sub> export occurs (Skyring et al., 1989; Hoehler et al., 2001), are an exception. Earlier, we found that, when subjected to the standard H<sub>2</sub> production assays in presence of excess reductants, two different patterns were observed (Chapter II). The strains from marine intertidal microbial mats exhibited higher rates, steady state H<sub>2</sub> concentrations and a lack of H<sub>2</sub> uptake (Pattern 2 H<sub>2</sub> production), in comparison to those from fresh water, which exhibited lower rates and steady state H<sub>2</sub> concentrations followed by uptake of most of the produced H<sub>2</sub> (Pattern 1, as was known from standard strain of *Synechocystis* sp. 6803) (Kothari et al., 2012). The fresh water strain *Anabaena* sp. PCC 7120 also conformed to Pattern 1 H<sub>2</sub> production. Thus, the cyanobacteria inhabiting the microbial mats (Pattern 2 H<sub>2</sub> production) must have evolved extraordinarily powerful hydrogenogenic abilities to produce/sustain H<sub>2</sub> under the unusually high concentrations of H<sub>2</sub> prevailing in their micro-environment. Of the Pattern 2 cyanobacteria, *L. aestuarii* BL J had the highest

rates and reached the highest steady state H<sub>2</sub> concentrations (Kothari et al., 2012). Additionally, this strain also displayed an inducible, strong natural hydrogenogenic capacity under dark fermentative conditions (Chapter III). Infact, the rate of fermentative H<sub>2</sub> evolution in the strain BL J was ten times higher than that reported for the closely related strain *Oscillatoria limosa* (= *Lyngbya aestuarii* PCC 8106) (Heyer et al., 1989). Hence it was of interest to study the genome of this strain, with a special emphasis on the H<sub>2</sub> producing system and the ecophysiological constraints imposed by the environment of origin.

## **2. Material and Methods**

### **2.1 Strains and culture conditions**

*Lyngbya aestuarii* strain BL J, a recent isolate from marine intertidal microbial mats in Baja California (Kothari et al., 2012), was grown in IMR medium set at 3% seawater salinity (Eppley et al., 1968), modified to incorporate a commercially available seawater salt mixture (Instant Ocean), instead of natural seawater, and supplemented with 0.5 μM (final concentration) NiSO<sub>4</sub>. The strain was maintained in axenic form on IMR media 1% agar plates (since it is less susceptible to contamination than liquid media) at room temperatures and also cryopreserved for long-term storage. Since the strain grew faster in liquid media, it was grown in 250 ml Erlenmeyer flasks, with 100 ml media in presence of 100 μmol photon m<sup>2</sup>s<sup>-1</sup> light at room temperature to obtain cyanobacterial biomass for microscopy and DNA extractions.

## **2.2 Confocal microscopy**

A small pellet from liquid culture was washed and resuspended in 300 µl of fresh IMR medium. To stain the DNA, 4', 6-diamidino-2-phenylindole, DAPI (2 µg/ml final concentration) was added. To stain the exopolysaccharide sheath, Fluorescein-labeled lectin (wheat germ agglutinin; 5 µg/ml final concentration) was added. The preparation was incubated for 1 h in dark at room temperature, and the filaments were washed thrice with fresh IMR medium. Cells were then imaged on glass slides under sealed glass coverslips using a Leica SP5 LASER scanning confocal microscope under a 63 X oil immersion objective. Excitation wavelength for DAPI was at 405 nm, excitation for Fluorescein-labeled lectin was at 488 nm, and photosynthetic pigments were excited at 561 nm. The corresponding emissions were detected at 445 - 465 nm, 520-535 nm and 675 - 715 nm. The images presented were maximum Z projections with corrected background (to eliminate background noise). All images were acquired at 1024 X 1024 pixel resolution. All images were manipulated using the image J software suite (Schneider et al., 2012). Imaging of hormogonia, which showed very fast gliding motility, required the use of carbonyl cyanide m-chlorophenyl hydrazine (10 µM final concentration, for 15 min, Santa Cruz biotech) as an uncoupler of proton motive force, to render them immotile.

## **2.3 Transmission electron microscopy (TEM)**

Unless stated all steps were carried out at room temperature. Samples were primarily fixed in 2.5% glutaraldehyde in IMR medium for 2 hours, followed by 4 washes in seawater medium over a period of approximately 1 h. Samples were then secondarily

fixed with 1% osmium tetroxide in IMR medium for 2 h. Osmium tetroxide was removed by washing with several changes of deionized water over a period of approximately 1 h, followed by block-staining of the cells with 2% aqueous uranyl acetate for 1 h. Uranyl acetate was removed by thorough washing in deionized water. Due to poor preliminary results thought to be caused by incomplete dehydration and resin penetration of the cells, the standard TEM preparation procedure was modified to incorporate increased resident-time in dehydrating agent and epoxy resin, as well as additional gradient steps. This involved: 10% (v/v of reagent grade acetone/deionized water), 20%, 40%, 60%, 80% and 100% anhydrous acetone for four consecutive changes, with each step lasting 30 min. A similar modified approach was employed during infiltration with Spurr's epoxy resin (Spurr, 1969): 10% (v/v of resin/anhydrous acetone), 20%, 30%, 50%, 75%, and four consecutive changes of pure resin. Each step was under rotation for 12 h except for the 10% step, which was 3.5 h. Samples were flat-embedded in fresh resin on Teflon-spray coated glass slides and overlaid with a solid Teflon strip, then polymerized for 24 h at 60 °C. Small regions of the pellet were selected and excised from the flat resin layer with a razor, then glue-mounted on a blank resin block in the desired orientation for sectioning. Ultra-thin sections (70 nm) were obtained with a Leica Ultracut-R microtome and collected on formvar-coated 1 mm x 2 mm slotted copper grids. Sections were post-stained for 5 min with 2% uranyl acetate in 50% ethanol solvent followed by 3 min with Sato's lead citrate (Hanaichi et al., 1986). Images were generated on a Philips CM-12 TEM operated at 80 kV and acquired by a Gatan model 791 slow-scan CCD camera.

#### **2.4 DNA extraction, quantification and library construction**

Genomic DNA preparations were obtained by PCI (phenol; chloroform; isoamyl alcohol) extraction (Countway et al., 2005). DNA was quantified using fluorometry of ethidium bromide-stained 1% agarose electrophoresis gels and sent for commercial MiSeq 250 Illumina sequencing at the University of Maryland School of Medicine, Institute for Genome Sciences, Genome Resource Center. Genomic DNA libraries were constructed for sequencing on the Illumina platform using the NEBNext® DNA Sample Prep Master Mix Set 1 (New England Biolabs, Ipswich, MA) using the protocol provided, and after DNA fragmentation with an ultrasonicator (Covaris E210). The DNA was purified between enzymatic reactions and the size selection of the library was performed with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA).

#### **2.5 DNA sequencing, assembly and annotation.**

Libraries were sequenced using the 250 bp paired-end protocol on an Illumina MiSeq sequencer. Raw data from the sequencer was processed using Illumina's RTA and CASAVA pipeline software, which includes image analysis, base calling, sequence quality scoring, and index demultiplexing. Data were then processed through both in-house pipelines for sequence assessment and quality control and FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). These pipelines report numerous quality metrics and perform a megablast-based contamination screen. By default, the quality control pipeline assesses basecall quality and truncates reads where the median Phred-like quality score falls below Q20 (implying more than 99% accuracy in base calling). Data was randomly sampled to create datasets at 100 X, 150 X and 200



X coverage. The different datasets were assembled with MaSuRCA (Zimin et al., 2013). The genome assembled by MaSuRCA was subjected to the Institute of Genomic Science (Burja et al., 2001) prokaryotic annotation pipeline that forms the core of the IGS Annotation Engine. The pipeline includes gene finding, protein searches, and the pFunc evidence hierarchy that produces automated functional annotation. The output of this pipeline was stored in a Chado relational database and accessed by Manatee for annotation visualization and curation (Galens et al., 2011). The genome was also annotated using RAST -Rapid Annotation using Subsystem Technology (Aziz et al., 2008).

## **2.6 Genomic analyses**

The genome annotated by Manatee and RAST was also analyzed manually. Homologs of certain key genes of interest were searched as queries of Psi blast of homologs from phylogenetically close protein sequences from NCBI against the entire genome. Given that the genome is not closed, there is a small probability that the genes reported as missing might be present in the unsequenced part of the genome. The Kyoto Encyclopedia of Genes and Genomes(KEGG) was employed by RAST to gain insight into the various metabolic pathway maps.

Since the bidirectional hydrogenase enzyme was central to the powerful H<sub>2</sub> production exhibited by the BL J strain, the architecture of the bidirectional hydrogenase (*hox*) gene cluster and hydrogenase accessory genes (*hyp*) was studied in detail. For the strain BL J, Manatee helped in viewing the genomic organization of the *hox* and associated ORFs.

The physical map of the bidirectional hydrogenase gene cluster and associated ORFs was manually re-constructed to scale in the strains *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 (exemplary of Pattern 1), *Microcoleus* (= *Coleofasciculus*) *chthonoplastes* PCC 7420 and *L. aestuarii* BL J (exemplary of Pattern 2), *Lyngbya aestuarii* PCC 8106 and *Lyngbya majuscula* CCAP 1446/4 (closely related to the strain BL J; H<sub>2</sub> production capacity unknown). Protein Psi Blast searches were employed to reveal if any ORFs associated with the *hox* cluster in BL J were also present in any of the other three strains.

To characterize the phylogenetic placement of this strain in reference to other strains in the same cyanobacterial subsection, 16S rRNA sequence (1322 bp) based phylogenetic tree was constructed. The sequences from 83 bacterial species were aligned using ClustalW. The alignment was manually curated and GTR (General Time Reversal model) model with GI (Gamma distributed with Invariant sites) was used to construct maximum likelihood trees with 1000 bootstrap replicates using MEGA 5.2.2 (Tamura et al., 2011).

### **2.7 Bidirectional hydrogenase sequence analysis and protein modeling**

The amino acid sequences of the bidirectional hydrogenase from representatives of Pattern 1 (fresh water strains: *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803) and Pattern 2 (marine intertidal strains: *M. chthonoplastes* PCC 7420 and *L. aestuarii* strain BL J) H<sub>2</sub>-producing cyanobacteria were used for this analysis. The protein sequences of the subunits HoxY and HoxH were individually aligned using Muscle. If the type of amino acid changed significantly between the two Patterns but remained consistent within a Pattern, it was marked as a significant change (Supplementary

Information. 1). The hydrogenase moiety (*hoxYH*) in *Synechocytis* sp. 6803 (Pattern 1) and *L. aestuarii* BL J (Pattern 2) were modeled to study the potential structural importance of these significant amino acid positions, which might have implications for the function of the bidirectional hydrogenase. Homology models were constructed using the [NiFe]- and [NiFeSe]-hydrogenase templates available in the Protein Data Bank, PDB (Bernstein et al., 1977). Multiple templates were chosen from the PDB for HoxH and HoxY based on the relationship to other bacterial hydrogenases. Both subunits of 1H2A (Higuchi et al., 1997), 1E3D (Matias et al., 2001), 1FRV (Volbeda et al., 1995), 1YQW (Volbeda et al., 2005), and 1CC1 (Garcin et al., 1999) were superimposed using STAMP (Russell and Barton, 1992), and an alignment prepared based on the result using MULTISEQ (Roberts et al., 2006). Each cyanobacterial hydrogenase was profile aligned using CLUSTALW (Larkin et al., 2007) without disturbing the structure-based alignment. The PDB structure files were edited to include protein, the proximal and medial FeS clusters, the [NiFe] center, CO and CN ligands and the Fe or Mg ion at the C-terminus. The previous alignments and edited PDBs were used as inputs for MODELLER (Sali and Blundell, 1993; Eswar et al., 2002) producing 50 independent models. The resulting models were ranked by energy and those with the lowest combined energies were considered in detail. All structures were viewed and figures prepared in Visual Molecular Dynamics software, VMD (Humphrey et al., 1996). The amino acid positions changing consistently between the two patterns were highlighted to study their potential functional significance in the 3D hydrogenase model.

### **3. Results**

#### **3.1 Strain morphology, untrastructure, and development**

*L. aestuarii* BL J is a marine filamentous cyanobacterium belonging to the cyanobacterial subsection III according to the classification of *Bergey's Manual of Systematic Bacteriology* (Boone and Castenholz, 2001). The *circa* 17- $\mu\text{m}$ -wide sheathed filaments appeared in various hues of green-brown shades under the light microscope (Figure 1A) as cylindrical, unbranched, and up to 2 cm in length. The trichome consists of short disk shaped stacked cells (1.6 – 1.8  $\mu\text{m}$  long). The cells are 14  $\mu\text{m}$  wide. Confocal microscopy imaging helped to visualize the DNA and nucleoids (in blue), the exopolysaccharide sheath (in green) and the photosynthetic pigments (in red) (Figure 1E, F). A distinct mucilaginous sheath about 1.6  $\mu\text{m}$  in thickness covers the trichome (Figure 1E). As evidenced by confocal microscopy, the main photosynthetic area is arranged parallel to the cross walls, and the nucleoid is central (Figure 1E). This strain often produces short, motile hormogonia as dispersal mechanisms, with little sheath (Figure 1F). The filaments develop necridic cells as a means of filament separation to make new trichomes or to aid in the formation of hormogonia (Figure 1C). Both the vegetative filaments and hormogonia have rounded terminal cells. Sometimes, single disk shaped cells were observed within the sheath and also free in the media (Figure 1D). Cell division was by formation of transversal centripetal growth of cross-walls was observed (Figure 1E), often with several consecutive rounds proceeding simultaneously. As expected, heterocysts, akinetes or any other type of specialized cells were absent.

The presence of the thick, laminated exopolysaccharide sheath can be easily visualized in

the transversal and longitudinal sections of TEM (appears wider than that observed in fluorescence microscope, perhaps due to the TEM sample preparation, Figure 2A-D). TEM imaging revealed that the thylakoid membranes were stacked and randomly oriented and present close to the periphery of the cells (Figure 2A, 2B). The thylakoid membranes were associated with glycogen granules (Figure 2B). Carboxysomes (Figure 2B) and cyanophycin (Figure 2A) granules were observed in the cytoplasm. Formation of new trichomes along with necridial cells was also observed (Figure 2D).

### **3.2 Whole genome sequence analysis**

#### **3.2.1 Quality of the whole genome sequence**

The draft genome contained 439 contigs of which 124 were large contigs (>10,000bp size). Based on the assembler MaSuRCA, the total contig base pair size was estimated to be 6.87 Mb. About 6.44 Mb of the entire genome was present in large contigs. The statistical measure of the median contig size or the N50 value was 80,423 Mb. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AUZM00000000. The version described in this paper is version AUZM01000000.

#### **3.2.2 General genomic features**

The chromosome includes 6515 potential protein-encoding genes. 43% encode proteins with assigned functional role categories, 26% encode proteins with domain or family assignments, 19.6% encode conserved hypothetical proteins, which are hypothetical proteins with similarity to other hypothetical proteins and 11.3% encode hypothetical

proteins, with no significant sequence similarity to other proteins. The average size of each gene was 893 bp. The rRNA and tRNA were coded by five and 48 genes, respectively.

In terms of whole genome DNA sequence similarity, RAST predicts that *Arthrospira maxima* strain CS-328 and *Lyngbya aestuarii* PCC 8106 (=strain CCY 9616, formerly referred to as *Oscillatoria limosa*) are the closest known strains to *L. aestuarii* BL J. Based on 16S rRNA sequence alone, the strain *L. aestuarii* PCC 8106 was the closest (99% similarity). The GC percentage of the genome *L. aestuarii* BL J was estimated to be 41.2%. This was closest to the GC percentage of *L. aestuarii* PCC 8106 (41.0%). Certain general features of the genome of sequenced strains closely related to *L. aestuarii* BL J have been tabulated (Table 1). In comparison to the closely related strains, the genome size, percent GC, predicted protein encoding genes and total predicted genes are in the expected range.

### **3.2.3 Energy metabolism**

As expected, this strain had the homologs of the complete sets of genes coding for photosystem I (14 genes; some with additional homologs) and photosystem II (22 genes; some with additional homologs). It also had homologs of genes coding for phycobilisome proteins, phycocyanin and allophycocyanin. Homologs of genes coding for phycoerythrocyanin and phycoerythrin were not detected. A complete set of genes required for the Calvin cycle (12 genes; some with additional homologs) along with the presence of key enzymes RuBisCo (*RbcL*), phosphoribulokinase (*PRK*), and

sedoheptulose-1,7-bisphosphatase (*SBP*), were present, as were the essential genes involved in the Carbon-dioxide Concentrating Mechanism, or CCM (15 genes; some with additional homologs). The gene *hat/hatR* (high affinity carbon uptake protein) has 31 homologs in BL J. In strains *L. aestuarii* PCC 8106, *M. chthonoplastes* PCC 7420, and *Acaryochloris marina*, similarly high number of homologs of the same gene can be found, while other strains like *Prochlorococcus marinus* MIT 9215, *Synechococcus* sp. WH 8102, *Synechococcus* sp. CC9311, *Cyanothece* sp. PCC 8801 and *Synechocystis* sp. PCC 6803, just contain none to two at most.

Homologs of genes coding photoprotective proteins such as flavodiiron proteins (FPD's) and orange carotenoid proteins were also present. The genome of *Synechocystis* sp. PCC 6803 contains four putative flavodiiron protein-coding genes (CyanoBase: <http://bacteria.kazusa.or.jp/cyanobase/>) of which two (*sll0219* and *sll0217*) have a role in photoprotection of the cells and in the sustenance of the photosystem II (PSII) complex (Zhang et al., 2009). In comparison, the strain BL J has hosts only two putative flavodiiron protein coding genes and with no corresponding homolog to the gene *sll0219* in *Synechocystis* sp. PCC 6803. It has the homologs of genes coding for the photoprotective orange carotenoid protein, also present in closely related strains such as *A. maxima* CS-328 and *L. aestuarii* PCC 8106.

With respect to dark carbon catabolic metabolism, it has the homologs of all genes required for glycolysis, Entner-Doudoroff pathway, and the pentose phosphate pathway. The TCA cycle has homologs of genes similar to that reported in other cyanobacteria,

including those (namely 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase) reported to be involved in a cyanobacterial type of TCA cycle (Zhang and Bryant, 2011). The ortholog of the gene coding for succinic semialdehyde dehydrogenase in the strain BL J is reduced (59% query coverage) compared to that observed in *Synechococcus* sp. PCC 7002. Similar results were observed in the orthologs of the same gene in closely related strains such as *L. aestuarii* PCC 8106 (68% query coverage) and *Trichodesmium erythraeum* IMS101 (64% query coverage) when compared to the succinic semialdehyde dehydrogenase gene observed in *Synechococcus* sp. PCC 7002.

This strain has all the genes required for mixed acid fermentation (8 genes) for surviving through dark anaerobic conditions (which, in fact, it carries out; (Chapter III)). Even though a capacity for anoxygenic photosynthesis is typical from microbial mat cyanobacteria (Garcia-Pichel and Castenholz, 1990), homologs of the enzyme sulfide:quinone oxidoreductase that catalyses the initial step in sulfide-dependent donation of electrons to PSI, was not detected.

### **3.2.4 Nitrogen metabolism**

Cyanobacteria have the ability to use various organic and inorganic sources of nitrogen from the environment. (Luque and Forchhammer, 2008). BL J has all the homologs required for fixing atmospheric nitrogen into ammonium. This includes the structural genes *nifD* and *nifK* encoding the dinitrogenase moiety and *nifH* encoding the dinitrogenase reductase. As observed earlier in filamentous strains like *Anabaena* sp.



PCC 7120 (Haselkorn et al., 1998) and *L. aestuarii* PCC 8106 the genes *nifBSUHDKENX* are clustered and oriented in a single direction. The homologs of genes coding for the uptake hydrogenase enzyme involved in consumption of the H<sub>2</sub> produced by the nitrogenase, are also present in this strain. The genes of coding for the uptake hydrogenase (*hupSLW*) are generally clustered and oriented in the same direction. In this strain *hupW*, the gene coding for the putative C terminal endopeptidase lies several kb downstream of the main locus. Interestingly, this strain possesses homologs of the gene *hetR* involved in the formation of heterocysts (Buikema and Haselkorn, 1991), even though it does not develop heterocysts. All genes required for reducing inorganic nitrate into ammonium, including nitrate reductase (*nar*) and nitrite reductase (Panda et al., 2008), are present. This strain also hosts homologs of genes corresponding to uptake of organic sources of nitrogen (urea) and amino acids (see below), and a homolog of the urease gene.

Ammonium-ion assimilation constitutes a central metabolic pathway in cyanobacteria wherein glutamine synthetase (Burja et al., 2001) and an NADPH-dependent glutamine 2-oxoglutarate amidotransferase (GOGAT) play the primary role for ammonium ion incorporation into glutamine and glutamate (Muro-Pastor et al., 2005). This strain has the homologs of glutamine synthetase and a NADPH-dependent GOGAT. The homolog of the gene coding for *ntcA* (Vega-Palas et al., 1992) involved in global nitrogen control is also present in this strain.

### **3.2.5 Signal transduction**

The sensory kinases (involved in sensing the environmental changes) and the response regulators (involved in regulating gene expression) together constitute the “two-component system”. This signal transduction system aids bacteria in adapting to their environmental changes. Only the orthologs of genes coding for the classic two-component systems were detected. This strain has 100 genes coding for the two-component systems (similar values reported in other strains). Of the 100 genes 42 encode histidine kinase A domain protein and the rest code for response regulators. Additionally, 57 other ORFs were detected with putative role in signal transduction. Of these, about 51 ORFs were assigned as the diguanylate cyclase domain protein-coding gene which participates in the formation of the ubiquitous second messenger cyclic-di-GMP (Ross et al., 1987). The other six ORFs were assigned to the EAL domain protein-coding gene, which is associated with the diguanylate cyclase protein domain. It is a conserved protein domain, proposed to function as diguanylate phosphodiesterase (Galperin et al., 2001).

### **3.2.6 Transport and binding proteins**

This strain has multiple ORFs with predicted function as binding protein-dependent transport systems. 590 ORFs are predicted to have a role in coding for transport and binding proteins for amino acids, peptides and amines (9), anions (24), carbohydrates, organic alcohols and acids (15), cation (56), nucleosides, purines and pyrimidines (2), Porins (4), other substrates such as heme or polysaccharides (17) and unknown substrate (463). The ORFs involved in anion binding and transfers were homologs of ABC transporter coding genes proposed to transfer phosphate, sulfate, nitrite, phosphonate,

phosphite and molybdate. The ORFs involved in cation binding and transfer were proposed to transport the cations- sodium, copper, ferrous, cadmium, cobalt, magnesium, calcium, potassium and nickel.

### 3.2.7 Organic osmotic solutes

The solute trehalose is characteristic of low-salt tolerant cyanobacteria (Oren et al., 1994), such as *Scytonema* sp. (Page-Sharp et al., 1999), *Anabaena* sp. PCC 7120 (Higo et al., 2006). But it is also present in some marine cyanobacteria such as *Crocospaera watsonii* WH8501 (Pade et al., 2012). The genome of this strain had homologs of the enzymes trehalose synthase and trehalose-6-phosphate synthetase involved in trehalose synthesis. However, the enzyme trehalase, involved in its breakdown, was not detected. *L. aestuarii* PCC 8106, seems to use trehalose as a storage compound (Heyer et al., 1989). Glucosylglycerol is an osmolyte commonly seen in moderately halotolerant cyanobacteria (*Synechocystis* PCC 6803 (Hagemann and Erdmann, 1997), *Arthrospira* (= *Spirulina*) *platensis* (Warr et al., 1985), *Synechococcus* sp. strain 7002 (= *Agmenellum quadruplicatum* PR6) (Tel-Or et al., 1986), *Microcystis firma* strain Gromow 398 (Erdmann et al., 1992) and *Oscillatoria* sp. SAG 3192 (Moezelaar et al., 1996)). However, the genes required for the synthesis of glucosylglycerol were not detected in this strain. Glycine betaine is characteristic of highly halotolerant cyanobacteria *Halothece* (*Aphanothece*) *halophytica* (Reed et al., 1984), *Halospirulina tapeticola* (Nubel et al., 2000), *Spirulina subsalsa* (Gabbay-Azaria et al., 1988), *Halothece* (*Dactylococcopsis*) *salina* (Moore et al., 1987) and *Synechocystis* sp. DUN52 (Mohammad et al., 1983). This solute has also been previously detected in

*Oscillatoriamats* (Oren et al., 1994) inhabiting the hypersaline sulfur hot springs at Hamei Mazor. In cyanobacteria, the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase catalyze the formation of this osmolyte (Oren et al., 1994). Homologs of both of these genes were present in the strain BL J indicating that this strain has the genetic capacity to make glycine betaine.

### **3.2.8 Storage compounds**

Glycogen is a major carbohydrate reserve molecule in cyanobacteria. Homologs of all the genes involved in glycogen metabolism were detected. In Chapter III, we showed that glycogen is stored in the light and mobilized in the dark either aerobically or anaerobically. Cyanophycin (multi-L-arginyl-poly-L-aspartate) is a water-insoluble, high nitrogen reserve polymer (Ziegler et al., 1998) quite commonly encountered as a carbon and nitrogen storage polymer in cyanobacteria. (Huang and Chou, 1991; De Philippis et al., 1992; Miller and Espie, 1994). Homologs for the cyanophycin synthetase were also found in BL J. Two homologs of the cyanophycinase, a peptidase degrading cyanophycin were present, one of which followed the cyanophycin synthetase gene.

Polyhydroxyalkanoates or PHAs are linear polyesters storage carbon and energy compounds seen in many cyanobacteria (Hai et al., 2001; Asada et al., 1999; Stal, 1992; Panda et al., 2008; Shrivastav et al., 2010). However, this strain seems to lack the homologs for poly (3-hydroxyalkanoate) synthase (*phaC*), the key enzyme for PHA synthesis.

### 3.2.9 Genes of biotechnological importance

*Secondary metabolites:* Polyketide synthases (PKSs) are a family of multi-domain enzymes that produce polyketides, a large class of secondary metabolites. The strain *L. aestuarii* BL J has genes homologous to the putative polyketide synthase module-related protein PKS in *Moorea producens* 3L (Jones et al., 2011). Homologs of genes involved in Curacin A and Barbamide synthesis (Jones et al., 2011) were also present as were homologs of genes coding for Hemolysin-type calcium-binding toxin seen in *L. aestuarii* PCC 8106. Three putative homologs of genes coding for the putative RTX toxin (a type of cytotoxin) along with toxin secretion ABC transporter ATP-binding protein seen in *L. aestuarii* PCC 8106 were detected as well. Certain cyanobacteria synthesize a protective pigments in response to UV irradiation (Gao and Garcia-Pichel, 2011b; Karsten et al., 1998). Among them, scytonemin, has been directly detected in *L. aestuarii* mats (Garcia-Pichel and Castenholz, 1991). Previously it has been reported that the scytonemin genes *scyABCDEF* are clustered with all the genes oriented in the same direction in a few cyanobacterial strains (Soule et al., 2009). Similar arrangement of *scy* genes was observed in this strain. Homologs of all the genes essential for the biosynthesis of tryptophan from chorismate (*trpE*, *trpC*, *trpA*, *trpB*, *trpD*) are present and they are oriented in the direction opposite to that of the *scy* gene cluster. In fact, the scytonemin gene cluster in the strain BL J is exactly the same as seen in *L. aestuarii* PCC 8106 (Soule et al., 2009). In response to UV-B irradiation, certain cyanobacteria synthesize mycosporines. This strain has the homologs of all the 3 genes involved in the formation of mycosporine-glycine from sedoheptulose-7-phosphate. All these 3 genes are clustered and oriented in the same direction as reported in *Anabaena*

*variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133. There is a homolog of the gene involved in the conversion Mycosporine-glycine to Shinorine, present elsewhere in the genome (Gao and Garcia-Pichel, 2011a).

Certain cyanobacterial strains (Schirmer et al., 2010; Starkenburg et al., 2011) have the genes coding for the synthesis of heptadecane and pentadecane alkanes; a major constituent of gasoline, diesel, and jet fuel. The homologs of genes acyl-[acyl carrier protein] (ACP) reductase and aldehyde decarbonylase involved in the synthesis of heptadecane and pentadecane alkanes (Schirmer et al., 2010) are present in this strain. Homologs of these genes are also present in closely related strains such as *L. aestuarii* PCC8106, *Microcoleus vaginatus* FGP-2 and *Trichodesmium erythraeum* IMS101.

### **3.2.10 Other genes of interest**

This strain has genes for resistance to copper, cobalt, zinc, cadmium, mercury, fluoroquinolones, arsenic and beta-lactam antibiotics. The homolog of the gene involved in biotin synthesis (*bioA*) could not be detected in this strain. However, it did encode genes for biotin uptake from the environment (and biotin was a constituent of the IMR medium), a trait also seen in other closely related strains. The genome has genes coding for *hipBA*, which are proposed to have a role in formation of persister cells (dormant cells with antimicrobial resistance) in response to antibiotic and other stresses (Jayaraman, 2008). The genome has genes corresponding to Internalin- putative, *inl* and Internalin A, *inlA* that are implicated internalization or virulence in *Listeria* (Cossart and Lecuit, 1998).

### **3.3 Comparative analysis of the bidirectional hydrogenase and accessory proteins.**

#### **3.3.1 Bidirectional hydrogenase**

We compared the bidirectional hydrogenase and hydrogenase accessory gene cluster of strains displaying either Pattern 1 (*Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120) or Pattern 2 (*L.aestuarii* BL J and *M. chthonoplastes* PCC 7420) H<sub>2</sub> production, and included comparisons with closely related *Lyngbya* species, *L. aestuarii* PCC 8106 and *L. majuscula* CCAP 1446/4 (of untested H<sub>2</sub> production capacity). *L. aestuarii* strain BL J hosts a Ni-Fe bidirectional hydrogenase enzyme the locus of which is a 6.89 kb gene cluster. The genes coding for the bidirectional hydrogenase (*hoxEFUYH*) are often grouped together as in the strain PCC 7420, but a few other ORFs are interspersed in the cluster of *Synechocystis* sp. PCC 6803 (Schmitz et al., 1995). In *Synechococcus elongatus* and in *Anabaena* sp. PCC 7120 (Boison et al., 1998; Kaneko et al., 2001), the two clusters, *hoxEF* and *hoxUYH*, are separated by several kb. The clusters *hoxEF* and *hoxUYH* are separated by a single gene coding for *hcp* (encoding a putative hybrid cluster protein) in *Lyngbya* strains CCAP 1446/4, PCC 8106 (Ferreira, 2009) and BL J. In fact, the overall arrangement of genes and ORFs in the hydrogenase cluster in *L. aestuarii* BL J is undistinguishable from that seen in PCC 8106 (Figure 3).

In terms of protein sequence, the bidirectional hydrogenase from *L. aestuarii* PCC 8106 was the closest to that of *L. aestuarii* BL J. Identities in the HoxE, F, U, Y and H between the two strains were 97%, 97%, 97%, 96% and 95%, respectively, and gene lengths for each subunit were the same in both strains. Similar to what was previously reported for *L. majuscula* CCAP 1446/4 and *L. aestuarii* PCC 8106 (Ferreira, 2009), the ORF before

*hoxE* is annotated as a pyruvate ferredoxin oxidoreductase in the strain BL J. Pyruvate ferredoxin oxidoreductase is a key enzyme in fermentation and is typically active in dark anaerobic conditions along with the bidirectional hydrogenase (Kletzin and Adams, 1996). Homologs of the ORF M595\_4252 (belonging to BL J strain) are also found in PCC 8106 (L8106\_07436) and CCAP 1446/4 (ORF 14) strain. This ORF codes for a hypothetical protein with three predicted transmembrane helices along with homology to cyanobacterial genes coding for putative membrane proteins. Similarly, homologs of other hydrogenase-cluster-associated-ORFs in BL J strain (M595\_4253, M595\_4255 and M595\_4256) were seen in PCC 8106 and CCAP 1446/4 strains (Figure 3).

The gene, *hoxW*, codes for a carboxyl-terminal protease that releases a short peptide from HoxH prior to progression of subunit assembly (Thiemermann et al., 1996). *HoxW* is found immediately downstream of *hoxH* in *S. elongatus* PCC 6301, *S. elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002. However *hoxH* and *hoxW* are separated by several kb in *Synechocystis* sp. PCC 6803 (Kaneko and Tabata, 1997) and *M. chthonoplastes* PCC 7420. Three ORFs separate the *hoxH* and *hoxW* in *Anabaena* sp. PCC 7120. A single ORF separates the *hoxH* gene from *hoxW* in the *L.aestuarii* strains (*L. majuscula* CCAP 1446/4, *L.aestuarii* PCC 8106 and *L.aestuarii* BL J).

The genes *hypFCDEAB* code for the maturation of bidirectional hydrogenase in cyanobacteria (Lutz et al., 1991; Jacobi et al., 1992). Amongst the Pattern 1 exhibiting strains, the *hyp* genes are dispersed in the genome of *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996) whereas they are clustered and oriented in the same direction in



*Anabaenas* sp. PCC 7120 with an additional ORF (coding for probable 4-oxalocrotonate tautomerase) between the *hypD* and *hypE* genes. In PCC 7420, one finds two clusters (*hypAB* and *hypFCDE*) with additional hypothetical ORFs between *hypF* and *hypC* and another one between *hypD* and *hypE*. In the strain BL J, *hypFCDEAB* are clustered, with all genes oriented in the same direction and encompassing two additional ORFs coding for hypothetical proteins. A similar arrangement has been observed in *L. aestuarii* PCC 8106. In BL J, *hypC* has one additional homolog in the genome, as does *hypF*, but the latter is highly truncated (13% query coverage). No additional homologs of *hyp* genes are found in *Lyngbya* strains (CCAP 1446/4 and PCC 8106). In *Synechocystis* PCC 6803, additional homologs *hypA2* and *hypB2* were clustered but they don't seem to play a role in maturation of the bidirectional hydrogenase (Hoffmann et al., 2006). Homologs of the gene *hypX*, with a proposed role in oxygen tolerance of soluble Ni-Fe hydrogenases in *Ralstonia eutropha* H16 (Bleijlevens et al., 2004), could not be detected in the strain BL J. Thus, at the level of the physical map of the bidirectional hydrogenase and accessory gene cluster we did not observe any congruent changes consistent within a H<sub>2</sub> production Pattern.

### **3.3.2 Protein modeling of the bidirectional hydrogenase**

The 3D structure of the hydrogenase subunit of the bidirectional hydrogenase from *L. aestuarii* BL J (as an exemplary of Pattern 2) and *Synechocystis* sp. PCC 6803 was modeled (as an exemplary of Pattern 1) based on the genomic sequence. The model was constructed based on five related bacterial [NiFe]-hydrogenases from the Protein Database. In general, the overall fold and length of the large subunit, HoxH, was similar

to the heavy chain of the bacterial [NiFe]-hydrogenase model templates. The C-terminus of the [NiFe]-hydrogenase aids in nickel insertion prior to its cleavage to allow a structural reorganization of the whole molecule, and the consequent assembly of the holoenzyme (Fritsche et al., 1999). It has been suggested that in the cyanobacterial bidirectional hydrogenases, the last 25-32 C-terminal amino acids are cleaved (Tamagnini et al., 2007). The alignments leading to the homology models presented in this work demonstrate strong homology in the C terminal region and both the strains are consistent with an excised C-terminal portion of 25 amino acids. As reported earlier, the small subunit, HoxY, is significantly shorter in the cyanobacterial bidirectional hydrogenases than in the light chain of the bacterial [NiFe]-hydrogenase model templates. The light chain template structures of [NiFe]-hydrogenases that corresponded to HoxY consisted of two folded domains connected through a linear unstructured sequence. Only the first domain of the light chain template is homologous to the cyanobacterial HoxY sequence. The amino acids corresponding to HoxY only house one of the three FeS, which corresponds to the proximal FeS cluster observed in the light chain of the bacterial [NiFe]-hydrogenase model templates.

The two-fold purpose of the structural comparison was to determine if there were significant structural differences in the enzyme itself and if the consistent amino acid substitutions between representatives of the two H<sub>2</sub> production Patterns were located in proximity to active sites in the enzyme. Our results indicate that the overall fold and domain structures between the hydrogenase subunits of *L. aestuarii* BL J and *Synechocystis* sp. PCC 6803 were very similar (Figure 4). In comparing protein

sequences, we found 15 positions in HoxH and five in HoxY where the type of amino acid remained consistent within a Pattern but varied amongst the two Patterns (see Supplementary Information. 2). However, all of these significant amino acid changes between the two Patterns lie on the exterior loops in the model (Figure 4) indicating they were not crucial in explaining the differences in the H<sub>2</sub> producing physiologies observed between the two Patterns.

#### **4. Discussion**

Our genomic and cellular description of *L. aestuarii* BL J shows that this strain shares phylogenetic placement, morphological and life history traits, with certain other environmentally and biotechnologically relevant cyanobacteria. This clade (Supplementary Information. 2) encompasses globally important marine cyanobacteria like *Trichodesmium* spp. and globally relevant terrestrial forms such as *Microcoleus vaginatus*, estimated to be the 3<sup>rd</sup> and 4<sup>th</sup> most abundant cyanobacteria on the planet, respectively (Garcia-Pichel et al., 2003). The group includes strains in the genus *Arthrospira* of importance for large scale production of biomass (Hu, 2004) and used commercially as “*Spirulina*” as a health food additive (Milledge, 2011) and, of course many other strains of *Lyngbya* in the so-called halophilic/brackish/freshwater cluster of biotechnological fame because of their rich and diverse set of secondary metabolites (Engene et al., 2011). All of them being filamentous, non-heterocystous, with discoidal, rather large cells that undergo several rounds of division simultaneously by invagination of cross-walls, and that develop necridial cells for the formation of dispersive hormogonia and for filament separation (Figures 1 and 2). Unfortunately, given their importance,

there are currently no members of this clade for which a system of genetic manipulation has been developed, heavily curtailing biotechnological advancement and bringing and added value to genomic investigations of their members. Given this lack, genomics provides an opportunity to identify and transform genes of interest into other model organisms.

The strain *L. aestuarii* PCC 8106, isolated from a similar intertidal habitat (a microbial mat in the German Wadden Sea island of Mellum), was the closest to BL J in terms of the 16S rRNA sequence similarity and phylogeny and many of the other genomic features (the genome size, percent GC, predicted protein encoding genes and total predicted genes; Table 1). However, significant differences exist between these two strains.

Perhaps the most conspicuous being that BL J is almost 50% larger than PCC 8106, with reported cell width around 10  $\mu\text{m}$  (Stal and Krumbein, 1981). Other features like the arrangement of the thylakoid membranes in stacks does not occur in PCC 8106 either (Stal and Krumbein, 1981). We also report the occasional presence of loose, disk-shaped cells within the sheath and the in the media, indicating that cell-to-cell linkages in our strain can be weak, in what can potentially be relevant as an additional means of dispersal that will require focused study.

Our genomic predictions found confirmation in a variety of traits that could be independently assessed. For example, electron microscopy (Figure 2) confirmed the predicted presence of glycogen as a major carbohydrate storage molecule in this strain to the exclusion of polyhydroxyalkanoates. It did also confirm the genomic predictions of

cyanophycin synthesis (Figure 2A) and the formation of carboxysomes. Light microscopy revealed the presence of scytonemin in the sheaths of BL J (and we could confirm its preferential synthesis under added UV-A radiation; data not shown), supporting the finding of the entire scytonemin operon in the genome. This all lends credence to other yet to be supported predictions.

A reading of *L. aestuarii* BL J's genome also speaks directly to some of the environmental constraints of this species in its environment of origin. Known to inhabit exposed intertidal surfaces and the topmost layers of the microbial mats, a high-light phenotype can clearly be surmised from the presence of many photoprotective mechanisms: from extra and intracellular sunscreens, to flavodiiron proteins that regenerate excess electrons by reducing molecular oxygen to water (Goncalves et al., 2011), to orange carotenoid protein, which helps decouple the light-harvesting systems from the reaction centers (Wilson et al., 2006), as well as from the absence of genes coding for light harvesting pigments that can be considered adaptations to low light intensity like phycoerythrin (Kana and Glibert, 1987), phycoerythrocyanin (Prufert-Bebout and Garcia-Pichel, 1994) or chlorophyll *d* (Swingley et al., 2008). Intertidal habitats are recurrently exposed to cycles of desiccation and rewetting. Not much is known about the genes involved in desiccation resistance in cyanobacteria, but recent transcriptomic studies on the terrestrial strain *Microcoleus vaginatus* indicate that this is a complex response that involves large sets of genes (Rajeev et al., 2013) and that includes complex DNA repair responses, up-regulation of reactive oxygen detoxification mechanisms, the production of osmolytes and upregulation of orange carotenoid protein.

Many of the genes involved in these adaptations are also present in this strain, but its mere presence cannot necessarily be linked to desiccation stress resistance. This strain has clearly acquired mechanisms to hold on to moisture, however. In fact, it was very difficult to dehydrate the filaments of this strain for the purpose of TEM preparation. Its thick sheath and the predicted presence of glycine betaine, unusual for a non-halophilic strain, in addition to trehalose, might help in providing desiccation resistance. Finally, a condition typical of the mat habit is that diffusion becomes the major transport mechanism for substrates and products of metabolism. This tends to create diffusion limitations to metabolic activities like photosynthesis and respiration (Garcia-Pichel et al., 1994), which gives relevance to the presence of homologs of genes coding for high affinity carbon uptake protein(*hat*) and carbon concentrating mechanism (*ccm*) along with abundant carboxysomes (Figure 2). It also promotes the establishment of anaerobiosis at night within the mat due to consumption by respiration. Under these conditions fermentation of internal reserves through a mixed acid pathway is the only energy-generating metabolism available to strain BL J. Interestingly, this strain lacks the capacity to perform anoxygenic photosynthesis using hydrogen sulfide as an electron donor (homologs of the gene coding for sulfide quinone oxidoreductase were missing) common in microbial mat cyanobacteria. Perhaps this is linked to the low concentrations of sulfide in the upper layers of these intertidal mats compared to mats that are constantly submerged, and where *Lyngbya* never dominates (Rothrock and Garcia-Pichel, 2005). The presence of recurrent anaerobic conditions will also make soluble ferrous iron available, perhaps leading to the fact that adaptations to iron deficiency (such as the

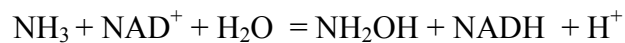
products of 'iron-stress-induced' gene, *isiA*; (Straus, 1994; Park et al., 1999) were not detected in BL J.

On the biotechnological potential of this strain, we have to note its apparently very rich set of secondary metabolites that range from toxins like Curacin A, Barbamide, Hemolysin-type calcium-binding toxin, to sunscreens like scytonemin and mycosporines, to biofuel prospects like heptadecane and pentadecane alkanes. But clearly, biohydrogen is the most promising product of biotechnological importance from this strain (Kothari et al., 2012). Since the standard assays for H<sub>2</sub> production were performed in the presence of nitrate, a condition in which nitrogenase is not known to be inactive (Ferreira et al., 2009), the role of nitrogenase in the production of H<sub>2</sub> is ruled out. The uptake hydrogenases are known to produce little H<sub>2</sub> in presence of reduced methyl viologen (Houchins and Burris, 1981). In contrast, reduced methyl viologen is commonly used to assay the bidirectional hydrogenase activity and is likely the enzyme majorly contributing to the strong H<sub>2</sub> producing capacity of the strain BL J described previously (Kothari et al., 2012). Therefore, the bidirectional hydrogenase gene cluster in this strain is studied in detail with comparisons drawn to other H<sub>2</sub> producing strains. The organization of the bidirectional hydrogenase (*hox*) and accessory hydrogenase (*hyp*) gene cluster was unique in all the four strains (*Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, *L. aestuarii* BL J and *M. chthonoplastes* PCC 7420). *A priori*, the comparative analysis of the organization of the bidirectional hydrogenase and accessory genes locus revealed no major changes consistent within a Pattern but varying between the two Patterns. A comparative analysis of the organization of the bidirectional hydrogenase locus in the

strain BL J revealed that it was similar to that of *L. aestuarii* PCC 8106 and showed only minor differences with that of *L. majuscula* CCAP 1446/4 strain (Figure 3).

In the *Lyngbya* strains CCAP 1446/4, PCC 8106, and BL J, a homolog of the *hcp* genes found between genes *hoxF* and *hoxU* (Figure 3). Interestingly, in cyanobacteria (*M. chthonoplastes* PCC 7420 and *L. aestuarii* BL J (Chapter III) and *Arthrospira maxima* CS-328 (Ananyev et al., 2008)) that are strong H<sub>2</sub> producers and display sustained concentrations of H<sub>2</sub> for more than 24 h in dark anaerobic conditions, the *hcp* gene is present. This gene is absent from the genomes of Pattern 1 strains, namely, *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120. This coincidence may provide a hypothesis worth elucidating the high hydrogenogenic capacity of the Pattern 2 strains. Hcp has homology to the following two enzymes.

- 1) Hydroxylamine reductase, catalyzing the following reaction



- 2) Carbon monoxide dehydrogenase, catalyzing the following reaction



Since Hcp is similar to hydroxylamine reductase (Wolfe et al., 2002; Cabello et al., 2004), it could be a potential source of NADH or protons, the substrates for H<sub>2</sub> production via the bidirectional hydrogenase enzyme, resulting in higher steady-state H<sub>2</sub> production in Pattern 2 strains. Alternatively, since Hcp is similar to carbon monoxide dehydrogenase, it could be providing the required CO ligand (Pierik et al., 1999) in the active site of the bidirectional hydrogenase in Pattern 2 strains. The source of the CO ligand in the [NiFe]-hydrogenases continues to be unknown (Burstel et al., 2011). Further experiments on



subjecting *hcp* mutants to H<sub>2</sub> production assays, might help to gain a better understanding of the role of the *hcp* gene in Pattern 2 strains.

At the level of HoxYH sequence comparison, we could detect some substitutions in the type of amino acids that were consistent within a Pattern but differed amongst the two Patterns. However, none of these amino acids mapped close to the enzyme's active sites, when located on 3D structural models of the hydrogenases of *L. aestuarii* BL J or *Synechocystis* sp. PCC 6803 (Figure 4), implying that they are unlikely to modify reaction rates. This suggests that polypeptide differences of the hydrogenase enzyme between the two Patterns are unlikely to explain the functional differences detected previously, necessitating, further study of the biochemistry and regulation of the bidirectional hydrogenase enzymes in these strains. Heterologous expression of the bidirectional hydrogenase from *L. aestuarii* BL J in model strains such as *Synechocystis* sp. PCC 6803 might help in gaining a better understanding of the enzyme system.

## **Tables/Figures**

Table 1. The genome size, the percent GC, the number of protein encoding genes and the total number of predicted genes in *L. aestuarii* BL J and other closely related strains.

| Strain                                     | Genome Size <sup>a</sup><br>(Mb) | Percent GC<br>(%) | Protein | Gene |
|--------------------------------------------|----------------------------------|-------------------|---------|------|
| <i>L. aestuarii</i> BL J                   | 6.70                             | 41.2              | 6515    | 6568 |
| <i>L. aestuarii</i> PCC 8106               | 7.04                             | 41.1              | 6142    | 6185 |
| <i>Trichodesmium erythraeum</i> IMS101     | 7.75                             | 34.1              | 4451    | 5126 |
| <i>Arthrospira maxima</i> CS-328           | 6.00                             | 44.7              | 5690    | 5728 |
| <i>Arthrospira</i> sp. PCC 8005            | 6.17                             | 44.6              | 5951    | 6094 |
| <i>Arthrospira platensis</i> NIES-39       | 6.79                             | 44.3              | 6630    | 6676 |
| <i>Arthrospira platensis</i> C1            | 6.09                             | 44.8              | 6108    | 6153 |
| <i>Arthrospira platensis</i> str. Paraca   | 5.21                             | 44.4              | 4674    | 4718 |
| <i>Microcoleus vaginatus</i> FGP-2         | 6.70                             | 46.0              | NA      | NA   |
| <i>Microcoleus chthonoplastes</i> PCC 7420 | 8.68                             | 45.4              | 8294    | 8347 |
| <i>Microcoleus</i> sp. PCC 7113            | 7.97                             | 46.2              | 6441    | 6821 |

NA Data unavailable on the NCBI website

<sup>a</sup> denoting the total contig bp sequenced for draft genomes

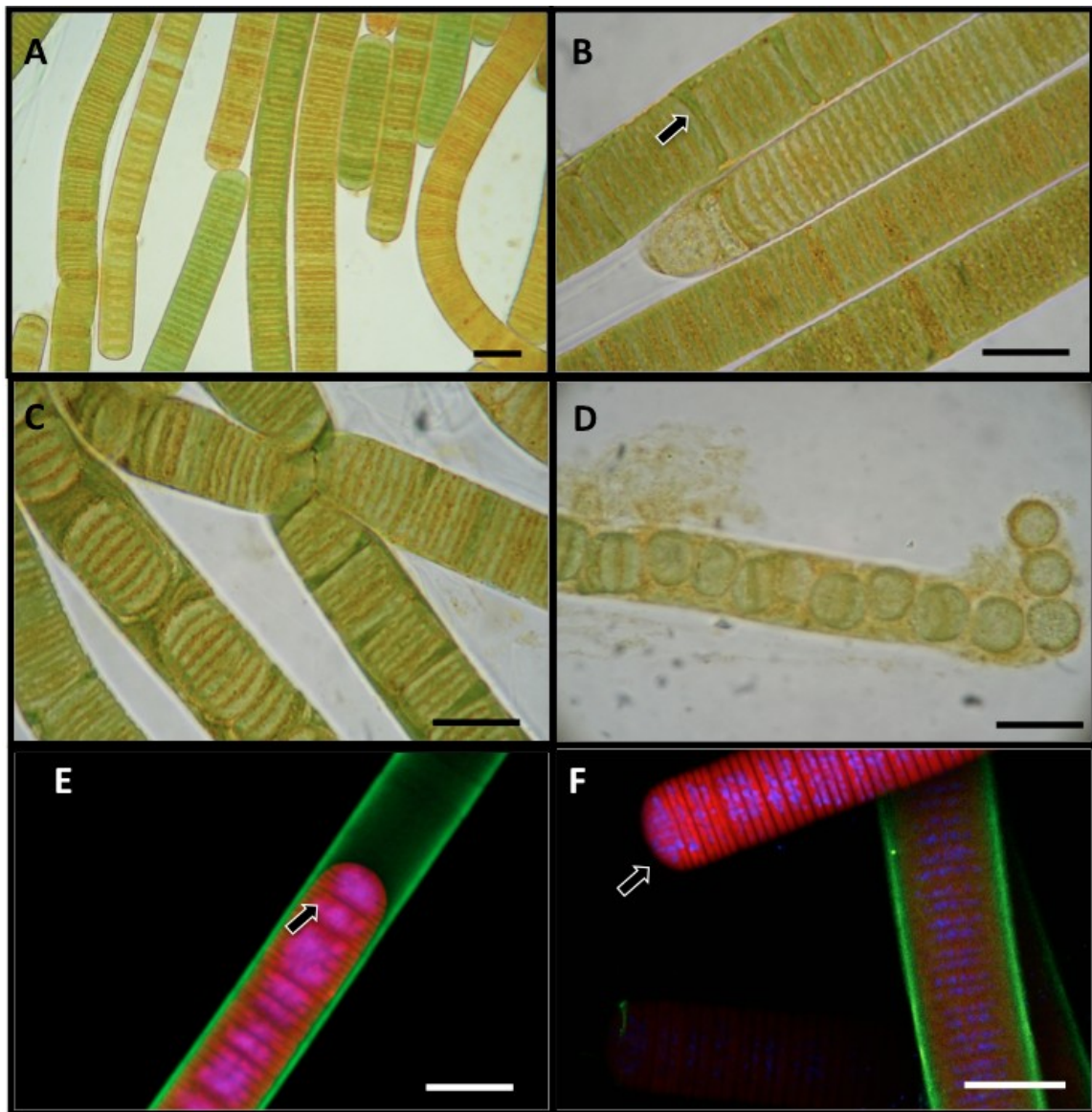


Figure 1. Light microscopy images (A-D) of *L. aestuarii* BL J. (A) The filaments display heterogeneity in pigmentation. (B) Formation of necridial cells (arrow). (C) Short filaments formed by cell division. (D) Breakage of trichome into individual cells or pairs of cells. Fluorescence microscopy images (E-F) depicting the exopolysaccharide sheath stained green, the photosynthetic pigments in red and the nucleic acids stained blue. (E) Cell division by transversal centripetal growth of cross-walls. Arrow marks nascent cell walls. (F) hormogonia (arrow) can be identified by the lack of the exopolysaccharide sheath and motility. A sheathed trichome is in the background. Bar 15 $\mu$ m.

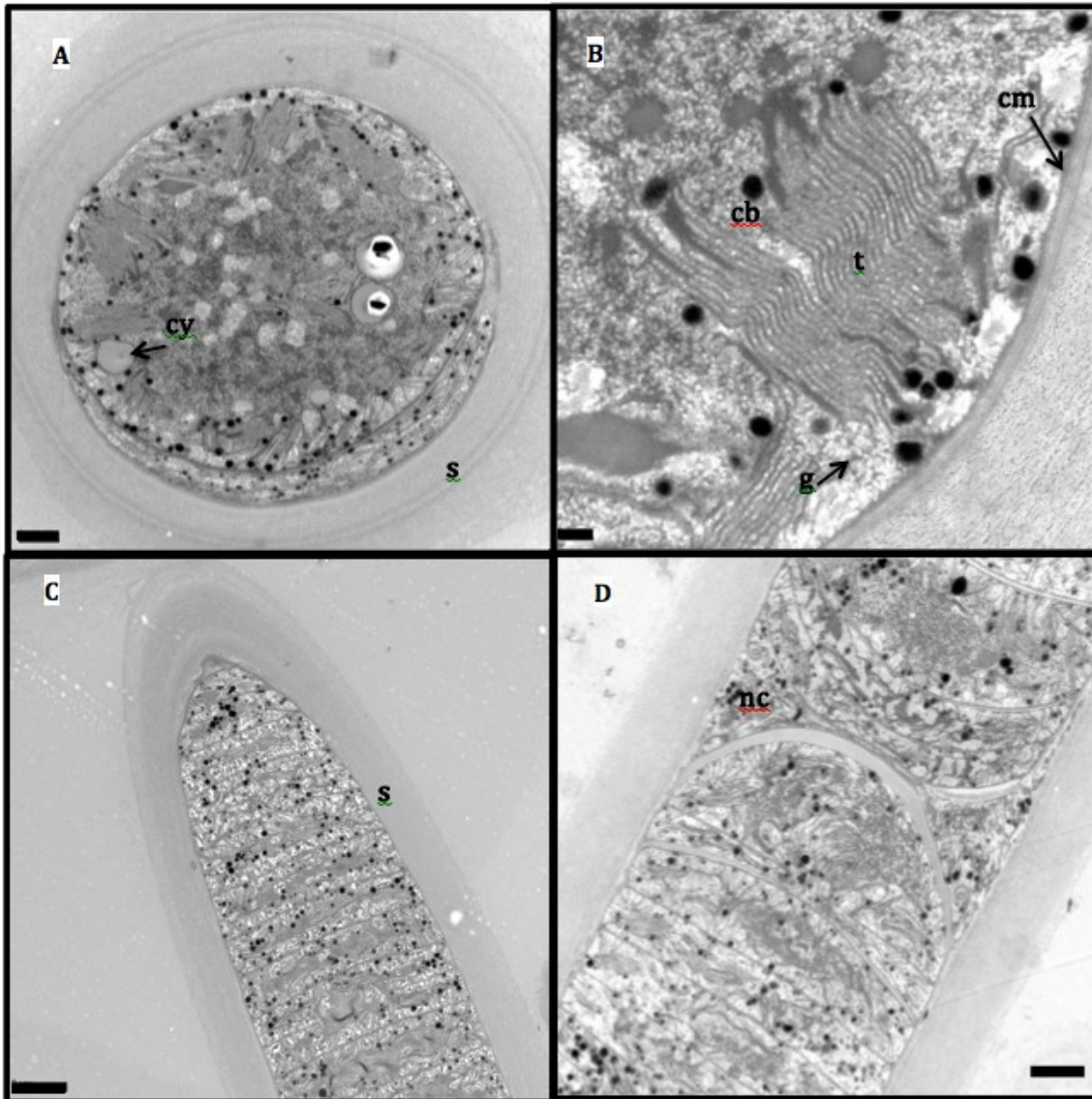
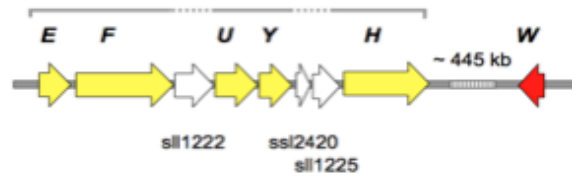


Figure 2. Transmission electron microscopy images of *L. aestuarii* BL J filaments. (A) The transversal section with the stacked thylakoid membranes, randomly oriented, close to the periphery of the cells. The cell contains cyanophycin granules (cy) and thick sheath (s) surrounds the cell; bar 1  $\mu\text{m}$ . (B) Detailed image of the transversal section, displaying the parallel thylakoid membranes (t) along with the polyhedral carboxysomes (cb). A distinct cell membrane (cm) can be observed; bar; 0.2  $\mu\text{m}$  (C) Longitudinal section of the filament, displaying the trichome and the thick sheath (s) around it; bar 2  $\mu\text{m}$ . (D) Longitudinal section displaying necridial cells and newly formed trichomes within the sheath; bar 2  $\mu\text{m}$ .

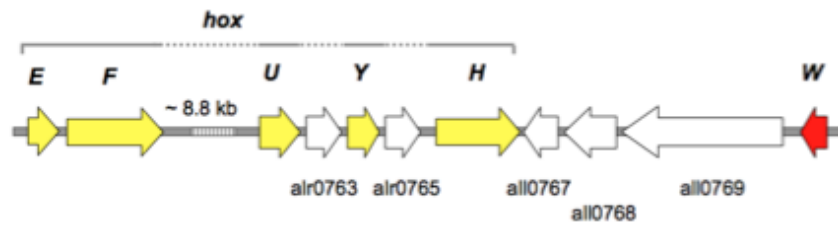
**Pattern 1**

1000 bp

*Synechocystis* sp. PCC 6803

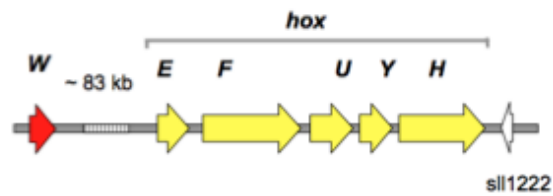


*Anabaena* sp. PCC 7120

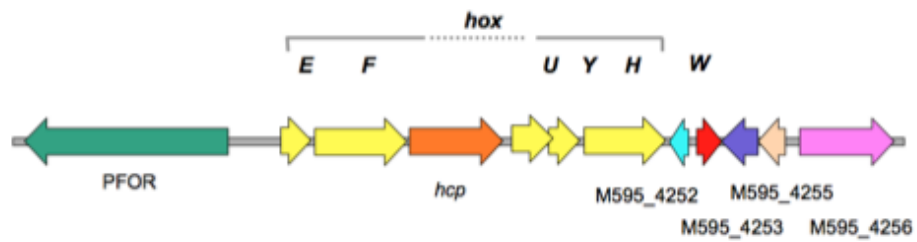


**Pattern 2**

*Microcoleus chthonoplastes* PCC 7420

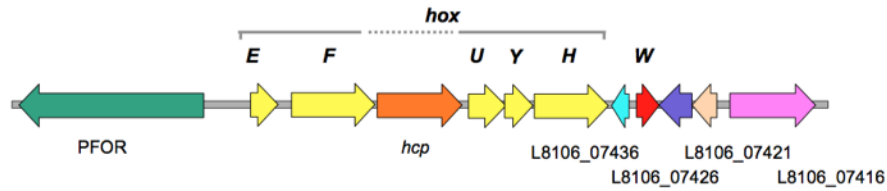


*Lyngbya aestuarii* BL J



## Pattern Unknown

*Lyngbya aestuarii* PCC 8106



*Lyngbya majuscula* CCAP 1446/4

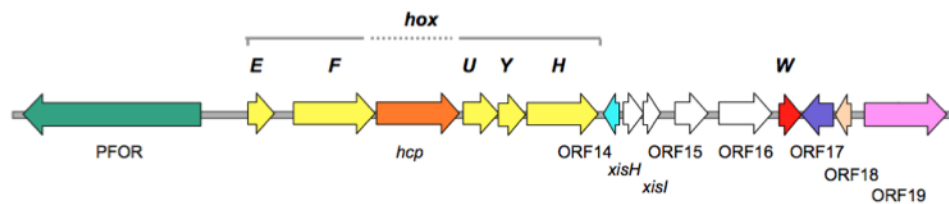


Figure 3. Comparison of the physical map of the bidirectional hydrogenase gene cluster and associated ORFs in Pattern 1 (*Synechocystis* sp. 6803 and *Anabaena* sp. PCC 7120) and Pattern 2 (*M. chthonoplastes* PCC 7420 and *L. aestuarii* BL J) H<sub>2</sub> production displaying strains. The genomic regions from other closely related *Lyngbya* species (*L. aestuarii* PCC 8106 and *L. majuscula* CCAP 1446/4) are included for reference (Hydrogen production Pattern unknown) (Ferreira, 2009). The following ORFs are depicted: *hox* genes (yellow ORFs), *hoxW* (red ORFs), and some additional ORFs (shown as white ORFs, or colored ORFs (including Pyruvate Formate Oxido Reductase PFOR; Hybrid Cluster Protein, hcp) when homologous to the ones in *L. aestuarii* BL J).

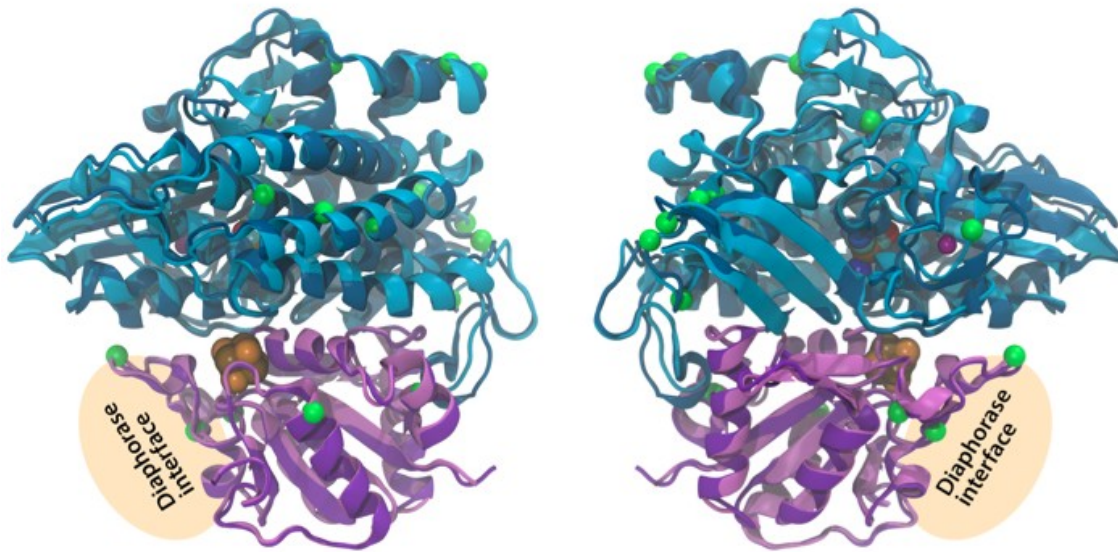
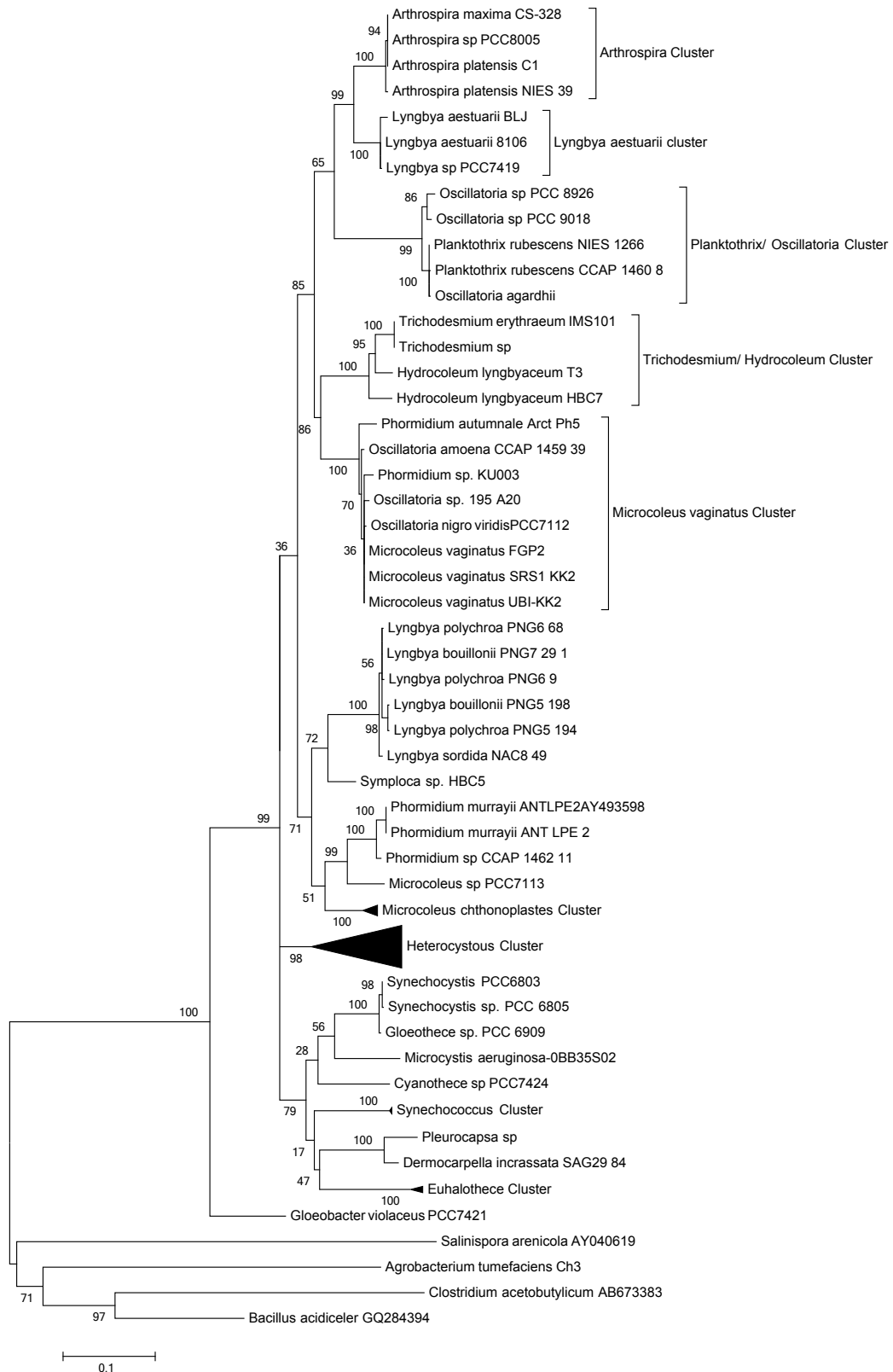


Figure 4. Three-dimensional homology model of the HoxYH subunits from *L. aestuarii* BL J and *Synechocystis* sp. PCC 6803. The backbone of each protein is depicted in ribbons with *Synechocystis* in dark blue (HoxH) and dark purple (HoxY) and *L. aestuarii* in light blue (HoxH) and light purple (HoxY). The images are related by a 180 ° rotation of the model along the Y-axis. The positions of the amino acids that vary significantly between the pattern 1 and pattern 2 have their alpha carbon depicted in space-filling green. The proposed diaphorase interface is also depicted in the figure. The cofactors shown at their Van der Waals radius and are colored as follows: Orange, sulfur; brown, iron; blue, nickel; cyan, carbon; red, oxygen; and maroon, magnesium.

**Supplementary Information**





Supplementary Information 1. Maximum likelihood tree based on the 16S rRNA sequence of 76 bacterial strains. Bootstrap values (%) are indicated, obtained from analyses of 1000 replicate trees. *Microcoleus chthonoplastes* cluster consists of strains WW2, CCY9608, NDN, 7420 and CHI; Heterocystous cluster contains strains *Scytonema* sp. IAM M26, *Scytonema* sp. u-3-3, *Scytonema hofmanni* PCC 7110, *Calothrix* sp. XP9A, *Rivularia* sp. XP 16B, *Rivularia* sp. PCC 7116, *Calothrix deserica* PCC7102, *Rivularia* IAM M26 1, *Calothrix* sp. PCC 7714, *Mastigocladus laminosus* Ja, *Nodulariaspumigena* AV63, *Nodularia spumigena* PCC 9350, *Nodularia sphaerocarpa* PCC 7804, *Nostoc* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, *Tolypothrix* sp. PCC 7415, *Tolypothrix* sp. PCC 7504, *Tolypothrix* sp. PCC 7101; *Synechococcus* cluster contains strains PCC 7310, 7117, 7002 and the *Euhalothece* cluster contains strains MPI95AH10, MPI96N303, MPI96N304.



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## CONCLUSION

The aim of this work was to exploit the biodiversity in Nature to find a cyanobacterium that has already evolved such that it has a better H<sub>2</sub>producing capacity than the standard cyanobacterial strains. Additionally, I had ecological evidence that the marine intertidal mats in Guerrero Negro export H<sub>2</sub> in the night (Hoehler et al., 2001). Therefore, I was specifically interested in characterizing the H<sub>2</sub> production capacities of cyanobacteria isolated from these mats.

A set of 36 phylogenetically diverse strains isolated from terrestrial, freshwater and marine environments were probed for the presence of *hoxH* gene and their potential to produce H<sub>2</sub> from excess reductant. This was the first study to encompass concurrent assessment of the presence of the *hoxH* gene and the potential H<sub>2</sub> evolution capacity from newly isolated cyanobacterial strains. Interestingly, none of the cyanobacteria isolated from the desert showed the presence of the *hoxH* gene or H<sub>2</sub> evolution (in presence of excess external reductants). Currently, no incontrovertible explanation is available as to why cyanobacteria from terrestrial environments are virtually devoid of bidirectional hydrogenases. It is possible that terrestrial environments, which tend to be oxic, may select against enzymes that are involved in anaerobic pathways. However, the epilimnion of freshwater lakes is also an oxygenated environment, and yet the incidence of *hoxH*-positive strains there was quite large (Allahverdiyeva et al., 2010). Perhaps, the cyanobacterial strains from terrestrial environments employ a different metabolism for redox regulation that does not involve a bidirectional hydrogenase.

A majority of the cyanobacterial strains from freshwater and marine microbial mats had the *hoxH* gene, and the correlation between the presence of *hoxH* and ability to produce H<sub>2</sub> in presence of excess external reductants was absolute. Typically only the rates of H<sub>2</sub> production ( $R_H$ ) are reported in the literature for assays with excess external reductants. In this work, additional parameters of maximum steady-state H<sub>2</sub> concentration ( $[H_2]_M$ ) and the time ( $T_R$ ), after which the enzyme reversed direction and started consuming H<sub>2</sub>, were also characterized. Together, these three parameters provide a more comprehensive comparison of the H<sub>2</sub> production capacities of different cyanobacterial strains.

Based on the H<sub>2</sub> production parameters, two distinct patterns in H<sub>2</sub> production were detected amongst the strains isolated from freshwater and intertidal mats. Strains displaying Pattern 1, as previously known from standard cyanobacterial strains such as *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, produced H<sub>2</sub> only temporarily, reverting to H<sub>2</sub> consumption within a short time and after reaching only moderately high H<sub>2</sub> concentrations. By contrast, Pattern 2 cyanobacteria, in the genera *Lyngbya* and *Microcoleus*, displayed high production rates, did not reverse the direction of the reaction and reached much higher steady-state H<sub>2</sub> concentrations. Similar results were obtained in presence of internal reductants in dark anaerobic conditions wherein the Pattern 2 cyanobacteria had a better H<sub>2</sub> production capacity with a lack of H<sub>2</sub> uptake in comparison to the Pattern 1 strains. The highest  $R_H$  and  $[H_2]_M$  in presence of excess external and internal reductants were exhibited by the strain *L. aestuarii* BL J, an isolate from the intertidal mats. Thus, this study highlights the importance of bioprospecting methods in finding novel strains with high H<sub>2</sub> production capacities. The magnitude of the steady-



state H<sub>2</sub> produced in both assays were comparable for *L. aestuarii* BL J, indicating that in the optimized fermentation assays, the amount of H<sub>2</sub> produced was close to its potential capacity to produce H<sub>2</sub> in presence of excess reductants. It is likely that *L. aestuarii* BL has highly efficient means of accumulating and catabolizing glycogen, presumably an ecologically important trait for the mat-dwelling cyanobacteria.

We propose that the powerful H<sub>2</sub> producer, *L. aestuarii* BL J, produces H<sub>2</sub> at the expense of internal reductants using a mixed acid fermentation pathway in dark anaerobic conditions. The high carbon- and H-available-recovery ratios indicate that no product or substrate of fermentation is missing. The strain BL J was average (in comparison to other cyanobacteria) in terms of the amount of H<sub>2</sub> produced per mole of glucose, thus pointing to the differences in the bidirectional hydrogenase enzymatic system itself. We hypothesize that the bidirectional hydrogenase enzyme is responsible for the H<sub>2</sub> production in *L. aestuarii* BL J. When the hydrogenase moiety of the bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 and *L. aestuarii* BL J were compared on the genomic and 3D protein structural levels, no significant differences were found.

The only difference observed between the Pattern 1 and Pattern 2 strains was the presence of a gene encoding hybrid cluster protein (*hcp*) in the Pattern 2 strains. Based on its homology to hydroxylamine reductase and carbon monoxide dehydrogenase, it could either provide the hydrogenase with NADH and protons or provide the required CO ligand present in the active site of the bidirectional hydrogenase. Further experiments on subjecting *hcp* mutants to H<sub>2</sub> production assays, might help to gain a better understanding

of the gene's role in Pattern 2 strains. Since dataset for Pattern 1 and 2 includes just five cyanobacterial strains at this point (Pattern 1 exhibiting *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120; Pattern 2 exhibiting *L. aestuarii* BL J, *M. chthonoplastes* PCC 7420 and *Arthrospira maxima* CS-328), it is necessary to increase the data set before reading too much into the results. This can be done by subjecting the fully sequenced cyanobacteria with genes coding for bidirectional hydrogenase (with or without presence of *hcp* in the genome) to H<sub>2</sub> production assays in presence of external reductants to verify if they fall under Pattern 1 or Pattern 2.

It is of interest to uncover the reason for the strong H<sub>2</sub> production in *L. aestuarii* BL J (exemplary of Pattern 2) in comparison to that of *Synechocystis* sp. PCC 6803 (exemplary of Pattern 1). The initial rate of H<sub>2</sub> production depends on the amount of bidirectional hydrogenase enzyme in the cell and/or the Michaelis constant ( $K_M$ ) of the enzyme (given other factors like pH, temperature and substrate concentrations are unaltered). The Michaelis constant is a measure of the affinity of the enzyme for the substrate, which would likely be the affinity towards NAD(P)H in case of cyanobacterial bidirectional hydrogenase. In comparison to *Synechocystis* sp. PCC 6803, the initial rates of H<sub>2</sub> production were five-fold higher in *L. aestuarii* BL J in presence of external reductants. This could result from slightly higher amounts of hydrogenase and/or  $K_M$  in the strain BL J. In comparison to *Synechocystis* sp. PCC 6803, the initial rates of H<sub>2</sub> production were 17-fold higher in *L. aestuarii* BL J in presence of internal reductants in the optimized fermentation assays. Most likely, this is due to increased amounts of bidirectional

hydrogenase in the strain BL J or because *Synechocystis* sp. PCC 6803 employed alternative strategies in dark anaerobic conditions to get regenerate NAD(P)<sup>+</sup>.

The steady state H<sub>2</sub> concentrations are determined by the concentration of NAD(P)H/NAD(P)<sup>+</sup> (source of electrons) and protons. Therefore, it is expected that when excess reductants (electrons and protons) are provided by reduced methyl viologen, the steady-state H<sub>2</sub> concentrations would be similar in *Synechocystis* sp. PCC 6803 and *L. aestuarii* BL J. However, in presence of excess externally provided reductants, the steady-state H<sub>2</sub> concentration in *Synechocystis* sp. PCC 6803 was 15-fold lower than the strain BL J. The only possible explanation for this behavior is some sort of regulation of the hydrogenase enzyme in the strain PCC 6803. In optimized fermentation assays, the steady-state H<sub>2</sub> concentration in *Synechocystis* sp. PCC 6803 was 45-fold lower than the strain BL J. This could possibly result from lower internal pH, high NAD(P)H/NAD(P)<sup>+</sup> ratio, higher amounts of glycogen or more efficient means of metabolizing glycogen in the strain BL J. It is also possible that *Synechocystis* sp. PCC 6803 employs alternative strategies in fermentative conditions to regenerate NAD(P)<sup>+</sup> thus not producing much H<sub>2</sub>.

NADPH and NADH are known to be electron donors of the bidirectional hydrogenase in *Synechocystis* sp. PCC 6803. A disadvantage of using NAD(P)H as an electron donor for hydrogenase is that it has several sinks such as carbon fixation, nitrate assimilation and lipid synthesis. Additionally, presence of alternate electron donors with a more negative redox potential than NAD(P)H (- 0.320 V), would make the H<sub>2</sub> production reaction more feasible. It is possible that *L. aestuarii* BL J is better at producing H<sub>2</sub> than

*Synechocystis* sp. PCC 6803 because it employs an alternate electron donor. Flavin adenine dinucleotide (FAD) (-0.219 V to -0.400 V) (Nelson and Cox, 2008; Faro et al., 2002), thioredoxin (-0.200 V to -0.350 V) (Krause et al., 1991) and ferredoxin (-0.432 V) (Nelson and Cox, 2008), could all act as potential electron donors for the bidirectional hydrogenase enzyme. *In vitro* assays of the enzyme in the presence of different electron donors will shed some light on this matter.

The major difference between the two patterns was that unlike Pattern 2, the Pattern 1 cyanobacteria exhibited a decline in the concentrations of H<sub>2</sub> leading to the consumption of almost all the H<sub>2</sub> produced. This decline was seen in presence of excess external reductants (Chapter II) and internal reductants (Chapter I), implying that the concentration of reductants was not the limiting factor. The observed decline also has little to do with the loss of enzyme activity, since the bidirectional hydrogenase works in the direction of hydrogen consumption (still active). It perhaps, has something to do with the regulation of enzyme. It is likely that the cells do not prefer bidirectional hydrogenase as means of regenerating the NAD(P)<sup>+</sup> and probably employ other enzymes such as acetaldehyde dehydrogenase, alcohol dehydrogenase and/or lactate dehydrogenase for the same.

Heterologous expression of the bidirectional hydrogenase gene cluster from *L. aestuarii* BL J in the model cyanobacterium *Synechocystis* sp. PCC 6803 may eventually help provide a better understanding of the role of the enzyme itself and the associated *hcp* in H<sub>2</sub> production. Therefore, the strain *L. aestuarii* BL J can be used as a source of useful

genetic information or even as a potential alternate platform for large-scale biohydrogen production.

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## APPENDIX



APPENDIX A  
PUBLICATION CITATIONS

### **Publications used in this dissertation**

- Kothari, A., Potrafka, R., and Garcia-Pichel, F. (2012). Diversity in hydrogen evolution from bidirectional hydrogenases in cyanobacteria from terrestrial, freshwater and marine intertidal environments. *Journal of Biotechnology* 162, 105-114.
- Kothari, A., Parmeswaran, P., and Garcia-Pichel, F. (*in review- Environmental Microbiology*). Powerful fermentative hydrogen evolution of photosynthate in the cyanobacterium *Lyngbya aestuarii* BL J.
- Kothari, A., Vaughn, M., and Garcia-Pichel, F. (2013). Comparative genomic analyses of the cyanobacterium, *Lyngbya aestuarii* BL J, a powerful hydrogen producer. *Frontiers in Microbiology* TBA

APPENDIX B  
AUTHOR PERMISSIONS

## **Author Permissons**

All co-authors have all granted their permission for the use of the papers as chapters in this dissertation.