Turun yliopisto
University of Turku

EXTENDED HYDROGEN PHOTOPRODUCTION BY NITROGEN-FIXING CYANOBACTERIA

Hannu Leino

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. AI osa - tom. 503 | Astronomica - Chemica - Physica - Mathematica | Turku 2014

University of Turku

Faculty of Mathematics and Natural Sciences
Department of Biochemistry
Laboratory of Molecular Plant Biology

Supervised by

Professor Eva-Mari Aro
Dr. Yagut Allahverdiyeva
Laboratory of Molecular Plant Biology
Department of Biochemistry
University of Turku
FI-20014 Turku, Finland

Reviewed by

Professor Matti Karp
Department of Biochemistry and Bioengineering
Tampere University of Technology
FI-33101 Tampere, Finland

Dr. Kari Skjånes
Bioforsk - Norwegian Institute for Agricultural and Environmental Research
Soil and Environment Department
NO-1430 Ås, Norway

Opponent

Dr. Jens Appel
Botanisches Institut
University of Kiel
D-24118 Kiel, Germany

ISBN 978-951-29-5971-6 (PRINT)
ISBN 978-951-29-5972-3 (PDF)
ISSN 0082-7002
Painosalama Oy - Turku, Finland 2014

Tyttärelleni

ABSTRACT

Cyanobacteria are the only prokaryotic organisms performing oxygenic photosynthesis. They comprise a diverse and versatile group of organisms in aquatic and terrestrial environments. Increasing genomic and proteomic data launches wide possibilities for their employment in various biotechnical applications. For example, cyanobacteria can use solar energy to produce H_2. There are three different enzymes that are directly involved in cyanobacterial H_2 metabolism: nitrogenase (*nif*) which produces hydrogen as a byproduct in nitrogen fixation; bidirectional hydrogenase (*hox*) which functions both in uptake and in production of H_2; and uptake hydrogenase (*hup*) which recycles the H_2 produced by nitrogenase back for the utilization of the cell.

Cyanobacterial strains from University of Helsinki Cyanobacteria Collection (UHCC), isolated from the Baltic Sea and Finnish lakes were screened for efficient H_2 producers. Screening about 400 strains revealed several promising candidates producing similar amounts of H_2 (during light) as the Δ*hupL* mutant of *Anabaena* PCC 7120, which is specifically engineered to produce higher amounts of H_2 by the interruption of uptake hydrogenase. The optimal environmental conditions for H_2 photoproduction were significantly different between various cyanobacterial strains. All suitable strains revealed during screening were N_2-fixing, filamentous and heterocystous. The top ten H_2 producers were characterized for the presence and activity of the enzymes involved in H_2 metabolism. They all possess the genes encoding the conventional nitrogenase (*nif*HDK1). However, the high H_2 photoproduction rates of these strains were shown not to be directly associated with the maximum capacities of highly active nitrogenase or bidirectional hydrogenase. Most of the good producers possessed a highly active uptake hydrogenase, which has been considered as an obstacle for efficient H_2 production. Among the newly revealed best H_2 producing strains, *Calothrix* 336/3 was chosen for further, detailed characterization. Comparative analysis of the structure of the *nif* and *hup* operons encoding the nitrogenase and uptake hydrogenase enzymes respectively showed minor differences between *Calothrix* 336/3 and other N_2-fixing model cyanobacteria.

Calothrix 336/3 is a filamentous, N_2-fixing cyanobacterium with ellipsoidal, terminal heterocysts. A common feature of *Calothrix* 336/3 is that the cells readily adhere to substrates. To make use of this feature, and to additionally improve H_2 photoproduction capacity of the *Calothrix* 336/3 strain, an immobilization technique was applied. The effects of immobilization within thin alginate films were evaluated by examining the photoproduction of H_2 of immobilized *Calothrix* 336/3 in comparison to model strains, the *Anabaena* PCC 7120 and its Δ*hupL* mutant. In order to achieve optimal H_2 photoproduction, cells were kept under nitrogen starved conditions (Ar atmosphere) to ensure the selective function of nitrogenase in reducing protons to H_2. For extended H_2 photoproduction, cells require CO_2 for maintenance of photosynthetic activity and recovery cycles to fix N_2. Application of regular H_2 production and recovery cycles, Ar or air atmospheres respectively, resulted in prolongation of H_2 photoproduction in both *Calothrix* 336/3 and the Δ*hupL* mutant of *Anabaena* PCC 7120. However, recovery cycles, consisting of air supplemented with CO_2, induced a strong C/N unbalance in the Δ*hupL* mutant leading to a decrease in photosynthetic activity, although total H_2 yield was still higher compared to the wild-type strain. My findings provide information about the diversity of cyanobacterial H_2 capacities and mechanisms and provide knowledge of the possibilities of further enhancing cyanobacterial H_2 production.

TIIVISTELMÄ

Syanobakteerit ovat ainoita prokaryoottisia eliöitä, jotka kykenevät fotosynteesiin. Syanobakteerit ovat monimuotoisia ja niitä löytyy lähes jokaisesta elinympäristöstä. Lisääntyvä tieto eri lajien genomeista ja niiden ilmentymisestä avaa laajoja mahdollisuuksia syanobakteerien biotekniseen soveltamiseen. Syanobakteerit pystyvät muun muassa valoenergiaa hyväksikäyttäen tuottamaan vetyä. Niillä on kolme entsyymiä, jotka ovat suoraan yhteydessä vetymetaboliaan: nitrogenaasi (*nif*) tuottaa vetyä typen sitomisen sivutuotteena; kaksisuuntainen hydrogenaasi (*hox*) sekä kuluttaa että tuottaa vetyä; ja lisäksi vetyä kuluttava hydrogenaasi (*hup*) kierrättää nitrogenaasin tuottaman vedyn takaisin solun energiakäyttöön.

Tässä tutkimuksessa seulottiin Itämerestä ja Suomen järvistä eristettyjen syanobakteerien (Helsingin yliopiston kantakokoelma, UHCC) joukosta hyvin vetyä tuottavia kantoja. Neljän sadan seulontaan valitun kannan joukosta löytyi muutama villityypin syanobakteerikanta, joiden vedyn tuotanto on samalla tasolla kuin geenitekniikan avulla muokattujen kantojen tehostettu vedyntuottokyky – kuten *Anabaena* 7120 -kannan *hup* poistogeeninen mutantti (Δ*hupL*). Ihanteelliset olosuhteet vedyn valotuotannolle vaihtelevat eri kantojen välillä. Kaikki seulonnassa valikoituneet kannat ovat ilmakehän typpeä sitovia, rihmamaisia ja heterokystejä muodostavia. Vedyn tuotantoon osallistuvat kartoitettiin kymmeneltä parhaalta kannalta, joilta kaikilta löydettiin tavallista nitrogenaasia (*nif*HDK1) koodaavat geenit. Valittujen kantojen korkea vedyn tuotantokyky ei kuitenkaan osoittautunut olevan suoraa seurausta nitrogenaasin tai kaksisuuntaisen hydrogenaasin korkeasta aktiivisuudesta. Suurimmalla osalla parhaista vedyntuottajista osoittautui olevan hyvin aktiivinen vetyä kuluttava hydrogenaasi, jonka on ajateltu olevan este tehokkaalle vedyn tuotannolle. Kymmenen parhaan vedyntuottajakannan joukosta valittiin *Calothrix* 336/3 tarkempaan tarkasteluun. Nitrogenaasin ja vetyä kuluttavan hydrogenaasin *nif* ja *hup* operonien vertaileva analyysi paljasti pieniä poikkeamia *Calothrix* 336/3:n ja muiden typpeä sitovien mallikantojen välillä.

Calothrix 336/3 on rihmamainen, typpeä sitova syanobakteeri, jolla on soikion muotoiset, rihmojen päissä olevat heterokystit. *Calothrix* 336/3:lla on kyky tarttua kiinni erilaisiin pintoihin ja kasvaa niiden pinnalla. Tätä ominaisuutta ja immobilisointi-tekniikkaa hyödyntämällä pystyttiin edelleen parantamaan vedyn valotuotantokapasiteettia. Alginaatilla immobilisointia vedyn valotuotantosysteeminä tutkittiin *Calothrix* 336/3:lla ja verrattiin sitä *Anabaena* 7120:een ja sen Δ*hupL*-mutanttiin samassa systeemissä. Ihanteellisen vedyn valotuotannon saavuttamiseksi, soluja pidettiin typpivapaissa olosuhteissa (Ar kaasufaasina), näin varmistaen nitrogenaasin toiminnan protonien pelkistämisessä vedyksi. Lisäksi syanobakteerisolut tarvitsevat pitkäaikaiseen vedyn valotuotantoon myös hiilidioksidia fotosynteettisen aktiivisuuden säilyttämiseksi sekä palautumissyklit mahdollistaakseen typen sitomisen. Säännöllisten vedyn valotuotantojaksojen ja palautumissyklien avulla, joissa Ar tai ilma toimivat kaasufaaseina, saatiin sekä *Calothrix* 336/3:n ja *Anabaena* 7120 Δ*hupL*-mutantin vedyn valotuotantoa huomattavasti pitkitettyä. On huomionarvoista, että ilman ja lisätyn hiilidioksidin toimiessa palautumissyklissä, Δ*hupL*-mutantin fotosynteettinen aktiivisuus väheni C/N epätasapainon johdosta, vaikka sen vedyn tuotto olikin parempi kuin vastaavan villityypin. Tutkimukseni lisää tietoa syanobakteerien vedyntuotannon moninaisuudesta ja mekanismeista, sekä lisää ymmärrystä syanobakteerien vedyn tuotannon lisäämiseksi tulevaisuudessa.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABC-transporter	ATP-binding cassette transporter
ADP	adenosine diphosphate
ATCC	American type Culture Collection
ATP	adenosine triphosphate
Ar	argon
BG-11	growth medium for reference strains
$bioH_2$	biological H_2 production
CCM	carbon concentrating mechanism
Chl	chlorophyll
Ci	inorganic carbon
cyt b_6f	cytochrome b_6f complex
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fd	ferredoxin
FNR	ferredoxin-$NADP^+$ reductase
GC	gas-chromatograph
G6PD	glucose-6-phosphate dehydrogenase
HPLC	high-performance liquid chromatograph
kDa	kilodalton
Mo	molybdenum
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCBI	National Center for Biotechnology Information
NDH-1	type-1 NADP(H) dehydrogenase
NIR	near-infrared radiation
Nm^R	neomysin resistant cassette
OCP	orange carotenoid protein
OD_{750}	optical density at 750 nm
OPP	oxidative pentose phosphate pathway
P700	reaction center chlorophyll of PSI
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation
PBS	phycobilisome
PC	plastocyanin
PCC	Pasteur Culture Collection

PCR	polymerase chain reaction
PHB	polyhydroxybutyrate
PSI	photosystem I
PSII	photosystem II
PQ	plastoquinone
rRNA	ribosomal ribonucleid acid
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
sp.	species
Sp^R	spectinomysin resistant cassette
UHCC	University of Helsinki Cyanobacteria Collection
UV	ultraviolet (radiation)
V	vanadium
WT	wild type
Z8	growth medium for UHCC strains

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to by their Roman numerals in the text

I Yagut Allahverdiyeva*, Hannu Leino*, Lyudmila Saari, David P. Fewer, Sumathy Shunmugam, Kaarina Sivonen, and Eva-Mari Aro. 2010. Screening for biohydrogen production by cyanobacteria isolated from the Baltic Sea and Finnish lakes. *International Journal of Hydrogen Energy* 35 (3) (2010): 1117-27.

II Hannu Leino, Sumathy Shunmugam, Janne Isojärvi, Paulo Oliveira, Paula Mulo, Lyudmila Saari, Natalia Battchikova, Kaarina Sivonen, Peter Lindblad, Eva-Mari Aro, and Yagut Allahverdiyeva. 2014. Characterization of ten H_2 producing cyanobacteria isolated from the Baltic Sea and Finnish lakes. *International Journal of Hydrogen Energy* 39 (17) (2014): 8983-91.

III Hannu Leino, Sergey N. Kosourov, Lyudmila Saari, Kaarina Sivonen, Anatoly A. Tsygankov, Eva-Mari Aro, and Yagut Allahverdiyeva. 2012. Extended H_2 photoproduction by N_2-fixing cyanobacteria immobilized in thin alginate films. *International Journal of Hydrogen Energy* 37 (1) (2012): 151-61.

IV Sergey N. Kosourov, Hannu Leino, Gayathri Murukesan, Fiona Lynch, Kaarina Sivonen, Anatoly A. Tsygankov, Eva-Mari Aro, and Yagut Allahverdiyeva. 2014. Hydrogen photoproduction by immobilized N_2-fixing cyanobacteria: Understanding the role of the uptake hydrogenase in the long-term process. *Applied and Environmental Microbiology* 80 (18) (2014): 5807-17.

*-equally contributing authors

1 INTRODUCTION

1.1 Hydrogen as a source and carrier of energy

The depletion of easily accessible fossil fuels and the detrimental environmental effects of their utilization have caused the need to find new renewable and environmentally friendly energy alternatives. According to the IPCC (Intergovernmental Panel on Climate Change 2013), if greenhouse gas emissions continue to rise as they do now, the global climate might rise up to 5 °C by the end of this century. However, if emissions are reduced starting from 2020, the temperature of climate would rise only one degree (IPCC 2013). In order to address this problem, the European Union has committed to increase the share of renewable energy from 11%, recorded at year 2009, to at least 20% of the total energy consumption by 2020 (European Renewable Energy Council, EREC 2010). Finland has made a statement committing to cut greenhouse gas emissions by at least 80% from the 1990 level by 2050 (EREC 2009). Fulfillment of these targets requires the utilization of all possible renewable energy sources.

Molecular hydrogen (H_2), also called "the fuel of the future", has potential to be a clean, renewable energy source since it provides energy without CO_2 emission. Indeed, the production of H_2 by photosynthetic organisms can actually capture carbon. H_2 has the highest gravimetric energy density of any known fuel. Most of the current commercial H_2 production is based on steam reforming of natural gas. Some portion of commercial H_2 is produced through the electrolysis of water, which requires electricity generated by burning fossil fuels. Thus large scale H_2 production from the fossil fuels is not a sustainable option.

The ultimate goal is to develop technologies, where H_2 is produced from renewable and sustainable resources. This could be achieved by direct water splitting by photoactive materials during illumination (Herrero et al. 2011, Graetzel et al. 2012, Kenney et al. 2013, Kim & Choi 2014). Progress in this direction has been achieved using inexpensive earth-abundant elements as catalysts for construction of an "artificial leaf" for electrolysis of water into H_2 and O_2 (Nocera 2012, Kibsgaard et al. 2014, Jackson et al. 2014). The electrolysis process, where electricity obtained from other renewable technologies, like geothermal, wind or bioenergy, can be further routed to the artificial, bioinspired catalyst to produce H_2 (Ott et al. 2004, Magnuson et al. 2009, Hammarström et al. 2011, Wang et al. 2011, Lubner et al. 2011, Helm et al. 2011, Brown et al. 2012). Recently, a bio-photoelectrolysis cell system for H_2 production, which utilizes cyanobacteria, has been devised (McCormick et al. 2013).

H_2 is also used as an energy carrier for electricity. H_2 can be converted into electricity via fuel cells, electrochemical devices that combine H_2 with O_2 to generate electricity. Fuel cell engines are believed to be a future replacement for combustion engines since they are approximately twice as efficient as the traditional internal combustion engine (van der Zwaan et al. 2013). If widely utilized, H_2 fuel cell technology will provide a link between renewable energy sources and sustainable energy services (Levin et al. 2004). However, the future utilization of solid state H_2 storage will uncover new possibilities for applications, since it would increase the gravimetric energy density of H_2 significantly (Hanlon et al. 2012).

The European Union has developed a H_2 energy roadmap, HyWays, which aims to establish the impacts of the large scale introduction of H_2 on environment, society and economy (HyWays 2008). The prospects for a future H_2 economy will depend on developing competitively priced fuel cells for stationary applications or vehicles. Multi-fuel integrated energy systems could co-produce electricity, H_2 and liquid fuels with overall high-conversion efficiencies and low emissions, whilst also facilitating CO_2 capture and storage (Floudas et al. 2012). H_2 can be produced independently from other countries, thus being of national interest in energy security.

There are several strategies to produce bioH_2 by living organisms, direct water biophotolysis (as performed by green algae and cyanobacteria), indirect water biophotolysis (by green algae and cyanobacteria), photo-fermentation (performed by purple non-sulfur bacteria), water-gas shift reaction (by photo-heterotrophic bacteria), and dark-fermentation (as performed by anaerobic bacteria, cyanobacteria and green algae) (Kerby et al. 1995, Levin et al. 2004, Azwar et al. 2014). Cyanobacteria and green algae are the only organisms capable of performing oxygenic photosynthesis with simultaneous production of H_2 in sunlight from water via hydrogenase and nitrogenase enzymes linked directly or indirectly to the photosynthetic electron-transport chain (Houchins 1984, Happe & Naber 1993, Tamagnini et al. 2007, Tsygankov 2007).

The focus of this thesis is the sustainable photoproduction of H_2 by using solar energy, water and cyanobacteria.

1.2 Cyanobacteria

Cyanobacteria are classified as Gram-negative prokaryotes, although many species have cell envelopes similar to a Gram-positive envelope, for example in the thickness of the peptidoglycan layer and composition of lipids. Cyanobacteria are covered with a gelatinous sheath followed by an outer membrane, a peptidoclycan layer and a cytoplasmic or plasma membrane (Liberton & Pakrasi 2008). Cyanobacterial cells range in size from those of typical bacteria (0.5-1 μm in diameter) to cells as large as 60 μm in diameter (Madigan et al. 2008). Most species are obligate phototrophs, being unable to grow in the dark on organic compounds. Some can grow photomixotrophically (You et

al. 2014). However, most cyanobacteria can use simple organic compounds, such as glucose, if light is not present (Rippka et al. 1979).

Cyanobacteria exhibit a carbon-concentrating mechanism (CCM), which allows the cells to raise the concentration of CO_2 in the carboxysome, in near proximity to Rubisco up to 1000-fold over that in the surrounding environment (Giordano et al. 2005, Price et al. 2008). The carboxysome is a special compartment consisting of thousands of proteins. The CCM compensates for the low affinity of Rubisco for CO_2, depresses photorespiration, and also helps the cell in dissipating excess light energy (Tchernov et al. 2003, Giordano et al. 2005, Price et al. 2008). The CCM is induced under low inorganic carbon (C_i as CO_2 and HCO_3^-) conditions, and under non-limiting C_i, the CCM declines to a basic constitutive level with mainly CO_2 uptake (Price et al. 2008).

Whilst cyanobacteria share the basic cellular features of other bacteria, they are unique in that they have evolved coupled photosystems which are able to remove electrons from water and produce O_2 as a byproduct (Knoll 2008). These photosystems are localized to a complex and organized system of internal membranes, called thylakoids. Thylakoids consist of a membrane surrounding a thylakoid lumen. Chlorophyll *a* in thylakoid membranes is an essential pigment for photosynthesis (Björn et al. 2009), but chlorophylls absorb only a certain range of the sunlight spectrum. Accessory and protective pigments such as phycobilins and carotenoids give an extended ability to harvest a wide spectrum of light for photosynthesis and, in some cases, protect cells from UV and other light-induced cell damage. The major light-harvesting antenna complexes in cyanobacteria are the water-soluble phycobilisomes, which surround the reaction centers (DeRuyter & Fromme 2008) and cyanobacteria have been characterized by their ability to form high amounts of the phycobilin pigment, phycocyanin, giving the blue color that lead to their naming as blue-green algae (Whitton & Potts 2012).

Major protein complexes, Photosystem (PS) II, PSI, Cytochrome b_6f complexes and ATP-synthase are also embedded in the thylakoid membrane. During photosynthesis, light is captured by the antenna complexes and excitation energy is transferred to PSI and PSII reaction centers where charge separation occurs. PSII uses absorbed light energy to split water to O_2 and protons and passes electrons down the electron transport chain – through plastoquinone, Cytochrome b_6/f and plastocyanin. Meanwhile, PSI is also excited by light and performs a charge separation at P700, a primary electron donor of PSI, and passes electrons through the iron-sulfur clusters to ferredoxin (Fd), which acts as a soluble electron carrier. Finally, Fd transfers electrons to the Fd-dependent $NADP^+$ oxidoreductase enzyme (FNR) that reduces $NADP^+$ to NADPH. Plastocyanin, a small mobile electron carrier, recovers oxidized P700 by donating electrons originated mainly from water splitting in PSII. This process is known as linear electron transport. During linear electron transport, protons are transferred from cytosol to lumen through the thylakoid membrane, thus producing an electrochemical proton gradient across the membrane. This drives ATP production via the ATP syn-

thase complex (**Figure 1**). Photosynthetically generated NADPH and ATP are utilized primarily by the Calvin-Benson-Bassham cycle for fixation of CO_2, but may also fuel many other metabolic pathways. In addition, Fd, FNR and NADPH which are reduced during light reactions, can distribute electrons to various alternative reactions. A common alternative is cyclic electron transport around PSI, where the electron flow from NADPH or Fd is re-directed back to plastoquinone, leading to ΔpH generation and additional ATP production (DeRuyter & Fromme 2008). In some cases, Fd can also directly donate electrons to nitrogenase (Bothe 1970, Smith et al. 1971) and bidirectional hydrogenase (Gutekunst et al. 2014) enzymes. Under specific conditions, these enzymes may contribute to efficient H_2 generation (Tamagnini et al. 2007).

Despite the prokaryotic nature of cyanobacteria, they have also contributed significantly to the eukaryotic kingdom, since it is likely that the engulfment of an ancient cyanobacterium resulted in the plant chloroplast (Sagan 1967, Mulkidjanian et al. 2006). In the Earth's history, cyanobacteria have been important primary producers, playing a significant role in both carbon and nitrogen cycles. Fossil traces of the ancestors to present cyanobacteria have been found dating back ~3.5 billion years. The evolution of cyanobacterial PSII (2.8-2.3 billion years ago) gave rise to increasing atmospheric oxygen which changed the life conditions on Earth, yielding an explosion in biodiversity (Knoll 2008, Schopf 2012). Cyanobacterial ecological plasticity, physiological flexibility and long evolutionary history are possible reasons for their easy adaptation to numerous ecological niches. Diversity is also seen in the sizes of their genomes, with sequenced genomes ranging from 1.44 (marine *UCYN-A* organism) to 15.87 Mbp (*Mastigocoleus testarum*) (Tripp et al. 2010, Dagan et al. 2013). Cyanobacteria can be found in environments ranging from fresh water to oceans, from terrestrial to arctic environments and from rocks to soil. Symbiotic interactions with other organisms are diverse, including fungi (lichens), sponges, protists and plants (Whitton & Potts 2012).

Cyanobacteria are promising microorganisms for the production of biofuels: they directly convert solar energy into chemical energy using water, CO_2 and some minerals as a substrate. Under certain conditions cyanobacteria can produce non-organic (H_2) and organic biofuels. Cyanobacteria have many positive aspects regarding biofuel production: (i) they can double their biomass in 6-12 hours at their exponential growth phase (Mori et al. 1996, Kondo et al. 1997); (ii) under suitable weather they can be cultivated year around; (iii) the culture systems demand less water than terrestrial crops; (iv) they can be grown in brackish water on non-arable land, avoiding the competition with the production of food crops; (v) they can utilize most nutrients from industrial or household waste; (vi) they can produce useful side-products, like bioactive compounds for pharmaceutical use or biomass used as feed or fertilizer, or fermented to produce different biofuels (Abed et al. 2009, Rastogi & Sinha 2009, Ortiz-Marquez et al. 2013).

A number of enzymes from different organisms have been transferred to cyanobacteria to engineer novel pathways for the targeted production of different organic biofuels. By means of genetic and metabolic engineering the following fuels have been successfully produced in cyanobacteria: (i) alcohol-derived fuels, like ethanol, isobutyraldehyde and butanol (Dexter & Fu 2009, Atsumi et al. 2009, Lan & Liao 2011, 2012, Gao et al. 2012, Savakis et al. 2013); (ii) isoprenoid-derived fuels, isoprene and monoterpenes (Lindberg et al. 2010, Bentley & Melis 2012, Bentley et al. 2013); (iii) ethylene and other alkenes (Takahama et al. 2003, Converti et al. 2009, Eser et al. 2011, Ungerer et al. 2012, Guerrero et al. 2012); and (iv) fatty acids and alcohols (Kaczmarzyk & Fulda 2010, Liu et al. 2011, Tan et al. 2011). Cyanobacteria have also been metabolically engineered to produce and secrete sugars and lactate (Niederholtmeyer et al. 2010, Angermayr et al. 2012).

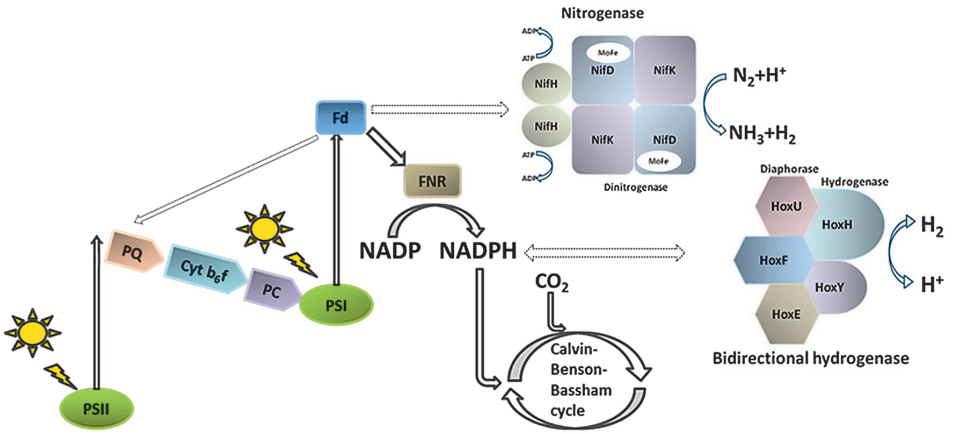

Figure 1. BioH_2 production pathways in cyanobacteria (modified from Hallenbeck 2012).

1.2.1 Morphology of filamentous cyanobacteria

Among cyanobacteria there are a wide range of morphologies including unicellular and multicellular forms. In some multicellular cyanobacteria, four different cell types can be found: the vegetative cells, heterocysts, hormogonia and akinetes. Under favorable growing conditions, filamentous cyanobacteria form mainly vegetative cells, also called as photosynthetic cells. Many filamentous strains in response to the deprivation of combined nitrogen, differentiate some vegetative cells into heterocysts. Heterocysts are decribed in more detail below. In response to certain environmental impulses, some filamentous cyanobacteria also produce hormogonia, which are short and mobile filaments that can be transiently used for short-range dispersal and the establishment of symbiosis. Hormogonia do not fix N_2 and are smaller and more cylindrical than vege-

tative cells. The smaller cell size is a result of the hormogonia being formed by cell division without cell growth or DNA replication. This also results in fewer copies of the chromosome in each cell (Meeks et al. 2002, Meeks & Elhai 2002).

When environmental conditions become extremely harsh, some heterocystous strains can differentiate vegetative cells into akinetes, which are spore-like cells. Akinetes are bigger than vegetative cells, very resistant to both cold and desiccation and can survive for a long time. In this cell type, high levels of valuable substances such as nitrogen and carbohydrates are stored in the form of cyanophycin and glycogen. When the environment becomes more favorable, the akinete germinates into a vegetative cell (Meeks et al. 2002).

1.2.1.1 Heterocysts

In N_2-fixing filamentous cyanobacteria, the absence of a combined nitrogen source allows some vegetative cells to differentiate into specialized cells, known as heterocysts. Heterocysts are microoxic compartments where nitrogenase catalyzed N_2 fixation occurs. These specialized cells are usually distributed at a frequency of 5-10% of the total cells in a semi-regular pattern, either individually along a filament or at one end of a filament.

Heterocysts can be morphologically identified by special nodules at their polar regions which connect them to adjacent vegetative cells. This polar region also contains membranes with high respiratory activity to consume residual O_2 that, in addition to N_2, enters the heterocyst from adjacent vegetative cells through the terminal pores (microplasmodesmata). These channels are thought to be a connection medium, allowing the exchange of different metabolites between cells in a filament. Heterocysts also accumulate granules of cyanophycin as a nitrogen reservoir to the polar regions of heterocysts (Fay 1992, Walsby 2007, Flores & Herrero 2010).

Heterocysts and vegetative cells are interdependent. As heterocysts lack Rubisco, and cannot reduce CO_2, they rely on carbon compounds supplied by the vegetative cells, mostly as sucrose (Lopez-Igual et al. 2010). Also, alanine is transported from vegetative cells into heterocysts where it is catabolized for reducing power (Pernil et al. 2010). In turn, heterocysts provide vegetative cells with fixed nitrogen. Glutamine and β-aspartyl-arginine produced from cyanophycin in the heterocysts is transferred intercellularly to vegetative cells (Flores & Herrero 2010, Burnat et al. 2014). PSI in the heterocyst provides nitrogenase with energy as ATP by cyclic photophosphorylation and light-reduced Fd as an electron donor (**Figure 2**) (Flores & Herrero 2010, Bothe et al. 2010). Carbohydrates are metabolized in heterocysts mainly through the oxidative pentose phosphate (OPP) pathway, which produces NADPH (Summers et al. 1995).

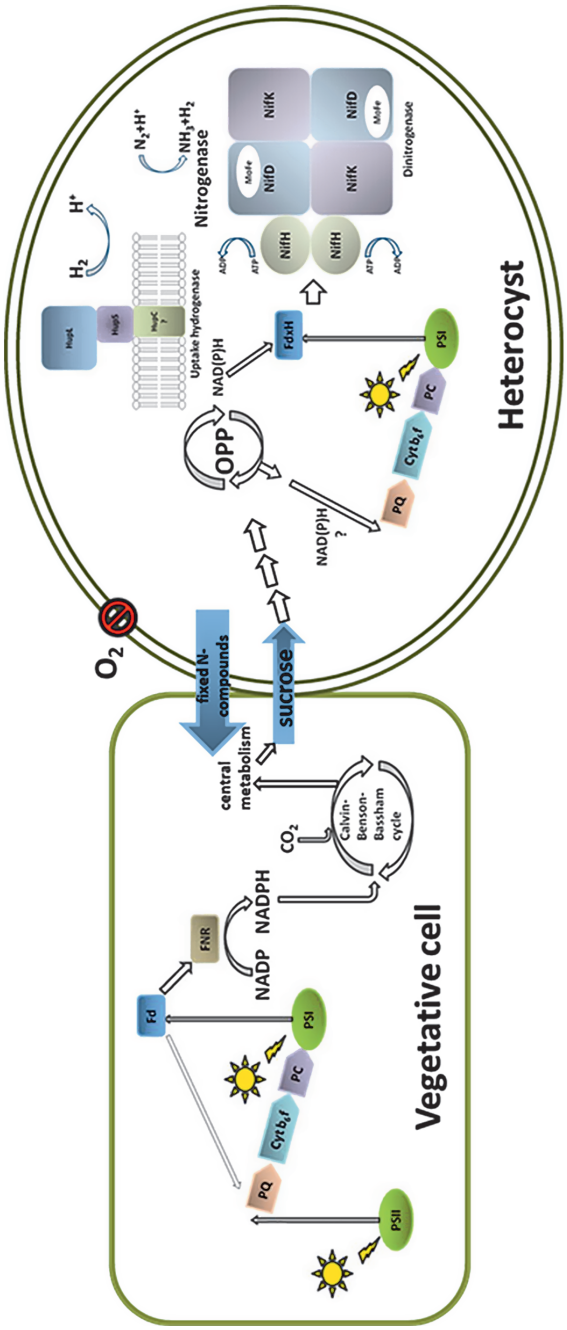

Figure 2. Metabolic interactions during biophotolysis by heterocysts and vegetative cells in N_2-fixing cyanobacteria (modified from Hallenbeck 2012).

The irreversible differentiation of a vegetative cell into a heterocyst takes about 24 hours during a nitrogen step-down (Yoon & Golden 1998, Huang et al. 2004). More than 500 proteins are differentially expressed in heterocysts during this differentiation

from a vegetative cell (Ow et al. 2008), demonstrating the complex regulation of this process. The transcriptional regulators NtcA and HetR are required for differentiation (Herrero et al. 2001, Flores & Herrero 2010). NtcA is highly conserved in cyanobacteria; it perceives nitrogen stress by responding to the internal 2-oxoglutarate (2-OG) level, which is an indicator of the C/N balance (Muro-Pastor et al. 2001, 2003, Flores & Herrero 2010). The NtcA response is to control nitrogen availability in cells by binding to the promoter region of target genes and activating or repressing their expression. An example of this is the binding of NtcA upstream of the site-specific recombinase *xisA*, resulting in the excision of an 11,5 kb DNA element located within *nifD* in *Anabaena* sp. PCC 7120 (hereafter, *Anabaena* 7120) (Ramasubramanian et al. 1994). Rearrangements also take place during the late stages of heterocyst differentiation of other cyanobacteria. These excision elements comprise of a 55-kb XisF within Fd (*fdxN*) and a 10.5-kb XisC within the large subunit of uptake hydrogenase (*hupL*) (detailed below) (Bothe et al. 2010). Recently, it has been found that NtcA can bind to more than 2000 DNA regions in *Anabaena* 7120, from which only a small portion of genes are involved in N metabolism, suggesting a wider role for NtcA in the physiology of the cells (Picossi et al. 2014).

NtcA and HetR, the master regulator of cell differentiation, demonstrate mutual dependency for expression. Mutants deficient of HetR do not develop heterocysts, while overexpression leads to an increase in heterocyst differentiation (Buikema & Haselkorn 2001). The activity of HetR is modulated by the products of *hetF* and *patS*. HetF is needed for HetR autoregulation and *patA* mutants form heterocysts only at the end of the filaments (Wong & Meeks 2001, Buikema & Haselkorn 2001). PatS and HetN are required for maintaining an established pattern of heterocysts (Yoon & Golden 2001, Callahan & Buikema 2001).

Ammonium, nitrate and urea are the most common sources of fixed nitrogen for cyanobacteria. Ammonium is the most preferred source, as it is the most energetically economical. The availability of fixed nitrogen sources reduces cyanobacterial N_2 fixation. In *Anabaena* 7120, the genes involved in the uptake and reduction of nitrate and nitrite are organized as a *nirA* (Fd nitrite reductase)-*nrtA*-*nrtB*-*nrtC*-*nrtD* (four genes encoding an ATP-binding ABC-transporter) -*narB* (Fd nitrate reductase) cluster. This gene cluster is highly expressed in the absence of ammonium but also in the presence of nitrate or nitrite. Upon transfer of *Anabaena* 7120 cells to ammonium deficient conditions, the transcription levels of the *nirA*-*narB* genes increased within approximately 30 minutes (Cai & Wolk 1997, Thiel 2005).

When conditions are unfavorable for N_2 fixation, mainly due to high O_2 concentrations, a modification of the nitrogenase Fe-protein occurs, which inactivates nitrogenase reversibly. Addition of ammonium at a high pH can lead to increasing O_2 levels in heterocysts by limiting the available reductant for respiration. This nitrogenase modification can similarly be induced during periods of low carbohydrate supply to heterocysts (Ernst et al. 1990).

Ammonium produced by nitrogenase is incorporated via the GS-GOGAT pathway. In this pathway, glutamine synthetase (GS) catalyzes the ATP-dependent amidation of glutamate, forming glutamine. Glutamate synthetase (GOGAT) catalyzes the reductive transamidation of 2-oxoglutarate (2-OG), producing two molecules of glutamate (Martin-Figueroa et al. 2000, Meeks et al. 1977, 1978, Flores & Herrero 2010). 2-OG directly enters the GS-GOGAT cycle in cyanobacteria, serving as a carbon skeleton for the incorporation of inorganic nitrogen. High levels of 2-OG indicate nitrogen starvation (a high C/N ratio) and induce nitrogen assimilation systems. Heterocysts contain high levels of GS and lack GOGAT. Isolated heterocysts produce glutamine when incubated in the presence of glutamate. This suggests that there is a glutamate-glutamine exchange between vegetative cells and heterocysts that results in a net export of nitrogen from the heterocysts (Wolk et al. 1976, Flores & Herrero 2010). Alternatively, glutamine can be converted to arginine which is incorporated into granule of cyanophycin (Gupta & Carr 1981).

1.2.2 *Anabaena* sp. PCC 7120

Anabaena 7120, also known as *Nostoc* sp. PCC 7120 or *Anabaena/Nostoc* sp. ATCC 27893 is a photoautotrophic, filamentous, heterocyst-forming and N_2-fixing cyanobacterium. This is the first filamentous cyanobacterium whose genome has been sequenced. The genome size is approximately 7.21 Mbp, and divided on one chromosome and six plasmids (Sazuka et al. 1999, Kaneko et al. 2001). The transcriptome (Ehira & Ohmori 2003, 2006) and proteome of the strain have been characterized (Stensjö et al. 2007, Ow et al. 2008). *Anabaena* 7120 has been a model organism for N_2-fixing cyanobacterial research. The strain is not symbiotically competent and lacks the ability to form either hormogonia or akinetes. Availability of genetic transformation tools, including an efficient conjugation system, have made this strain suitable for studies of cellular differentiation, pattern formation, N_2 fixation and H_2 production.

1.3 Cyanobacterial H_2 metabolism

An important approach to the sustainable production of H_2 from a variety of renewable resources, such as sunlight, water, organic wastes or biomass, is through using microorganisms. Early work on photobiological H_2 production was performed by Gaffron and Rubin, who in 1942 reported that the green alga *Scenedesmus* produced H_2 under light conditions which followed a dark, anaerobic treatment. In 1974, Benemann and Weare demonstrated that a N_2-fixing cyanobacterium, *Anabaena cylindrica,* produced H_2 and O_2 gas simultaneously in an argon (Ar) atmosphere for several hours. Later, the

cyanobacterium *Anabaena* 7120, which transiently produced H_2, was found to have a reversible, or bidirectional, hydrogenase (Houchins & Burris 1981b).

Since this early work, a total of three enzymes have been demonstrated to be directly involved in the process of H_2 metabolism in cyanobacteria (**Figure 3**): nitrogenase catalyzes the production of H_2 as a byproduct of the fixation of N_2 to ammonia; uptake hydrogenase catalyzes the utilization of H_2 produced by nitrogenase; bidirectional hydrogenase has the capability to evolve and consume H_2. Cyanobacterial strains can have a bidirectional hydrogenase, an uptake hydrogenase, or both.

Some cyanobacteria can produce H_2 by direct water biophotolysis. During direct biophotolysis, electrons for H_2 production are supplied directly from water-splitting PSII via linear electron flow. However, this production system is limited in cyanobacteria by the simultaneous evolution of O_2, which hydrogenases are highly sensitive to. For this reason, H_2 production via direct photolysis in cyanobacteria lasts only a few seconds during a dark to light transition (Cournac et al. 2004). During indirect biophotolysis the O_2-producing water splitting and O_2- sensitive H_2 production reactions are temporally or spatially separated. During indirect biophotolysis, electrons are supplied to H_2 via degradation of intracellular carbon compounds produced during photosynthetic CO_2 fixation.

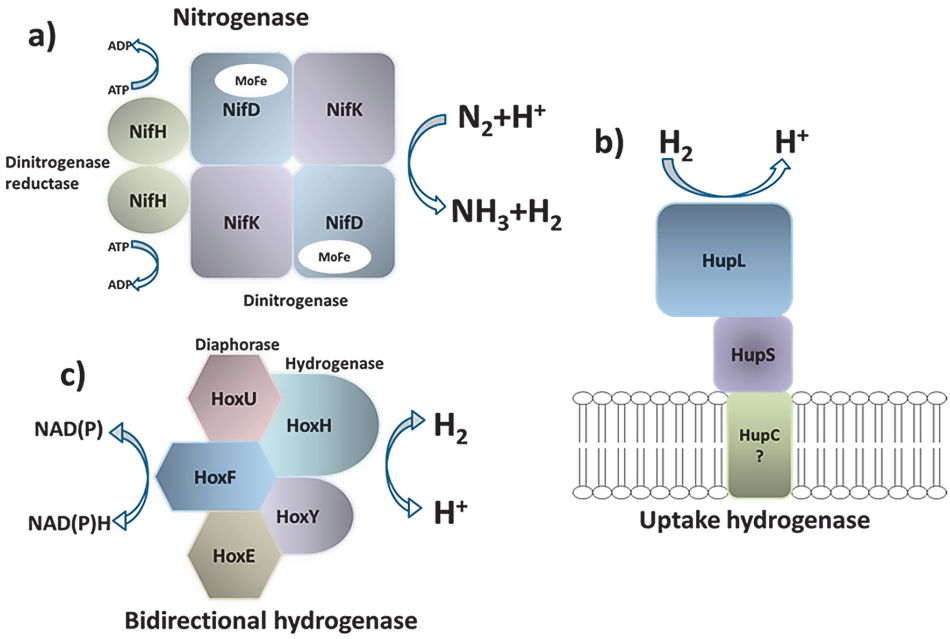

Figure 3. Enzymes involved in H_2 metabolism in cyanobacteria. (a) Nitrogenase, (b) Uptake hydrogenase, (c) Bidirectional hydrogenase (modified from Hallenbeck 2012).

1.3.1 Nitrogenases

There are different types of nitrogenase based on the metal composition in the active site. The molybdenum (Mo) containing nitrogenase fixes atmospheric nitrogen into ammonia (NH_3) and produces H_2 as a byproduct according to:

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi.$$

The NH_3 produced is later converted into proteins and nucleic acids (Meeks et al. 2002). This reaction is highly endergonic and costly for the cell, since 16 ATP molecules are consumed per nitrogen molecule fixed.

In the absence of atmospheric N_2, nitrogenase catalyzes the reduction of protons to H_2 (Masukawa et al. 2010):

$$2\ H^+ + 2\ e^- + 4\ ATP \rightarrow H_2 + 4\ ADP + 4\ Pi.$$

Reduction of protons to H_2 by the nitrogenase enzyme is a relatively slow reaction (6.4 s^{-1}) (Hallenbeck et al. 1979). The properties of the cyanobacterial Mo-nitrogenase, as shown by purification and characterization, are equivalent to those of heterotrophic organisms (Hallenbeck & Benemann 2002).

Mo-containing nitrogenase comprises of two subunits: the dinitrogenase, which is a MoFe protein, and the dinitrogenase reductase, which is a Fe-protein (**Figure 3**). The dinitrogenase is an $\alpha_2\beta_2$ heterotetramer of about 240 kDa. It is a substrate reducing active site and the α and β subunits are encoded by *nifD* and *nifK* genes. The dinitrogenase reductase is a homodimer of 64 kDa, encoded by the *nifH* gene (Bothe et al. 2010). It initiates electron transfer from the external donor, Fd or flavodoxin (Flv) to the dinitrogenase, where the actual reduction of N_2 occurs (Howard & Rees 1996). Reduced Fe-protein forms a complex with MoFe-protein, two Mg-ATP molecules are hydrolysed by the Fe-protein, and transfer of an electron to the MoFe-protein takes place. The complex dissociates, ADP is released and can be reduced again. The process is repeated until enough electrons have been transferred to N_2 to form NH_3 (Burgess & Lowe 1996, Bothe et al. 2010).

The reaction catalyzed by nitrogenase requires reducing power. Electrons derived from carbohydrates transported from vegetative cells are transferred to nitrogenase mainly via NAD(P)H and a heterocyst specific Fdx. *Anabaena* 7120 possesses two heterocyst-specific ferredoxins, encoded by *fdxN* and *fdxH* genes. The FdxH functions as a donor to nitrogenase, whereas the role of FdxN remains unclear. FdxH takes electrons from PSI in the light, but can also be reduced by FNR in darkness with the use of NADPH produced in OPP. Alternatively, electrons can pass to nitrogenase via NADH and a NADH-dehydrogenase to the plastoquinone pool, PSI, which can also reduce Fdx. ATP may be generated by either cyclic photophosphorylation or oxidative phos-

phorylation. Oxidative phosphorylation also helps in keeping oxygen levels low, using the same source of reductants as nitrogen fixation (Haselkorn & Buikema 1992, Bothe et al. 2010).

While most N_2-fixing strains have only one Mo-containing nitrogenase, some N_2-fixing *Nostoc* and *Anabaena* strains possess two Mo-containing nitrogenases (Thiel et al. 1995, 1997, Masukawa et al. 2009). One of the Mo-containing nitrogenases, encoded by the *nif1*-operon, is expressed in heterocysts, while the other, encoded by *nif2*-genes, is expressed only in vegetative cells under anaerobic conditions (Thiel et al. 1995, 1997). The structural genes of nitrogenase, *nifHDK*, are located together with a number of additional accessory *nif*-genes (Meeks et al. 2001).

Apart from the conventional Mo-containing nitrogenases, there are also alternative nitrogenases, V-containing and Fe-containing nitrogenases. V-containing nitrogenases have been found in different cyanobacterial strains (Thiel 1993, Masukawa 2009). The stoichiometry between NH_3 production and H_2 formation via Mo-nitrogenase is about 2:1, whereas the stoichiometry for V-containing nitrogenase is 2:3 (Masukawa et al. 2009, Bothe et al. 2010). The *vnf*-genes encoding the V-containing nitrogenase are transcribed in the absence of molybdenum (Thiel 1993). A Fe-containing nitrogenase has not been identified in cyanobacteria examined thus far.

Nitrogenases are sensitive to O_2. In order to protect these enzymes, cyanobacteria have developed different strategies based on either spatial or temporal separation, to separate O_2 evolving photosynthesis from N_2 fixation. Some filamentous or unicellular strains express nitrogenase activity only in the darkness to avoid inactivation by O_2 (Herrero et al. 2001). On the other hand, *Trichodesmium* sp., a N_2-fixing cyanobacterium of global ecological importance, fixes nitrogen aerobically and expresses nitrogenase activity in the light within special, protective compartments called diazocytes (Fredriksson & Bergman 1997).

1.3.2 Hydrogenases

Hydrogenase catalyzes the simple chemical reaction, the reversible formation of hydrogen from protons and electrons: $2H^+ + 2e^- \leftrightarrow H_2$, a reaction not requiring ATP but reductants. The redox-potential of the individual electron acceptors or donors capable of interacting with the enzyme determines the direction of the reaction (Böck et al. 2006, Vignais & Billoud, 2007). There are three phylogenetically distinct classes of enzymes, the [NiFe]-hydrogenases, the [FeFe]-hydrogenases and the [Fe]-hydrogenases. All have conserved functional cores (Volbeda et al. 1995, Peters 1999, Lyon et al. 2004, Vignais & Billoud, 2007). Cyanobacteria have two types of [NiFe]-hydrogenases, uptake and bidirectional hydrogenases (Tamagnini et al. 2007).

Cyanobacterial hydrogenases have not been crystallized, so most conclusions regarding the structure of the active site are depicted from the X-ray structure of [NiFe]-

hydrogenases from *Desulfovibrio gigas* (Volbeda et al. 1995). Results from other organisms fundamentally verify these structures and it is expected that the general features are the same for cyanobacteria.

The genes which are essential for the biosynthesis, maturation and processing of the [NiFe]-hydrogenases have all been recognized. Furthermore, their products have been biochemically and functionally characterized. The overall assembly of a [NiFe]-hydrogenase is a complex process, which involves auxiliary proteins, the products of the *hyp*-genes, namely HypABCDEF. These proteins dictate the synthesis and insertion of the metal center into the large subunit with ATP, GTP, and carbamoyl phosphate (Böck et al. 2006, Hoffmann et al. 2006, Forzi & Sawers 2007, Vignais & Billoud 2007, Shomura & Higuchi 2012). Other genes involved in the maturation of hydrogenases encode endopeptidases like *hupW/hoxW,* which cleave off the last amino acids of the C-terminal end of the large subunit of hydrogenase (Lindberg et al. 2012).

The presence of only one copy of most of the *hyp* genes in the genome of cyanobacteria indicates co-regulation of the *hyp*-genes on the maturation of both types of hydrogenases, regardless of whether they possess only the uptake hydrogenase (e.g. *Nostoc punctiforme* ATCC 29133, hereafter *N. punctiforme*), the bidirectional hydrogenase (e.g. *Synechocystis* PCC 6803) or both enzymes (e.g. *Anabaena* 7120). Factors that affect the regulation and maturation of uptake hydrogenases in cyanobacteria are not well studied. The present knowledge is mainly based on research conducted using *Escherichia coli* (Böck et al. 2006, Hoffmann et al. 2006, Forzi & Sawers 2007, Vignais & Billoud 2007). The *hyp*-genes are conserved and can either be clustered, like in *Anabaena* 7120 or spread in the genome, as in *Synechocystis* PCC 6803 (Kaneko et al. 2001). Details of the regulation system are not known, but the *hyp*-genes are most likely regulated differently depending on the species, the environment and the type of hydrogenase.

1.3.2.1 Uptake hydrogenase

In N_2-fixing cyanobacteria H_2 is mainly produced by the nitrogenase enzyme, however uptake hydrogenase consumes the H_2 produced by nitrogenase and redirects extracted electrons back to other cell functions. Uptake hydrogenase may also function as a protector of the micro-oxic environment of the heterocyst, since O_2 is reduced via the oxyhydrogen reaction (Tamagnini et al. 2007). Uptake hydrogenase has been found in almost all of the N_2-fixing cyanobacteria examined so far, with a few exceptions in unicellular N_2-fixing strains (Steunou et al. 2008). The inactivation of uptake hydrogenase in *Anabaena variabilis* ATCC 29413 (hereafter *A. variabilis)* reduced the growth rate and caused a lower rate of N_2 fixation when compared with the corresponding wild type (Happe et al. 2000). However, it is still thought that uptake hydro-

genase is not essential for diazotrophic growth (Happe et al. 2000, Lindberg et al. 2002, Masukawa et al. 2002).

All known cyanobacterial uptake hydrogenases consist of at least two subunits, encoded by the structural genes *hupSL* (hydrogen uptake, small, large) (Vignais et al. 2001) (**Figure 3**). The *hupSL* genes are transcribed together as an operon and are usually located in close proximity to the maturation genes. The active site is positioned in HupL and HupS harbors three FeS clusters (Volbeda et al. 1995; Vignais et al. 2001, Raleiras et al. 2013).

The localization of uptake hydrogenase is not fully clear. In *Anabaena* 7120, and in *N. punctiforme*, uptake hydrogenase was found to be exclusively localized in heterocysts (Peterson & Wolk 1978, Carrasco et al. 2005, Seabra et al. 2009, Camsund et al. 2011). Whereas in N_2-fixing non-heterocystous *Lyngbya majuscula* CCAP 1446/4, uptake hydrogenase was found in the vegetative cells (Seabra et al. 2009). It has been suggested that the uptake hydrogenase enzyme is a membrane-bound protein, with a putative HupC subunit playing an anchoring role (Tamagnini et al. 2007).

On a gene expression level, the transfer of non-N_2-fixing vegetative cells to N_2-fixing conditions has been demonstrated to induce *hupL* transcription in *Anabaena* 7120 and *A. variabilis* through the action of the cyanobacterial global nitrogen regulator NtcA (Carrasco et al. 1995, Happe et al. 2000, Weyman et al. 2008). It has also been demonstrated that the transcription of *hupSL* is increased following incubation under anaerobic conditions, incubation with addition of H_2 to the gas phase, or by addition of Ni to the culture medium (Axelsson & Lindblad 2002).

The regulation of uptake hydrogenase is further complicated in some species by the need for excision of an intervening sequence, a process linked to heterocyst development (Carrasco et al. 2005). Prior to expression of *hupSL* in *Anabaena* 7120, a 10.5 kb DNA fragment is excised from within *hupL*. In *Anabaena* 7120, two additional gene rearrangements occur (Golden et al. 1985). Each excision requires a site-specific recombinase. XisC is responsible for the excision of the 10.5 kb in *hupL* and its gene is located within the interrupting sequence. Studies of the upstream region of another site-specific recombinase, *xisA*, have revealed a binding site of the nitrogen regulator NtcA (Carrasco et al. 1995).

1.3.2.2 Bidirectional hydrogenase

Bidirectional hydrogenase can either consume or evolve H_2 depending on the redox-state of the cells (Tamagnini et al. 2007, Carrieri et al. 2011). Its physiological role is not fully understood, but there are three main possible functions: (i) it serves as a safety valve for excess electrons generated during the dark-light transition (Appel & Schulz 1998, Appel et al. 2000); (ii) it catalyzes oxidation of H_2 in the periplasm by redirecting electrons to the respiratory chain; or (iii) it removes surplus reductants under

anoxic conditions (Schmitz et al. 1995, Tamagnini et al. 2007, Carrieri et al. 2011, Gutekunst et al. 2014).

Bidirectional hydrogenase is a pentameric enzyme, consisting of a hydrogenase and a diaphorase part, encoded by the *hoxEFUYH*-genes (hydrogen oxidation) (**Figure 3**). The active site is positioned in the large subunit, HoxH, and the electron transporting 4Fe4S cluster is located in the small subunit, HoxY (Schmitz et al. 2002, Germer et al. 2009). *hoxEFU* are the structural genes for a diaphorase unit, which bidirectionally transports electrons to produce or reduce NAD(P)H.

In some cyanobacterial strains all *hox*-genes are clustered in one operon but with multiple transcription start points, like in *Synechocystis* PCC 6803, or are divided on two operons, like in *Anabaena* 7120 (Sjöholm et al. 2007). It has been demonstrated that the *hox*-genes have a circadian clock expression (Schmitz et al. 2001). LexA and AbrB-like proteins are members of the complex signaling cascade that regulates the expression of the bidirectional hydrogenase genes (Gutekunst et al. 2005, Oliveira & Lindblad 2009, Dutheil et al. 2012, Sakr et al. 2013).

It has been accepted that in cyanobacteria both NADH and NADPH can act as substrates for bidirectional hydrogenase. Some studies have shown that NADH is a preferential substrate, whereas NADPH is an efficient activator, of bidirectional hydrogenase (Serebryakova & Sheremetieva 2006, Aubert-Jousset et al. 2011). Interestingly, recent investigations demonstrated that Fd (or Flv) act as electron donor to the bidirectional hydrogenase in *Synechocystis* PCC 6803, under both dark to light transition and dark fermentation conditions (Gutekunst et al. 2014).

Bidirectional hydrogenase is found in vegetative cells as well as in heterocysts and its presence is not connected to N_2 fixation (Hallenbeck & Beneman 1978, Houchins & Burris 1981). Also, it seems that bidirectional hydrogenase does not play an essential role in those strains where it is present, with the inactivation of *hox* genes in *Synechocystis* PCC 6803 and *Anabaena* 7120 resulting in only small decreases in growth rate compared to wild type strains (Appel et al. 2000, Masukawa et al. 2002, Eckert et al. 2012).

Both aerobic and anaerobic conditions allow constitutive expression of bidirectional hydrogenase, but the enzyme is only active in dark, anoxic conditions, or during a transition from anoxic dark to light. Higher levels of *hox* transcripts have been detected under microoxic or anoxic conditions in *Synechocystis* PCC 6803 and *Anabaena* 7120 (Cournac et al. 2004, Schutz et al. 2004, Kiss et al. 2009). Following circadian rhythm, *hox* transcripts increase in light and decrease in darkness (Kucho et al. 2005). Bidirectional hydrogenase in *Anabaena* 7120 is transcribed in both vegetative cells and heterocysts under oxic conditions, with several fold higher activity observed in the microoxic heterocysts. When transferred to anoxic conditions, the activity of bidirectional hydrogenase increased about two orders of magnitude with approximately the same activities found in both vegetative and heterocyst cell types (Houchins & Burris 1981a). Interestingly, the activity of bidirectional hydrogenase in the unicellular strain

Gloeocapsa alpicola CALU 743 was found not to be directly dependent on O_2 (Sheremetieva et al. 2002).

1.4 Improvement of cyanobacterial H_2 production

There are many ways of enhancing the H_2 production capacity of cyanobacteria. To improve large-scale H_2 photoproduction using cyanobacteria, several research challenges must first be overcome: (i) the underlying limitations of photosynthetic efficiency and balance; (ii) the genetic engineering challenges of some cyanobacteria; (iii) the oxygen sensitivity of the hydrogenase and nitrogenase enzymes; (iv) the competition between nitrogenases, hydrogenases and other metabolic pathways for electrons; (v) the inhibitory effects of fixed nitrogen on nitrogenase activity; (vi) the low turnover of nitrogenase; and (vii) H_2 uptake occurring inside of the cells. The most promising approaches are regarded as biotechnical, such as bioprocessing engineering, but it is equally important to improve the efficiency of the technical engineering required for the whole production system.

Cyanobacterial conversion of total incident light energy to free energy of H_2 has been shown to be about 0.7 % under favourable laboratory conditions, where higher efficiencies can be expected (Yoon et al. 2006, Sakurai & Masukawa 2007, Berberoglu et al. 2008). Under natural sunlight, this efficiency drops to 0.1% (Tsygankov et al. 2002). Interestingly, calculated theoretical efficiencies of a nitrogenase based system range from 4.6% (Hallenbeck 2011) to 6.3-7.3% (Sakurai & Masukawa 2007), implying large scope for improvements.

Very high rates, 300 to 400 μmol H_2 mg Chl^{-1} h^{-1} (the latter when supplemented with 50 mM glycerol), of light-driven nitrogenase based H_2 production have recently been demonstrated by the unicellular cyanobacterium, *Cyanothece* ATCC 51142 (Min & Sherman 2010, Melnicki et al. 2012). Surprisingly, this strain produced even more H_2 (465 μmol H_2 mg Chl^{-1} h^{-1}) under aerobic conditions (Bandyopadhyay et al. 2010). However, these results have subsequently been questioned (Skizim et al. 2012). So far, the maximum rate published for heterocystous N_2-fixing cyanobacteria is 167.6 μmol H_2 mg Chl^{-1} h^{-1} by the uptake hydrogenase mutant of *A. variabilis* PK84 (Sveshnikov et al. 1997, Tsygankov et al. 1999). For heterocystous strains that produce H_2 only via the bidirectional hydrogenase route, a much lower maximum rate of 3.1 μmol H_2 mg Chl^{-1} h^{-1} was reported for *Arthrospira maxima* (Carrieri et al. 2008 according to Skizim et al. 2012) and 25 μl H_2 (mg dry weight) $^{-1}$ h^{-1} for *Gloeocapsa alpicola* CALU 743 (Troshina et al. 2002).

1.4.1 Improvement of the enzymes involved in H_2 production

Improving the efficiency of the enzymes involved in H_2 production can be achieved via a systems biology understanding of their function in cyanobacteria and the successful application of genetic engineering (Navarro et al. 2009). Although a lot of work has been completed towards engineering cyanobacteria for the production of biofuels (Angermayr et al. 2009, Lu 2010), relatively little has been focused on the genetic and metabolic engineering of cyanobacteria for improving H_2 production.

There are several strategies to prolonging the catalytic lifetime and negating the oxygen sensitivity of hydrogenases. These include: (i) computer pathway simulations and genetic engineering modifications into the catalytic site of hydrogenases for O_2 gas diffusion, possibly by applying steric hindrance to the gas channels to block O_2 from reaching the catalytic site; (ii) down-regulating PSII-catalyzed O_2 evolution; and (iii) screening for more O_2-tolerant hydrogenases from environment and importing those genes into cyanobacteria (Tamagnini et al. 2007, Vignais & Billoud, 2007, Ghirardi et al. 2009).

Cyanobacterial [NiFe] hydrogenases are O_2 sensitive, but can be reactivated when O_2 is removed. The exact mechanism for O_2 inactivation is not known, but it is proposed that O_2 uses the same gas channel as H_2 to reach the active site of hydrogenase, thus competively binding to the same site. Therefore, amino acids in the gas channel have been modified to prohibit the O_2 molecule from entering (Buhrke et al. 2005, Vignais & Billoud, 2007).

There have been several attempts to express a foreign O_2 tolerant hydrogenase in cyanobacteria. Oxygen-tolerant [NiFe] hydrogenases from *Alteromonas macleodii* and *Thiocapsa roseopersicina* were heterologously expressed in *Synechococcus elongatus* PCC 7942 and were found to be active by *in vitro* assay (Weyman et al. 2011). The expression of [FeFe] *Clostridial* hydrogenase in *Synechococcus elongatus* sp. 7942 resulted in > 500-fold increase of H_2 evolution in total of 2.8 μmol H_2 mg Chl^{-1} h^{-1} (Ducat et al. 2011). [FeFe] hydrogenase from *Chlamydomonas reinhardtii* was successfully expressed in *Synechocystis* PCC 6803 without additional maturation proteins (Berto et al. 2011). The [FeFe] hydrogenase operon from *Shewanella oneidensis MR-1* has been expressed in the heterocysts of *Anabaena* 7120 (Gärtner et al. 2012). Although the heterologous protein was active, supporting the O_2 protective function of heterocysts, the increase in H_2 production was disappointingly small, likely due to competition for reducing power with coexisting nitrogenase. So far, all of these attempts have been only partially successful due to hindrances in the electron transport chain compatibility.

A systems biology approach has been applied for the construction and verification of genome-scale metabolic models for *Synechocystis* PCC 6803 (Fu 2009, Montagud et al. 2010, Yoshikawa et al. 2011). This approach has led to the recently developed "Biobrick" shuttle vector system (Huang et al. 2010, Rokke et al. 2014) or SBPkb sys-

tem (Galdzicki et al. 2011) and the characterization of other synthetic biology tools, like promoters, ribosomal binding sites, terminators and regulatory elements. These features facilitate successful transformation and bioengineering in cyanobacteria leading to strains capable of producing higher amounts of H_2 (Shetty et al. 2008, Boyle & Silver 2009, Purnick & Weiss 2009).

There are at least two major problems in producing H_2 using cyanobacteria, the function of: (i) uptake hydrogenase and (ii) bidirectional hydrogenase in the consumption of H_2 as it is produced. In recent years, there have been numerous attempts to solve these problems. Several specified mutants have been created with reduced or deleted uptake hydrogenase activity. Uptake hydrogenase-deficient mutants of *A. variabilis* (Mikheeva et al. 1995), *N. punctiforme* (Lindberg et al. 2002), *Anabaena* 7120 (Masukawa et al. 2002), *Nostoc* sp. PCC 7422 (Yoshino et al. 2007) and *Anabaena siamensis* TISTR 8012 (Khetkorn et al. 2012) were generated and shown to produce H_2 at a higher level than wild-type strains. Except for *N. punctiforme*, other strains also possess bidirectional hydrogenase, which can also have H_2 uptake activity. Masukawa et al. (2002) have succesfully produced a *hox*-defective mutant (Δ*hoxH*) *of Anabaena* 7120 and also a mutant deficient in both hydrogenases (Δ*hupL/*Δ*hoxH*). Additionally they demonstrated that the double mutant produced H_2 at the same rate as other uptake hydrogenase-deficient mutants. This is contrasted by the bidirectional hydrogenase-deficient mutant (Δ*hoxH*), which produced less H_2 than the wild type (Masukawa et al. 2002). Unlike filamentous cyanobacteria, deletion of uptake hydrogenase in unicellular *Cyanothece* PCC 7822 led to inactivation of nitrogenase and therefore resulted in decreased cell viability (Zhang et al. 2014). The important role of uptake hydrogenase in unicellular cyanobacteria could be related to the fact that these organisms have to contend with oxygen on a continuing basis, without the advantage provided by the heterocyst (Zhang et al. 2014).

Genetic engineering has been applied to change the properties of the active site of the nitrogenase enzyme in order to produce more H_2 in air (N_2 atmosphere). Site-directed mutagenesis was applied to six amino acid residues close to the FeMo-cofactor of nitrogenase in *Anabaena* 7120 in order to selectively direct electron flow towards proton reduction (Masukawa et al. 2010). Indeed, several mutants exhibited a significantly increased H_2 production rate under the N_2 atmosphere. Site-directed mutagenesis at the α-75 site of *nif*2 in *A. variabilis* resulted in impaired acetylene reduction, but a four-fold higher H_2 production was observed in a N_2 atmosphere compared to the wild type (Weyman et al. 2010).

Another interesting approach to improve H_2 yield would be to increase heterocyst frequencies either by the use of chemicals (Bothe & Eisbrenner 1977) or by genetic modifications (Meeks & Elhai 2002). Increasing the heterocyst frequency to approximately 20-25 % is known to enhance nitrogenase activity (Meeks & Elhai 2002). However, it was found that nitrogenase activity of genetically modified *Anabaena* 7120 with increased heterocyst frequency up to 29% was comparable to a wild-type

strain with a heterocyst frequency of 10%, suggesting that at very high heterocyst frequencies the remaining vegetative cells were not able to provide enough carbohydrates to heterocysts (Buikema & Haselkorn 2001).

1.4.2 Funneling electron flow towards H_2 production

In order to improve the H_2 photoproduction rates, competing electron transport pathways must be identified and eliminated. The electron flow must be redirected to H_2 production instead of the production of biomass. To overcome these barriers of H_2 production, the entire electron flow, from water splitting to H_2, the transcriptional regulation, maturation and function of the enzymes involved in H_2 production and the metabolic and regulatory network in the target strains needs to be thoroughly characterized.

In general, electron transport is regulated by cyclic electron flow and nonphotochemical quenching (Cournac et al. 2002, Li et al. 2009). It has been suggested that inactivation of these routes would result in higher rates of H_2 photoproduction. Directing more electrons to hydrogenase by preventing cyclic electron transfer around PSI would maintain the high ratio of NADPH to ATP required for producing H_2. Supporting this theory, a *Chlamydomonas reinhardtii* mutant with low cyclic photophosphorylation has been shown to produce H_2 more effectively than ordinary cells (Kruse et al. 2005). A mutant strain of *Synechocystis* PCC 6803, deficient in cyanobacterial NDH-1 complex (M55), also produced higher levels of H_2, presumably because of low activity of cyclic electron flow (Cournac et al. 2004). However, a decrease in cyclic electron-transport rates in phycobilisome mutants had only a minor impact on linear photosynthetic electron-transport flow (Bernat et al. 2009).

Modifications made to the growth, development or nutrient uptake pathways of cyanobacterial cells can yield improved H_2 production. For example, the deletion of components involved in the respiration and nitrate assimilation of *Synechocystis* PCC 6803 has been shown to improve H_2 production (Gutthann et al. 2007, Baebprasert et al. 2011). Increased H_2 production has also been achieved using specific inhibitors to prevent biomass accumulation, possibly through the redirection of electron flow towards nitrogenase and bidirectional-hydrogenase (Khetkorn et al. 2012b). Finally, the overexpression of the sigma factor s*igE* has been a successful approach to increased H_2 production, whereby an increase in heterocyst frequency was also observed (Mella-Herrera et al. 2011, Osanai et al. 2013).

The low efficiency of light to H_2 conversion in photosynthetic systems is believed to be caused by the shading effect and non-photochemical quenching capacity of huge light-harvesting system of photosynthetic cells. In mass cultures, upper cell layers utilize the available illumination and shade the lower layers (Ort et al. 2011). Therefore, when the size of the photosynthetic antennae is reduced through genetic modifications,

it improves the usage of high light intensities by photosynthetic microorganisms (Mussgnug et al. 2007). Recently, modifications made to the light harvesting machinery of green algae have increased their photosynthetic capacity (Polle et al. 2002, Mussgnug et al. 2007, Beckmann et al. 2009, Mitra & Melis 2010, Oey et al. 2013) and improved H_2 production (Kosourov et al. 2011). However, truncation of the phycobilisome antennae of *Synechocystis* PCC 6803 resulted in decreased culture productivity, suggesting that antennae engineering for increased H_2 production is a complex matter (Page et al. 2012).

Most oxygen-evolving photosynthetic organisms are only capable of utilizing the visible 400–700 nm of the solar spectrum. The challenge for bio-energy applications based on photosynthesis is to engineer photosystems capable of utilizing a wider range of the solar spectrum. The photosynthetic efficiency of *Acaryochloris* sp. containing Chl *d* is as high as only Chl *a*-containing cyanobacteria and can effectively extend the utilized range of the solar spectrum to 750 nm (Swingley et al. 2008, Pfreundt et al. 2012). Co-culturing purple non-sulphur bacteria capable of utilizing near-infrared radiation (NIR) photons with cyanobacteria might improve overall efficiencies of H_2 production (Das & Veziroglu 2008). Converting NIR radiation to visible light available for photosynthesis with the photon up-conversion mechanism (Antal et al. 2012) might also be feasible to improve H_2 production by natural and artificial photosynthetic systems. Applying carbon nanotubes to the plant chloroplast has been demonstrated to increase photosynthetic capacity through augmented photoabsorption in NIR wavelengths and enhanced electron transport rates (Calkins et al. 2013, Giraldo et al. 2014)

Constructing mutant strains with several improvements is a strategy for improved H_2 production. An example of this is the deletion of the homocitrate synthese genes from Δ*hupL* mutant of *Anabaena* 7120. This double mutant demonstrated a higher H_2 production and increased heterocyst ratio in a N_2 atmosphere when compared to parental strain (Masukawa et al. 2007).

1.4.3 Immobilization

Cyanobacterial H_2 production is difficult to maintain in photobioreactor suspension cultures. Efficient production requires intensive mixing for better light utilization, which might cause damage to the filaments. Upscaling the suspension culture systems is also difficult. The immobilization of cyanobacteria and design of special photobioreactors with new materials (for example, gas barrier plastic) might solve these challenges (Das & Veziroglu 2001, Carvalho et al. 2006, Posten 2009, Show et al. 2011, Kumar et al. 2011, Guo et al. 2011, Kitashima et al. 2012). The purpose of immobilization is to maintain and control the biological activity and photosynthetic productivity of the cells while improving stability, density and reuse of cells. Cells entrapped in

films are more protected from contaminations and receive equally distributed light (Moreno-Garrido 2008, Kosourov & Seibert 2009, Meunier et al. 2011).

Immobilization of biomolecules and whole cells can be distinguished by their attachment to solid surfaces (biofilms, adsorption to porous (nano) materials) or entrapment in different organic and inorganic polymer matrixes (gels, foams, latex) (Olguín 2003, Moreno-Garrido 2008, de-Bashan & Bashan 2010, Meunier et al. 2011). Supporting structures inside the gel have been applied to increase mechanical stability (Markov et al. 1993, Kaya & Picard 1995, Kosourov & Seibert 2009) and nanoparticles added to protect cells from UV radiation and oxidative stress (Sicard et al. 2011). Immobilization has been used for biosensors, environmental remediation, biofuel production, and biosynthesis of chemicals. Key elements for cell immobilization are: (i) the diffusion properties of the immobilization matrix; (ii) the optimal thickness of the microbial layer; (iii) the physical and chemical stability of the matrix in the long term and the retention of the cells within the matrix; iv) low cost; v) neutrality of the matrix to cell metabolism; and vi) simplicity of the immobilization technique (Mallick 2002, Olguín 2003, Azbar & Kapdan 2011). Entrapped cells have limited gas diffusion and substrate acquisition, depending on the porosity of the matrix, which results in restrictions of the available applications. Natural and artificially induced biofilm formations have limited control of cell concentration and biofilm thickness, and the release of cells (Sekar et al. 2004). There are some examples of cyanobacteria being immobilized to enhance H_2 production (Brouers & Hall 1986, Phlips & Mitsui 1986, Kuwada & Ohta 1987, Sarkar et al. 1992, Markov et al. 1993, 1995, Serebryakova & Tsygankov 2007, Dickson et al. 2009, Rashid et al. 2009, 2012).

Combining different embedded microbes within biocomposite material structures, can lead to a more efficient or multifunctional hybrid material, such as a biomimetic leaf (Bagai & Madamwar 1998, Flickinger et al. 2007, Jenkins et al. 2013). Recently, a method for hydrated microbial latex coatings was developed, with the successful immobilization of cyanobacteria and green algae (Gosse et al. 2012). Implementing this technique, it could be possible to physically paint viable cyanobacteria onto a variety of surfaces for multipurpose applications.

Application of microalgae cultivation and co-culturing other microbes for wastewater treatment coupled with H_2 and biofuel generation as a biorefinery, could reduce energy, fertilizer and freshwater costs and reduce greenhouse gas emissions. This biorefinery approach could ensure the economic viability and sustainability of the whole biofuel production process and yield multiple possible products according to several Life Cycle Analyses (LCA) (Lardon et al. 2009, Clarens et al 2010, Kumar et al. 2010, Cherubini 2010, Subhadra 2010, Pittman et al. 2011, Shirvani et al. 2011, Pfromm et al. 2011, Ferreira et al. 2012, Ortiz-Marquez et al. 2013). Such symbiotic immobilization approaches have already been undertaken. For example, by the immobilization of *Nostoc* sp., which increased H_2 production in a system integrated with biosorption of biological waste (Kaushik et al. 2011, Mona et al. 2013).

1.4.4 Screening for more efficient H_2 producing strains

The genetic pool of cyanobacteria is enormous, with a wide range of possibilities for biofuel production. However, only a few studies have attempted to find cyanobacteria with high H_2 production from natural environments or from culture collections (Lambert & Smith 1977, Howarth & Codd 1985, Kumar & Kumar 1992, Vyas & Kumar 1995, Masukawa et al. 2001, 2009, Yeager et al. 2011, Kothari et al. 2012, Bandyopadhyay et al. 2013). It is possible to discover strains that have naturally higher capacities for H_2 production than the model organisms generally studied in laboratories. H_2 production capacities of these naturally good H_2 producing strains could be further improved by genetic modifications of the strains or manipulation of culture conditions (Shah et al. 2001, Antal & Lindblad 2005, Ananyev et al. 2008, Berberoglu et al. 2008, Burrows et al. 2008, 2009, Carrieri et al. 2008, Chen et al. 2008, Marques et al. 2011). Screening is possible not only by searching for the organism, but also by screening the genes for H_2 production for example from global ocean sampling metagenomic databases (Venter et al. 2004, Rusch et al. 2007). Barz et al. (2010) have undertaken such an approach to demonstrate that hydrogenases are common in marine cyanobacteria.

Screening many cyanobacteria for H_2 reliably is very labor intensive. For the best possible outcome, cyanobacteria need to be tested under various environmental culture conditions (including pH, temperature, cell density, light intensity and quality). Screening under standard conditions might result in under- or overestimated production of the strain, leading to distorted results (Yeager et al. 2011). Concentrating screening efforts to certain environments might be beneficial. For example, it has been suggested that cyanobacteria originating from marine intertidal and freshwater are more suitable in producing H_2 than terrestrial cyanobacteria (Kothari et al. 2012).

The selection of cyanobacterial strains most suitable for biofuel production is determined by both the process itself, and the end product. Agricultural style, open production systems are exposed to the surrounding environment and cyanobacteria need to tolerate variations in temperature, light, salinity, and contamination. Ideally, strains that produce high yields in a range of climates are identified in a screening process and then modified for optimized production in various geographical areas in different seasons. In closed, industrial production systems, similarly to laboratory conditions, the cost of the system is the main problem and production must be very efficient. It is clear that no one strain will be likely to have all desired characteristics, therefore domestication and modifications must be made for commercially viable production. Research performed with model cyanobacteria does not often reflect the complexity of microbial communities in nature and in open production systems. Understanding and controlling the complex communities is the key for economically viable use of cyanobacteria.

The Nagoya Protocol is a supplementary agreement to the Convention on Biological Diversity (CBD 2011). The European Union and most of its member states, includ-

ing Finland, have signed and ratified the Nagoya Protocol. According to the Protocol, the country in which a certain microbe has been isolated has equal rights to determine the usage of the microbe's genetic resources. Thus, finding important and useful local microbes, for example in the field of biofuels, is of national importance.

2 AIMS OF THE STUDY

The aim of my thesis has been to discover promising H_2 producing cyanobacterial strains that grow naturally in Finnish lakes and the Baltic Sea. The second aim has been to improve the H_2 producing capacity of those newly identified cyanobacteria, which demonstrate distinct production of H_2. Towards these goals the following approaches were taken:

(i) Screening of the University of Helsinki Cyanobacteria Collection for capacity of H_2 production (Paper I).

(ii) Characterization of the best H_2 producing strains for the presence and activity of the enzymes involved in H_2 production (Paper II).

(iii) Extending the period of active H_2 production by engineering an immobilization technique for good H_2 producers (Paper III).

(iv) Optimizing the immobilized cyanobacterial cultures for high H_2 production (Paper IV).

3 METHODOLOGY

3.1 Growing cyanobacteria

The University of Helsinki Cyanobacteria Collection (UHCC) contains about 1000 planktonic and benthic strains isolated mostly from the Baltic Sea and Finnish lakes. The strains from the UHCC collection are maintained at the Division of Microbiology, Department of Applied Chemistry and Microbiology, University of Helsinki in agar plates and/or in liquid Z8 medium at pH 7.5 (Kotai 1972), at room temperature (22 °C), under approximately 7 µmol photons m^{-2} s^{-1} PAR. Depending on the strain, different Z8 media with slightly modified content were used for maintenance: Z8 (non-N_2-fixing species); Z8x (without combined nitrogen – N_2-fixing species); or Z8xS (saline media without combined nitrogen – some Baltic Sea species). About 200 strains were axenic cultures. The axenicity was confirmed by microscopy and plating on R2A and LB plates at UH.

The strains held at the University of Turku were maintained in liquid medium of Z8 or BG11, at room temperature (22 °C) and under approximately 20 µmol photons m^{-2} s^{-1} PAR. The axenic strains were stored in 5% DMSO at -70°C, from which fresh patches were thawed when necessary.

For experiments, cultures were cultivated in liquid Z8 media (UHCC strains) and continuously aerated under an illumination of 40 µmol photons m^{-2} s^{-1} PAR at 23ºC. WT and the Δ*hupL*, Δ*hupL*/Δ*hoxH* and Δ*hoxH* hydrogenase mutants of *Anabaena* 7120 were grown under an illumination of 40 µmol photons m^{-2} s^{-1} PAR in $BG11_0$ media at pH 8.2 (Paper I) or Z8x media at pH 7.5 (Papers II, III, IV). Growing conditions for other reference strains (*N. punctiforme* and its Δ*hupL* mutant NHM5, *A. variabilis* and *Synechocystis* PCC 6803) are described in Papers I and II.

The taxonomic characteristics of some of the H_2 producing strains were resolved by PCR amplification and sequencing of a part of the 16S rRNA gene. The taxonomic identities were evaluated by comparing obtained sequences to other cyanobacterial 16S rRNA genes from the NCBI database. The taxonomic identity of other strains was determined based only on morphology.

Cell fitness after immobilization was determined by diluting the cells to adjust OD_{750} to values of 0.25 and transferred to 24-well cell culture plates (2 ml well volume). The cells were grown under standard growth conditions at 50 µmol photons m^{-2} s^{-1} for 6 days and the change in optical density was measured.

Table 1. Cyanobacterial strains used in the study. In addition, in total of 400 strains were screened in Paper I.

Cyanobacterial strain	Origin	Mutated genes (antibiotics, vector)	Individual paper	References
Anabaena variabilis ATCC 29413	U.S. Department of Energy Joint Genome Institute		II	
Anabaena 7120			I, II, III, IV	
Anabaena 7120 Δ*hupL*		*hupL*::Sp^R (pRL277)	I, II, III, IV	Masukawa et al. 2002
Anabaena 7120 Δ*hoxH*		*hoxH*::Nm^R (pUC4K)	I	Masukawa et al. 2002
Anabaena 7120 Δ*hupL*/Δ*hoxH*		*hupL*::Sp^R (pRL277) *hoxH*::Nm^R (pUC4K)	II, IV	Masukawa et al. 2002
Nostoc punctiforme ATCC 29133	cycad *Macrozamia*		I	
Nostoc punctiforme ATCC 29133 NHM5	cycad *Macrozamia*	*hupL*::Nm^R (pRneoK)	I	Lindberg et al. 2002
Synechocystis PCC 6803	Californian freshwater lake (Stanier et al. 1971)		II	
Calothrix 336/3	Lake Enäjärvi, Laukilanlahti		I, II, III, IV	
Calothrix XPORK 5E	Porkkala cape, the Baltic Sea coast		I, II	
Nostoc Becid 19	Gulf of Finland, Vuosaari, the Baltic Sea coast		I, II	
Calothrix Becid 33	Gulf of Finland, the Baltic Sea coast		I, II	
Nostoc XHIID A6	Lake Hiidenvesi, Kirkkojärvi		I, II	
Nodularia AV33	Brackish water, the Baltic Sea		I, II	
Nodularia TRO31	Brackish water, the Baltic Sea		I, II	
Anabaena XSPORK 7B	Porkkala cape, the Baltic Sea coast		I, II	
Calothrix XSPORK 36C	Porkkala cape, the Baltic Sea coast		I, II	
Calothrix XSPORK 11A	Porkkala cape, the Baltic Sea coast		I, II	

3.2 Immobilization

The cells for immobilization in Ca^{2+}-alginate films were harvested by centrifugation and re-suspended in fresh Z8x medium with 4% alginate. A template consisting of a webbed plastic screen, placed over a Scotch-type tape, was built. The mixture was pipetted onto the template and levelled even with glass rod. The alginate films were polymerized with a mist of 50 mM calcium chloride solution. The templates with entrapped cells were then cut into 3 cm^2 pieces. The thickness of the film was about 0.2-0.5 mm. When it was necessary, the cells from alginate films were recovered by solubilizing films with 5 mM EDTA.

3.3 H_2 and enzyme assays

3.3.1 H_2 production

Cells from the growth media were harvested and re-suspended in 5 ml of fresh Z8x medium at a concentration of 0.1-40 µg Chl ml^{-1} (Paper I), 5 µg Chl ml^{-1} (Paper II) and transferred into 23 ml vials. The sealed vials including cells were sparged with Ar and placed in a cultivation chamber at 26-30 °C under continuous light (cool white fluorescent lamps, 130-150 µmol photons m^{-2} s^{-1} PAR) and with shaking at 80 rpm for 24 h. For *Nodularia* TRO31 and *Nodularia* AV33 strains, 50 µmol photons m^{-2} s^{-1} PAR was used for illumination, due to their light sensitivity.

Immobilized cells in the 3 cm^2 pieces in vials were flashed with Ar for 20 min, supplemented with different concentration of CO_2 and incubated in a growth chamber at 26^{o}C under continuous light from above (130-150 µmol photons m^{-2} s^{-1} PAR). The atmosphere was returned back to Ar + CO_2 after a 5-7 days (Paper III) or 4-5 days (Paper IV) (a cycle). Films containing cells were periodically treated with sterilized gases, Ar or N_2 containing CO_2, air or air containing 6% CO_2 (Paper IV). In the case of air treatments, the headspaces in the vials were returned back to Ar with 6% CO_2 after 16 – 20 h. H_2 and O_2 were quantified taking 150 µl samples from the vials with a gas-tight syringe (Hamilton Co.) and measured with a Gas-Chromatograph (GC, Perkin Elmer Clarus 500) equipped with a thermal conductivity detector (TCD) and a Molecular Sieve 5A column (60/80 mesh). Calibration was performed using 1.0% H_2 (AGA, Finland). Ar worked as a carrier gas. H_2 production rates were calculated on the basis of the cells' Chl content or on the area of the films.

3.3.2 Nitrogenase enzyme activity

The most widely used method for measurement of nitrogenase activity is the acetylene reduction assay (Dilworth 1966). This method relies on the capability of cyanobacteria to reduce compounds with a triple bond. Besides reducing atmospheric N_2, cyanobacteria can also reduce acetylene (C_2H_2) to ethylene (C_2H_4), which can be detected using gas chromatography.

Sample preparation was similar to that used in the H_2 production assay; with the exception that the gas phase contained 10% acetylene. To quantify ethylene, 10 µl samples from the vials were measured with GC (Perkin Elmer Autosystem) equipped with a flame ionization detector (FID) and a CP-CarboBOND column (Varian). Helium worked as a carrier gas and the calibration was performed using 1% ethylene (AGA, Finland). Enzymatic activity was calculated on the basis of the cells' Chl content or the area of the films.

3.3.3 Hydrogenase enzyme activity

A French press (Cell Disrupter, Constant Systems) (30 kPSI, two rounds) was used to harvest and homogenize cells for hydrogenase assays. To determine bidirectional hydrogenase activity, these cells were transferred to a vial and incubated with 5 mM methyl viologen and 20 mM sodium dithionate in a N_2 environment for 2h in light. H_2 was quantified with GC measurements.

H_2 uptake assay was performed using homogenized cells, placed in a cuvette (Hellma Analytics) with a septum, and were first flushed with N_2, followed by H_2 and finally incubated with 1 mM benzyl viologen. The reduction of benzyl viologen was monitored as 5 min intervals for 30 min using spectrophotometry at an absorbance of 555 nm. Activities were calculated based on a molar absorption coefficient for benzyl viologen ε_{555}=7550 M^{-1} cm^{-1}.

3.4 Chlorophyll and protein analysis

Chl contents were determined after solubilization of the alginate films, using a spectrophotometer at 665nm after extraction from cells using 90% methanol (Meeks & Castenholz 1971). Protein concentrations were determined from homogenized cells using the Bio-Rad Protein Assay based on Bradford protein assay (Bradford 1976).

3.5 DNA isolation and southern hybridization

Genomic DNA was extracted according to the method of Neilan et al. (1995). The quality of DNA was ensured by agarose gel electrophoresis (Sambrook et al. 1989). 1-2 µg of DNA was digested using the restriction enzymes *Hind*III and *Eco*RI. The obtained fragments were separated on an agarose gel, followed by subsequent stages of Southern hybridization as described in Paper III.

3.6 Sequences of *nif* and *hup* operons in *Calothrix* 336/3

The genome of *Calothrix* 336/3 was commercially sequenced using Illumina Hi-Seq 2000 in the Beijing Genome Institute.

The *hup* and *nif* operons were automatically annotated by the DOE-JGI Microbial Annotation Pipeline (DOE-JGI MAP), while the partially sequenced *Calothrix* 336/3 genome was processed via the Integrated Microbial Genome Expert Review (IMG-ER) system (Markowitz et al. 2009).

3.7 Microscopy imaging

Images were obtained at 40x magnification using a Zeiss Axioskop 2 Plus fluorescence microscope, equipped with a Zeiss Axiocam HRc digital camera.

3.8 HPLC analysis

The Chl *a* was separated from 100% methanol cell extracts by HPLC device equipped with a diode array detector and a reverse phase C18 endcapped column as described in Paper IV. HPLC was used when special accuracy was needed to measure Chl *a*.

3.9 Photochemical activity

The photochemical performance of cells in films was evaluated utililizing the Dual-PAM 100. The steady-state Chl *a* fluorescence level (*Ft*) and the utmost fluorescence level at light (*Fm*') was determined to calculate the PSII yield Y(II) (*Fm*'-F_t/*Fm*') and described in Paper IV.

4 RESULTS AND DISCUSSION

4.1 Screening of the UHCC for naturally good H_2 producers

The fact that cyanobacteria strains from the Baltic Sea are genetically very diverse (Sihvonen et al. 2007, Halinen et al. 2008), prompted an idea to screen the University of Helsinki Cyanobacteria Collection for efficient H_2 photoproducers. About 400 cyanobacterial strains from the UHCC were subject to preliminary screening. Preliminary screening was undertaken on cells grown under 7 µmol photons m^{-2} s^{-1} PAR of light at the University of Helsinki. These were concentrated to a volume of 50 ml and transferred to the Turku laboratory for the H_2 assay. During pre-screening, the strains were subjected to four different conditions: microoxic and aerobic dark, aerobic and microoxic light. Half of the strains subjected to the screening process, mostly filamentous cyanobacteria, produced H_2, generally in very small amounts in light, microoxic conditions.

Approximately 25 promising strains were selected for a second stage screening process. During this process, cells were grown in the Turku laboratory at 40 µmol photons m^{-2} s^{-1} PAR with constant aeration, and H_2 assays were accordingly performed. The top ten strains were chosen for further investigation in a third stage where characterization of the strains and optimization of the H_2 production process was undertaken. In order to evaluate the H_2 production capacities of the studied strains, the wild-type (WT) control strain and Δ*hupL* mutant of *N. punctiforme,* and the wild-type (WT) control, Δ*hupL*, Δ*hoxH* and Δ*hupL/*Δ*hoxH* mutants of *Anabaena* 7120 were employed (Paper I).

Later studies demonstrated that the growth and selected H_2 production conditions strongly affected the results of the screening. Most likely, strains that grew well in pH 7.5 were represented better in the selection to be screened. Also, the growth medium for screening, Z8, has a specific composition that is preferred by some cyanobacteria. It is important to note that the screening of the UHCC was completed under specific conditions, in which the H_2 production rates were significantly lower due to the transportation of cells and the high cell density. Still, even with robust pre-screening conditions, UHCC strains with high H_2 production potential were identified (Paper I).

4.2 H_2 production and nitrogenase activities of the UHCC strains

Optimization of H_2 production process for each strain (optimization parameters are dicusssed below) in the third stage resulted in increased H_2 production rates of all control strains and only a few of the selected UHCC strains. The Δ*hupL* and Δ*hupL*/Δ*hoxH* deletion mutants of *Anabaena* 7120 showed a 4-7 times higher H_2 photoproduction rate compared to the WT, whereas the Δ*hox*H mutant of *Anabaena* 7120 demonstrated an even lower amount of H_2 production compared to the WT strain (**Figure 4**). Among the top ten UHCC strains, it was possible to optimize the H_2 production yields of *Calothrix* 336/3, *Nostoc* XHIID A6 and the *Calothrix* XPORK 5E strains. As a result, these strains produced H_2 at approximately the same level as Δ*hupL* and Δ*hupL*/Δ*hoxH* mutants and at a rate which was about 3-4 times higher than that of *Anabaena* 7120. The optimization process was not very successful for the remaining UHCC strains which were chosen for characterization, with H_2 production rates of these strains remaining at a level which was only comparable with *Anabaena* 7120 (Paper II).

H_2 production of all selected UHCC strains was based on nitrogenase activity. It is known that nitrogenase activity is affected by O_2 concentration and, to a lesser extent, temperature (Compaore & Stal 2010). The measured nitrogenase activity, expressed as the ethylene production rate, was similar in *Anabaena* 7120 and its Δ*hupL* mutant. *Calothrix* Becid 33, *Nostoc* Becid 19, and *Nodularia* AV33 showed higher ethylene production activity as compared to their H_2 production. This resembles the behavior of *Anabaena* 7120 and is likely related to the presence of active uptake hydrogenase (**Figure 4**). Indeed, Southern hybridization and enzyme activity confirmed the presence of uptake hydrogenase in these strains (see below). Contrasting this, the seven other UHCC strains, *Calothrix* 336/3, XPORK 5E, XSPORK 11A XSPORK 36C, *Anabaena* XSPORK 7B, *Nostoc* XHIID A6, and *Nodularia* TRO31, showed similar levels of ethylene and H_2 production rates, as was the case with the Δ*hupL* mutant (Paper II).

These ethylene production results might indicate that N_2 fixation and H_2-evolution are not strictly coupled in all cyanobacterial strains. In line with this, modification within the α-subunit of the vegetative cell-located Mo-nitrogenase of *A. variabilis* has previously been demonstrated to result in decreased activities of both N_2 fixation and ethylene-reduction, whilst having no impact on the H_2-formation of this nitrogenase (Weyman et al. 2010). Nitrogenase activity of the marine cyanobacterium *Trichodesmium erythraeum,* was saturated at a certain level of light intensity, but H_2-production and thus the H_2/ethylene ratio gradually increased with either increases in light intensity above this threshold value, or by alterations in the light quality (Wilson et al. 2012). It is therefore likely, that the measured nitrogenase activity of the UHCC strains does not describe the whole H_2 capacity of the nitrogenases.

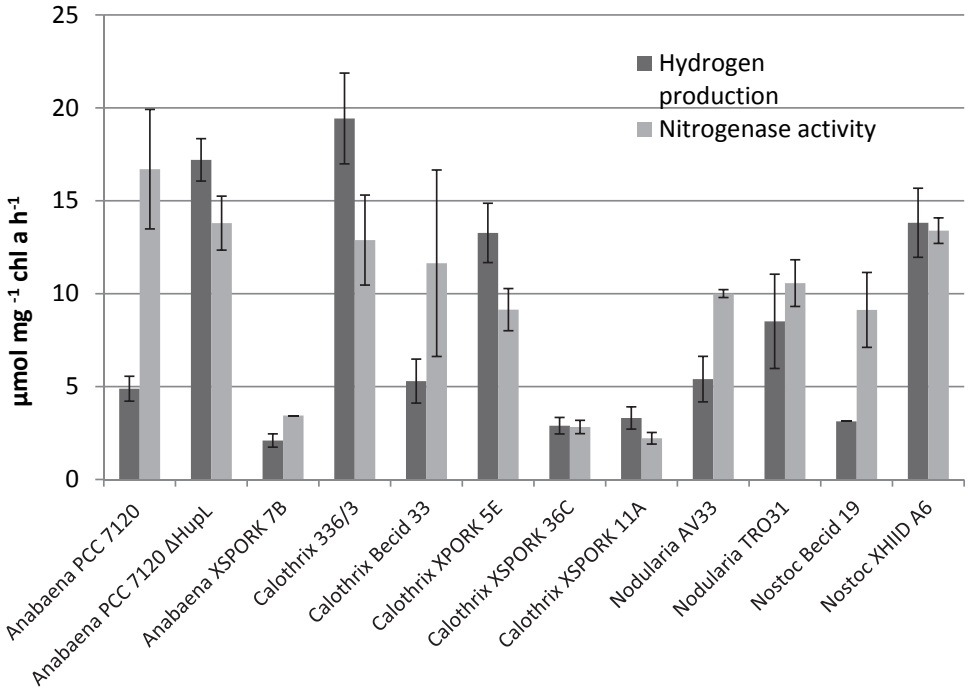

Figure 4. H_2 production and nitrogenase activity of reference strains and the top ten H_2 producers from the UHCC.

4.2.1 The effect of different environmental parameters on H_2 production

H_2 production of the studied cyanobacterial strains was found to be dependent on light. Light distribution to planktonic cells is balanced between light intensity, mixing and cell concentration. The capacity of cyanobacteria to efficiently utilization light is highly varied. In short-term H_2 photoproduction experiments, *Nodularia* AV33 and *Nodularia* TRO31 tolerated only 50 µmol photons $m^{-2}s^{-1}$ at a maximum, whereas *Calothrix* XPORK 5E could bear 250 and *Calothrix* 336/3 up to 500 µmol photons $m^{-2}s^{-1}$ before a decrease was observed in H_2 production. The optimal light intensity was assessed considering the compromise between viability and H_2 production and was species specific, ranging from 50 to 150 µmol photons $m^{-2}s^{-1}$ in UHCC strains. A light intensity of ~150 µmol photons $m^{-2}s^{-1}$ was found to be optimal overall UHCC strains (except *Nodularia*) and was thus used in protocols for H_2 production experiments.

Modulation of cell density (Chl concentration) similarly affected H_2 production rates of the cells. In fact, the H_2 production rate of the cells gradually increased with decreasing cell density (Chl concentration), with *Nodularia* strains the only exception.

Notably, the planktonic strains originating from the Baltic Sea demonstrated a higher sensitivity to light than those from Finnish lakes.

The growth and H_2 production rates of cyanobacterial strains studied were affected by pH in a strain specific manner. For example, *Anabaena* 7120 showed a significantly higher H_2 production rate at pH 7.5 than at pH 8.2, whilst *Calothrix* 336/3 was grown at pH 7.5 but was not able to grow in a medium of pH 6.8 or pH 8.2 (Paper I).

It is obvious that not only pH but also changes in the composition of the growth medium and/or H_2 production assay medium differently affects the rate of H_2 production depending on the strain. The *Calothrix* XPORK 5E strain demonstrated similar growth and H_2 production rates in both the $BG11_0$ and Z8x media, whereas *Calothrix* 336/3 strain demonstrated similar growth but lower H_2 production rates in $BG11_0$ when compared to the Z8x medium. *Nodularia* AV33, which usually grows in Z8xS medium at pH 7.5, could not survive in $BG11_0$ medium at pH 7.5, with salt supplement. (Paper I).

Ni^{2+} has been shown to enhance the activity of the bidirectional hydrogenase (Vignais et al. 2001) and to stimulate growth, heterocyst frequency, CO_2 fixation and nitrogenase activity of some cyanobacteria (Rai & Raizada 1986). In contrast to these results, addition of 1 μM Ni^{2+} to the growth and H_2 assay medium of the *ΔhupL* mutant of *Anabaena* 7120 resulted in only a slight increase in H_2 production rate. This is in line with previous results showing that Ni^{2+} supplementation does not improve the H_2 production of *Anabaena* 7120 (Marques et al. 2011). The nitrogenase activity of *Calothrix* sp. has been proposed to be maximal when the phosphorus (P) content of the medium is high (Livingstone and Whitton 1983). However, the addition of P to Z8X medium did not increase H_2 production rates in *Calothrix* 336/3 (data not shown). (Paper I).

The optimum temperature for H_2 production varies in different cyanobacterial species (Dutta et al. 2005). The effect of temperature on H_2 production was studied with *Calothrix* 336/3. An increase in the temperature of the H_2 assay from 23°C to 30°C stimulated the H_2 production rate of this strain about two times, with a temperature-dependent increase in H_2 production noted for most UHCC, and all reference strains (unpublished data). These results agree with those obtained in different laboratories. For example, maximal H_2 production of *Anabaena variabilis* SPU 003 was achieved at 30°C (Shah et al. 2001), whereas *Nostoc muscorum* SPU 004 demonstrated maximum H_2 production rate at 40°C (Shah et al. 2003). Decreasing temperatures below 15°C correspondingly resulted in severe reduction of the H_2 production of all strains (unpublished data). This is interesting, particularly given that the UHCC strains are isolated from habitats of cold climate and can be cultivated about at 4-12 °C. However, many cyanobacterial species isolated from colder habitats have been reported to have considerably higher optimal temperature for photosynthesis and growth than in their native cold water habitats (Nadeau & Castenholz 2000). In short term experiments the H_2 and nitrogenase assays were performed mostly at 30° C, since this temperature was

optimal compromise between the H_2 production and the cell viability. In long term experiments, in order to increase viability of cells, the treatments were performed at 26° C (Paper I).

The cyanobacteria selected from the UHCC are heterocystous, filamentous, N_2-fixing cyanobacteria. Heterocysts are special microoxic compartments where N_2 fixation takes place. The microoxygenic environment inside of heterocysts is created by: (i) the absence of active PSII; (ii) a thick cell envelope consisting of an inner laminated layer composed of densely packed glycolipids and an outer layer composed of specific polysaccharides protecting the cell from oxygen diffusion; and (iii) the high respiratory activity consuming any penetrated oxygen (Meeks et al. 2002, Meeks & Elhai 2002, Tamagnini et al. 2007, Bothe et al. 2010).

Taking into account these arguments, O_2 environment around the cells should not strongly affect H_2 production rates. To assess the impact of O_2 on the production of H_2 by the *Calothrix* 336/3 cells, H_2 production assays in both the presence and absence of O_2 were performed. In the presence of Ar + 21% O_2 the H_2 production rate of *Calothrix* 336/3 cells decreased by about 24% in comparison to Ar treated cells. It is possible that the decrease in H_2 production rate which was observed in the presence of O_2 occured due to the leakage of the heterocyst envelope, or due to the leakage of O_2 into heterocysts from neghbouring vegetative cells. Although an increase in the O_2 levels in the headspace of assay vials only inhibited nitrogenase to a small degree, the total H_2 production rate decreased substantially (93 %) in the presence of air, demonstrating a higher degree of inhibition of H_2 production by the presence of N_2 rather than O_2. Indeed, in the presence of N_2 most of the electrons are allocated to NH_3 production, where one molecule of H_2 is produced at the expense of 8 electrons. Importantly, in the absence of atmospheric N_2, nitrogenase allocates all electrons to H_2 (Masukawa et al. 2010) (Paper III).

It has always been problematic to find a uniform and appropriate strategy for the normalization and comparison of H_2 production data. Calculations of most of the obtained data were performed on the basis of cellular Chl content (Paper I, II, III) and on the area (m^2) of films (Paper III, IV). To analyse the reliability of the data obtained on Chl basis, a comparison of the H_2 production rate of *Calothrix* XPORK 5E and *Calothrix* 336/3 strains based on dry biomass was done. On the basis of Chl, the H_2 production rate of *Calothrix* XPORK 5E was only 70% of that of *Calothrix* 336/3, whereas on the basis of dry weight the H_2 production rate was only ~50%. Therefore, a detailed analyze of the properties of specific strains are required (Paper I).

4.3 Hydrogenases of the UHCC strains

Cyanobacteria possess two types of hydrogenases, uptake and bidirectional hydrogenases (Tamagnini et al. 2007). Among the selected UHCC strains only *Calothrix* Becid

33 demonstrated relatively high bidirectional hydrogenase activity when compared to the control *Anabaena* 7120 strain. Four of the other strains, *Calothrix* XSPORK 5E, *Nodularia* AV33, TRO31 and *Nostoc* XHIID A6, showed much lower enzyme activity than the control, whilst the activity of the bidirectional enzyme in all remaining UHCC strains was below the lower limit of detection. (Paper II).

Uptake hydrogenase has been found in almost all of the N_2-fixing cyanobacteria examined so far, with the only exception found in unicellular N_2-fixing strains (Steunou et al. 2008). The activities of the uptake hydrogenases of selected UHCC strains varied considerably. Since (i) non of the ten good H_2 producers selected from UHCC demonstrated better nitrogenase activity compared to the control *Anabaena* 7120, and (ii) net H_2 production yield is a combination of H_2 production by nitrogenase and H_2 consumption by uptake hydrogenase it was reasonable to expect that selected UHCC strains were either naturally missing, or possessing a very low uptake hydrogenase activity. In contrast to these expectations, nine of the selected ten UHCC strains demonstrated similar or higher levels of uptake hydrogenase activity than the control strain *Anabaena* 7120. Two UHCC strains, *Calothrix* Becid 33 and *Calothrix* 336/3 demonstrated twice as high uptake hydrogenase activity in comparison to the *Anabaena* 7120 strain. Importantly, *Calothrix* XSPORK 36C did not show any uptake hydrogenase activity, similar to the Δ*hupL*/Δ*hoxH* mutant of *Anabaena* 7120 used as a negative control. (Paper II).

Thus, the conclusion was made that the maximum capacity of nitrogenase or uptake hydrogenase enzymes cannot be taken as a main reason for the increased yield of H_2 photoproduction in the selected UHCC strains. However, it is important to consider that, for certain cyanobacterial strains, the activity of uptake hydrogenase can be influenced by different external factors such as nickel, H_2, carbon and nitrogen concentrations in the surrounding medium. An increase in H_2 uptake activity has been demonstrated when a fraction of headspace air was replaced with H_2 in *Anabaena* 7120 (Houchins & Burris 1981a) and *N. punctiforme* (Oxelfelt et al. 1995) cultures, or when cells were supplemented with Ni concentrations below 10 μM (Oxelfelt et al. 1995). However, addition of ammonium was shown to decrease the activity of both uptake hydrogenase and the nitrogenase in *N. punctiforme* (Oxelfelt et al. 1995). It is highly possible that *in vivo* conditions, the enzymes studied function with a different rate depending on the intracellular redox status of the cells.

4.4 Gene analysis

4.4.1 Southern hybridization of genes involved in H_2 production of UHCC strains

The genes involved in the H_2 production of UHCC strains were identified by Southern hybridization using the PCR products for the *nif1*, *nif2*, *vnf*, *hup* and *hox* genes, which were obtained from the genomic DNA of *A. variabilis* and *Calothrix* XPORK 5E. Positive hybridization signals from the *nifH1*, *nifD1* and *nifK1* probes indicated the presence of the conventional Mo-nitrogenase in all selected UHCC strains. Additionally, all of the selected UHCC strains lacked alternative nitrogenases, with no hybridization signals detected using *nifD2, nifH2, nifK2* and *vnfDG*, *vnfK* probes (Paper II).

Southern hybridization with *hupL*-, *hupS*- and *hoxY* specific probes resulted in positive signals from eight UHCC strains, whereas two strains, *Calothrix* XSPORK 36C and *Calothrix* 11A, lacked signals. The combined absence of signal from Southern hybridization of the *hupSL* and *hoxY* genes, and the lack of observable hydrogenase activity suggest that the *Calothrix* XSPORK 36C strain lacks both hydrogenase enzymes. The negative Southern hybridization result for the *hoxY* gene also suggests the absence of bidirectional hydrogenase in *Calothrix* XSPORK 11A. However, this cannot be certain due to the demonstration of low enzyme activity, slightly above the lower limit of detection of the assay (Paper II).

Interestingly, even though bidirectional hydrogenase activity was not observed in *Calothrix* 336/3, *Calothrix* XSPORK 36C, *Anabaena* XSPORK 7B, and *Nostoc* Becid 19, these strains demonstrated, a positive hybridization signal with the *hoxY* probe, which could indicate a possible presence of the bidirectional enzyme in these strains. Despite this, the analysis of the sequenced genome of *Calothrix* 336/3 did not indicate probable *hox* elements. However, it is still possible that these strains possess the bidirectional hydrogenase (except *Calothrix* 336/3), but it is not expressed and maturated under the tested conditions (Paper II).

4.4.2 Structures of the *hup* and *nif* operons in *Calothrix* 336/3

The most efficient H_2 producer among the screened UHCC strains *Calothrix* 336/3 was subjected to *de novo* genome sequencing (unpublished data). The structure of the *hup* operon in *Calothrix* 336/3 was compared with the corresponding regions in *Calothrix* PCC 6303, *Calothrix* PCC 7507, and *Anabaena* 7120 from the NCBI database. Typically, the *hupS* gene coding for the small subunit of the uptake hydrogenase is followed by *hupL* encoding the large subunit. The structure of the *hup* operon in *Calothrix* 336/3 is similar to that in *Calothrix* PCC 6303 and *Anabaena* 7120. In all these

strains, the *hupL* gene is interrupted by the *hupL* element, which is excised during heterocyst maturation by the internally encoded XisC recombinase. Interestingly, and in difference to *Anabaena* 7120, the *hupL* elements of both *Calothrix* 336/3 and *Calothrix* PCC 6303 contain a gene encoding the orange carotenoid protein (OCP). OCP in cyanobacteria acts as a non-photochemical quencher of energy and a photosensor for triggering a photoprotective mechanism through increasing thermal dissipation (Jallet et al. 2014). Gene clusters coding for HypA-HypF maturation factors responsible for the proper folding and activity of hydrogenases, were alike in all four strains. The distance between *hup* and *hyp* operons remains unclear for *Calothrix* 336/3 (Paper II).

The structural genes of nitrogenase, *nifD*, *nifH* and *nifK*, are conserved among cyanobacteria in the *nif* operon. In *Calothrix* 336/3 they were identified in a large gene cluster also encoding several accessory elements of nitrogenase. This cluster is moderately compact compared to the *nif* operons in the other two *Calothrix* strains, which are interrupted by long DNA segments. The excision element (14 kb) and genes for *xisA* recombinase were found in *Calothrix* PCC 6303. No known excision element was found in the *Calothrix* 336/3 *nif* operon (Paper II).

The presence of excision elements and genes for *xisF* and *xisA* recombinases specific for the *nif* operon varies among cyanobacteria. Within heterocystous cyanobacteria, three excision elements ranging from 11 to 55 kb have been found in *nifD, fdxN* and *hupL* genes (Henson et al. 2011). Recently, the presence of two excision elements (80 and 5.9 kb) in the *nifH* gene of *Anabaena* sp. 90 was demonstrated (Wang et al. 2012). Excision elements and genes for *xisF* and *xisA* recombinases are present in *Anabaena* 7120 (Paper II).

A compact *nif* operon without excision elements might be related to symbiotic relationships, like in '*Nostoc azollae'* 0708 (Ran et al. 2010, Wang et al. 2012). In many aspects, symbiotic cyanobacteria behave ideally for H_2 production performed in photobioreactors. Their growth rate is very low. Still, metabolic activities and nitrogenase activity of these cells are increased, as is the heterocyst frequency. *Calothrix* species are known as cyanobionts of lichens, liverworts and hornworts, cycads, green algae and diatoms (Rosenberg & Paerl 1981, Rasmussen & Nilsson 2002, Foster et al. 2011). Perhaps the right signals will yield cyanobacteria in an advantageous, artificial symbiotic state. For example, *Calothrix* symbionts with diatoms have yielded 171-420 times higher N_2 fixation rates than free living cells (Foster et al. 2011). Symbiotic cyanobacteria can also have heterocyst frequencies up to 60-80% (Franche et al. 2009). However, there are no known symbiotic relations with *Calothrix* 336/3 (Paper II).

The *fdxN* gene, within the *nif* gene cluster in *Calothrix* 336/3, encodes a "bacterial-type" ferredoxin (Mulligan & Haselkorn 1989). Although its role remains unclear, the disruption of the *fdxN* gene did not affect the diazotrophic growth of *Anabaena variabilis* (Masepohl et al. 1997).

4.5 Morphology of the selected H_2 producers of UHCC

All H_2 producers selected from the pre-screening of UHCC were N_2-fixing, filamentous cyanobacteria with the ability to form heterocysts. The taxonomic identities of the strains were based on the 16S rRNA gene sequence data and morphology of the strains. Eight of the ten best H_2 producers were found to be benthic cyanobacteria, with *Calothrix* 336/3 being periphytic. The remaining two strains are planktonic *Nodularia* strains. Morphologically, *Nodularia* strains have barrel shaped cells with intercalary heterocysts. *Calothrix* strains have terminal ellipsoidal heterocysts at basal end of filaments. *Nostoc* and *Anabaena* strains have terminal and intercalary heterocysts, with a beaded trichome appearance. The microscopic image of 6 week old, immobilized cells showed that heterocysts in the entrapped cultures were difficult to distinguish from vegetative cells (Paper I II, III).

Calothrix 336/3 was observed to have a ratio of about 5 % heterocysts to vegetative cells under normal growth conditions (unpublished data). Increasing the heterocyst frequency to approximately 20-25 % is known to increase nitrogenase activity and thus H_2 production (Meeks & Elhai 2002) and this is one of the biotechnological strategies towards further increases in H_2 production of the *Calothrix* 336/3 strain. One possible approach to increase heterocyst frequency might be to limit Mo, Ca and Fe nutrients, since defiencies of these nutrients has led to increases in heterocyst frequencies in other *Calothrix* species (Sinclair & Whitton 1977).

4.6 H_2 production from the immobilized cells

Like many other prokaryotes, cyanobacteria secrete small signal molecules into the environment that induce self and neighbouring cell gene expression, known as quorum sensing. Cyanobacterial growth density and particularly N_2 fixation activity of *Anabaena* 7120 are known to be dependent on quorum sensing (Romero et al. 2011). This molecular signaling prohibits high cell densities and H_2 production. In order to produce high amounts of H_2 in limited space, immobilization of cyanobacteria was applied.

4.6.1 Comparison of the H_2 production and growth by suspension and immobilized cells

Long-term experiments involving several H_2 production cycles (5-7 days each cycle) were performed to compare the H_2 photoproduction capacities of *Anabaena* 7120, the Δ*hupL* mutant of *Anabaena* 7120, and *Calothrix* 336/3. These were undertaken for both immobilized and suspension cell cultures. In order to release excess pressure and

to lessen the possible negative effect of accumulated O_2 and H_2, the culture vials were opened and the gas atmosphere of the headspace was refreshed at the end of each H_2 production cycle (Paper III).

The maximum specific rate of H_2 production was consist between the alginate-entrapped cells and the suspension cultures of the *Calothrix* 336/3 (25-35 μmol H_2 mg $Chl^{-1}h^{-1}$), *Anabaena* 7120 (9-13 μmol H_2 mg $Chl^{-1}h^{-1}$) and the Δ*hupL* mutant (about 30 μmol H_2 mg Chl^{-1} h^{-1}). The *Anabaena* 7120 produced significantly less H_2 than the Δ*hupL* mutant or *Calothrix* 336/3 (Paper III).

In suspension cultures, H_2 production reduced significantly after the first cycle: *Anabaena* 7120 did not produce any H_2 on the second cycle; Δ*hupL* decreased H_2 production dramatically, and did not produce H_2 after second cycle; and *Calothrix* 336/3 demonstrated a more gradual decrease in H_2 production yield. Differently to suspension cultures, all immobilized cells demonstrated a slower, more gradual loss of H_2 production activity over time; with production diminishing only after 4 to 6 cycles. Immobilized cyanobacterial cells not only produced more H_2 over a much longer time course, they also demonstrated high viability. After 84 days of entrapment, the cells were still able to produce similar amounts of H_2 compared to freshly immobilized cells. The cells were viable even after 6 months after the initial immobilization, without demonstrating outgrowth from the films. Entrapped cells could also be revived after several months of total dehydration of the films. Such long-term viability of entrapped cells could have a significant biotechnological value. (Paper III, IV).

Whilst cells can remain viable on recovery from the alginate films, one of the main advantages of immobilization is that cells that remain entrapped are strongly (under microoxic conditions) or partially (under oxic conditions) inhibited in cell growth (Paper III). This presents the opportunity to control cell growth during the H_2 production phase. Since the energy flow to biomass is proportional to the growth rate (Dickson et al. 2009), it is important to to keep cultures at their lowest growth rate to redirect most of the cell's energy to H_2 production, instead of biomass accumulation. The reduced growth observed after immobilization might result from the inability of filaments to reproduce through hormogonia distribution. It is possible that, due to the lack of space, signals transmitted by dying cells could cause death in living, neighbouring cells (Rooke et al. 2010). Whilst this was not evident for *Calothrix* 336/3, since cells stayed viable after 6 months of entrapment. It is also possible that growth limitations caused by encapsulation might lead to a distorted cell metabolism due to decreased activity, eventually leading to cell death in the longer term (Dickson et al. 2013). Constant exposure to light can result in photoinhibition and photodamage, to which cells must respond in order to live. Thus, the delicate balance between light utilization efficiency and photoinhibition of entrapped cells is important for future optimization of H_2 production by immobilized cyanobacterial cells (Paper III, IV).

Entrapment of cells in polymeric films causes significant changes in gene expression profiles, as recently described for *Synechocystis* PCC 6803 encapsulated in rigid,

aqueous silica gels (Dickson et al. 2012). Microarray experiments demonstrated down-regulation of photosynthetic genes responsible for linear electron flow from PSII to carbon fixation, upregulation of the genes involved in cyclic electron flow around PSI, several nitrite/nitrate transporters, and genes related to iron stress, particularly *isiA* and *isiB* (Dickson et al. 2012). Entrapment of cells in alginate films is also advantageous in reducing the possibility of culture contamination. This is important for long term H_2 production, when it is difficult to maintain axenic cell cultures. Also, storing the cells in harsh conditions is useful for the economical operation of the production system (Paper III, IV).

4.6.2 The effect of CO_2 additions on H_2 photoproduction

Cyanobacterial cells maintained under conditions without CO_2 additions stopped H_2 production after only the first cycle (5-7 days). This could be due to the rapid utilization of CO_2 during photosynthesis, resulting in a deficency of CO_2 available to the cells, thus inhibiting H_2 production. To avoid this limitation, CO_2 was regularly added to the headspace of Ar-purged vials. Supplementation of CO_2 increased the H_2 photoproduction yield in both suspension and immobilized cultures of the cyanobacterial strains studied. Different levels of CO_2 (2%, 6%, 10%) were applied to *Anabaena* 7120, its Δ*hupL* mutant and to *Calothrix* 336/3, although only *Calothrix* 336/3 was followed over several cycles in all CO_2 levels. The level of 6% CO_2 was found to be suitable for sustaining the H_2 production of all three strains. However, even with CO_2 supplementations, the H_2 production process could only be sustained for 25-38 days, depending on the utilized strain. After 38 days of immobilization, the entrapped *Calothrix* 336/3 cells supplemented with 2 % and 6 % CO_2 were still able to produce H_2 and O_2, indicating the viability of this strain, although variation between experiments was noted (Paper III, IV).

β-cyanobacteria, including filamentous N_2-fixing strains, have the capacity to express CCMs that allow photosynthesis and growth to be saturated by CO_2 at well below normal temperature-dependent air-equilibrium concentrations. This does not prevent increased CO_2 from increasing the photosynthetic rate and amount of carbon per cell, and may also increase the excretion of dissolved organic carbon (Price et al. 2008, Fu et al. 2007). Possibly, *Anabaena* 7120 and *Calothrix* 336/3 have different properties in their CCM –system, what can be seen from the prolonged H_2 and O_2 productions of the *Calothrix* 336/3 strain. There was, however, an upper threshold level for improvement of H_2 production by CO_2. The entrapped *Calothrix* 336/3 cells supplemented with 10% CO_2 already demonstrated a decrease in H_2 production after the second cycle. In this case, the decrease in H_2 production might also be due to a drop in pH, since the Z8-media is not buffered adequately to prevent this occuring. It is also

known that the extended incubation of cells with CO_2 increases their ratio of heterocysts compared to vegetative cells (Marques et al. 2011) (Paper III).

H_2 production activity was more prominent in the Δ*hupL* mutant due to the lack of uptake hydrogenase in this strain. In the best case, the Δ*hupL* cells in films supplemented with Ar + 6% CO_2 produced over 0.9 mol m^{-2} of H_2 gas for 33 days, while the films with *Anabaena* 7120 and *Calothrix* 336/3 cells produced only about 0.1 and 0.42 mol m^{-2} for 25 and 38 days. The film made with *Calothrix* 336/3 was thicker than the other films (~0.5-1 mm), which might result in a slow diffusion of H_2 from the film, making it easier for uptake hydrogenase to recapture H_2. It is also highly possible that, due to thickness of the film, the H_2 production capacity of the *Calohrix* 336/3 cells observed in alginate films during continuous culturing is underestimated (Paper IV).

One of the important findings in this thesis work is that immobilized cells which have significantly reduced growth are able to utilize levels of up to 10% CO_2. These conditions might be favorable for the production of different valuable carbon compounds. The intracellular glycogen level of cyanobacteria is on average 1-5% w/w dry weight and varies mainly as a function of light and macronutrient availability (Wilson et al. 2010). It has been demonstrated, that high light stress (De Philippis et al. 1992) and growing the cells in high CO_2 concentrations (Eisenhut et al. 2007) lead to an increase of the glycogen pool. In general, elevated temperature and higher than atmospheric CO_2 concentrations increase photosynthetic growth of cyanobacteria, also leading to higher C:P and N:P ratios (Fu et al. 2007), although there are considerable differences between species. The decreased levels of gas exchange observed at the end of long term experiments indicate down-regulation of photosynthesis and carbon assimilation activities in response to nitrogen starvation. When excess carbon is combined with nitrogen depletion, a rapid glycogen accumulation of up to 70% w/w dry weight is observed in cyanobacteria (Yoo et al. 2007). At the gene expression level, nitrogen deficiency results in a lack of transcripts from phycobilisome encoding genes and photosynthesis-related genes and reduced amounts of proteins involved in Chl synthesis, the Calvin-Benson-Bassham cycle, and sugar anabolism (Krasikov et al. 2012, Ehira & Ohmori 2014) (Paper III, IV).

The lack of ammonia in the GS-GOGAT cycle leads to an increase in the 2-OG level, which activates a signaling cascade resulting in the degradation of phycobilisomes. When nitrogen is available again, the phycobilisomes are resynthesized and glycogen is degraded (Karradt et al. 2008). The presence of chemically and osmotically inert stores of intracellular glycogen enables cyanobacteria to survive macronutrient-starvation conditions and to recover rapidly (Gründel et al. 2012). Microorganisms capable of closely adjusting the rate of glycogen degradation to their energy maintenance requirements survive for the longest periods (Dawes 1992). *Calothrix* 336/3 is able to survive long periods without added nutrients. Even after 25 days incubation in conditions of only Ar + CO_2, it's Chl *a* level was increased in contrast to *Anabaena*

7120. This might indicate that *Calothrix* 336/3 is uniquely adapted to variations in C/N levels, and is able to balance amounts of glycogen and phycobilisomes (Paper III, IV).

It is known that some cyanobacteria can also synthesize another carbon reserve, polyhydroxybutyrate (PHB) (Stal 1992). It is assumed, that PHB acts mainly as a regulator of the intracellular reduction charge (De Philippis et al. 1992). It is highly possible that UHCC strains also exhibit significant amounts of PHB and extracellular polymeric substances (EPS). EPS amounts increase when C/N metabolism is unbalanced in favor of fixed carbon (Otero & Vincenzini 2004).

4.6.3 N recovery during long-term H_2 photoproduction

Although all three studied strains were supplemented with CO_2, a clear decline of photosynthetic capacity and H_2 production capacity was still observed during the long-term H_2 production process. This decline was most likely due to a lack of N-sources. Measurements of photochemical activity from the surface of the films using the Dual-PAM 100 fluorometer at the beginning of, and after 19 days of H_2 production, demonstrated that photosynthetic activity significantly declined in all strains under long-term N-starvation (Ar +6% CO_2) conditions. In these films, the effective PSII yield, Y(II), dropped from 0.33 to 0.16 in *Anabaena* 7120, from 0.29 to 0.18 in *Calothrix* 336/3, and from 0.34 to 0.06 in Δ*hupL* after 19 days of the experiment. Photosynthetic performance of the cells was also evaluated by measuring the amount of O_2 in the headspace of the vials, and demonstrated a similar decrease (Paper IV).

To further stabilize H_2 photoproduction and to support the viability of the entrapped cyanobacterial strains, a recovery phase was introduced to the long-term experiments. During the recovery phase, the cells were periodically exposed to air + 6% CO_2, or air with atmospheric levels of CO_2 for about 16 - 20 h every fourth day. The air treatments, by supplying cells with N_2, allowed nitrogen-deficient cells to briefly fix N_2 to recover photosynthetic apparatus and other impaired metabolic activities. The regular treatment of entrapped *Anabaena* 7120 and *Calothrix* 336/3 strains with air and air + 6% CO_2 completely recovered photochemical activity after 19 days of the long-term H_2 production process. However, the Δ*hupL* mutant behaved differently, demonstrating recovery of PSII yield only during treatment with air, and not during treatment with air +CO_2 (Paper IV).

Under optimal conditions, the films with entrapped Δ*hupL* cells produced over 1.1 mol H_2 m^{-2} for 32 days when treated periodically with air, while the *Calothrix* 336/3 produced over 0.5 mol H_2 m^{-2} for the period of 27 days when treated periodically with air + 6% CO_2. The films with entrapped *Anabaena* 7120 cells did not produce more than 0.1 mol m^{-2} under any conditions tested. Thus, the recovery phase increased the H_2 production of Δ*hupL* mutant and *Calothrix* 336/3 strains, while having negligable impact on *Anabaena* 7120 (Paper IV).

Even the periodic treatments of the immobilized cultures with air or air + 6% CO_2, allowed the cells (except Δ*hupL* with air + CO_2 treatment) to recover photosynthetic apparatus and made O_2 production more stable. Still, all three strains stopped producing H_2, albeit after extended periods of time. Moreover the wild-type cells always produced less H_2 and usually for shorter periods of time. The decrease in H_2 yield could not be linked to the H_2 uptake process exclusively, since the Δ*hupL* mutant strain was also affected. Furthermore, the Δ*hupL*/Δ*hoxH* mutant deficient in both hydrogenases, showed almost the same kinetics of H_2 production as the Δ*hupL* strain (unpublished data), indicating that bidirectional hydrogenase was not involved in H_2 uptake. Despite this, the increase of H_2 uptake capacity over the time of the experiment could not be entirely ruled out as a factor in H_2 production for both wild-type strains. It is known that alginate matrix significantly restricts the diffusion of gases to and from the entrapped cells (Kosourov & Seibert 2009). Thus, increasing levels of H_2 and O_2 inside of the films may favor the oxyhydrogen reaction (Rao & Hall 1996, Tamagnini et al. 2007). The comparison of net H_2 and O_2 yields in the examined strains indicates that a direct connection between H_2 uptake and O_2 respiration may exist in long-term H_2 production process for these strains (Paper IV).

4.7 The role of uptake hydrogenase

Prolonged incubation of alginate films in the absence of nitrogen caused a significant loss of photochemical activity and the degradation of Chl *a* in the entrapped cells. Wild type strains recovered photosynthetic apparatus due to periodic treatments with air, independent of the presence or absence of CO_2 in air. In contrast, the Δ*hupL* mutant, treated periodically under air + 6% showed significant bleaching, decreased Chl *a*, and a decrease in PSII yield. The photosynthetic performance as measured by O_2 production confirmed this reduced photosynthetic capacity. Also, immobilized Δ*hupL* cultures showed reduced cell fitness, determined by suspension culture regrowth for 6 days following recovery from the alginate matrix (Paper IV).

These results indicate that uptake hydrogenase may protect, not only the nitrogenase system from O_2 inactivation in heterocysts, but also vegetative cells from photoinhibition by removing excess O_2 that may be accumulated inside the films. This condition can also accelerate the consumption of H_2 in the wild-type strains. Despite accelerating the H_2 production rate, the elimination of uptake hydrogenase in cells may thus reduce the length of the process.

When uptake hydrogenase is inactivated, the heterocysts compensate by increasing respiratory activity, modifying the heterocyst cell envelope and the upregulation of stress response proteins. There is a reduction in the amount of ATP and NADPH available to drive N_2-fixation, which leads to an increased C:N ratio. In order for heterocysts to maintain a high N_2-fixation rate they compensate by generating more re-

ducing power (Ekman et al. 2011). Alternative mechanisms, such as O_2 photoreduction pathway triggered by a heterocysts specific Flv3B flavodiiron protein (Ermakova et al. 2014), might be strongly upregulated to keep the O_2 concentrations low in heterocysts (Ekman et al. 2011).

CONCLUSION AND FUTURE PERSPECTIVES

Important parameters for photoautotrophic cyanobacterial H_2 production are efficiency, robustness, stability, and the cost efficiency of the production system. These are the parameters which have guided the work for this thesis. However, the task of fulfilling these parameters is not easy.

When characterizing the diverse H_2 producers in the UHCC, it was found that a high H_2 production rate is not due to the presence of highly active nitrogenase or bidirectional hydrogenase enzymes. It is noteworthy that the best H_2 producers in the UHCC possess a highly active uptake hydrogenase enzyme as compared to the reference strain, *Anabaena* 7120. Although, it should be kept in mind that regardless of the high activity determined *in vitro*, the uptake hydrogenase might not function *in vivo*. In order to enhance H_2 production, the the cell metabolism and intracellular redox status of the UHCC strains should be further studied.

In all experiments, the *Calothrix* 336/3 strain outperformed the wild-type of *Anabaena* 7120, both in H_2 photoproduction yields and in the duration of the process. Since both wild-type strains possess uptake hydrogenase, elimination of uptake hydrogenase in *Calothrix* 336/3 might result in higher H_2 photoproduction yields than the Δ*hupL* mutant of *Anabaena* 7120.

Factors including culture density, light intensity, pH, temperature, medium composition and the presence of O_2 affected the H_2 production of the studied strains. Accordingly, the productivity and economics of all photosynthesis-related processes is influenced by the light utilization efficiency. Factors that may impact the efficiency of H_2 production include light saturation and distribution, self-shading, and an inability of strains to utilize all wavelengths of light. Comparison of autotrophic systems to heterologous production systems is difficult in terms of economical viability.

Further usage of the UHCC strains for H_2 production might be possible with the best producing strains, *Calothrix* 336/3, *Nostoc* XHIID A6 and *Calothrix* XPORK 5E. For the rest of the UHCC strains, utilization appears implausible. Filamentous cyanobacteria could be utilized as host organisms to various hydrogenase enzymes to increase the nitrogenase based H_2 production.

Continuous H_2 production from immobilized cells is a more promising H_2 production system in larger scale than production from suspension cultures. Combining immobilization with recovery treatments demonstrated the great potential for gains to be made that could finally yield a completely continuous cyanobacterial H_2 production system.

The function of uptake hydrogenase was clarified in a metabolic context, with the role of this enzyme in protecting the photosynthetic apparatus of vegetative cells from photoinhibition being revealed. This information could be useful in future strain engineering approaches when introducing foreign hydrogenases into cyanobacteria or when attempting to enhance H_2 production rates in cyanobacteria using endogenous H_2 producing enzymes.

It has been claimed that H_2 production by the cyanobacterial nitrogenase systems is not efficient enough to be of practical use. Nitrogenase must be produced in large amounts, requiring a number of other proteins for assembly and the maintainance of a suitable environment. The reaction itself consumes much ATP, thus being energetically expensive for the cell. Whilst this work has demonstrated improvements towards the feasibility of cyanobacterial H_2 production, further optimizations of the nitrogenase process, including the continuing identification of optimal conditions and further genetic engineering of promising strains will certainly improve the situation.

The main goal in the future studies is to engineer sustainable integrated cyanobacteria based production system. Integration of efficient waste-water purification, and production of H_2, other biofuels, and valuable biocompounds, could be applied in order to make the system economically viable. The key scientific methods to develop such a system would be application of systems biology approach, high throughput RNA-sequencing, computational biomodels, proteomics, metabolic engineering, and genome sequencing of good producers.

ACKNOWLEDGEMENTS

This study was carried out in the Laboratory of Molecular Plant Biology at the University of Turku. Financial support from the Academy of Finland, Kone Foundation, the Finnish Cultural Foundation and the Fortum Foundation is gratefully acknowledged.

I wish to thank Prof. Eevi Rintamäki for guidance throughout my thesis. My supervisors Prof. Eva-Mari Aro and Dr. Yagut Allahverdiyeva are thanked for giving me the opportunity to exist in the life of science, and for providing scientific advice. I am also deeply grateful to Dr. Sergey Kosourov for all the help during my thesis. Without your never-ending positiveness, this thesis would have been much worse.

I am grateful to Prof. Matti Karp and Dr. Kari Skjånes for reviewing this thesis and providing constructive comments. Dr. Fiona Lynch is warmly thanked for revising the language of the thesis and giving excellent suggestions for editing.

All my co-authors are thanked for collaborating with me. I know, that it has not always been so easy.

I thank Kurt and Mika for solving all the endless technical issues. Ulla-Maija, Eve, Anniina and Mirja are thanked for keeping things in their right places in the lab, both mentally and physically.

Many thanks to all my workmates, present and past. You are too numerous to mention, but I will remember you all. Emeritus Prof. Marjatta Rauduskoski is specially acknowledged for inspiring the whole generation of not so hopeful new scientists.

Thanks for all my friends for the support; you maintain my sanity in doubtful times.

I am fortunate to have a large and happy family. Marja, and my daughter Enja, you are my life.

Turku, December 2014

Hannu

REFERENCES

Abed, R.M.M., Dobretsov, S. & Sudesh, K. 2009, "Applications of cyanobacteria in biotechnology", *Journal of Applied Microbiology,* vol. 106, no. 1, pp. 1-12.

Ananyev, G., Carrieri, D. & Dismukes, G.C. 2008, "Optimization of metabolic capacity and flux through environmental cues to maximize hydrogen production by the cyanobacterium "Arthrospira (Spirulina) maxima"", *Applied and Environmental Microbiology,* vol. 74, no. 19, pp. 6102-6113.

Angermayr, S.A., Hellingwerf, K.J., Lindblad, P. & de Mattos, M.J.T. 2009, "Energy biotechnology with cyanobacteria", *Current Opinion in Biotechnology,* vol. 20, no. 3, pp. 257-263.

Angermayr, S.A., Paszota, M. & Hellingwerf, K.J. 2012, "Engineering a Cyanobacterial Cell Factory for Production of Lactic Acid", *Applied and Environmental Microbiology,* vol. 78, no. 19, pp. 7098-7106.

Antal, T., Harju, E., Pihlgren, L., Lastusaari, M., Tyystjarvi, T., Holsa, J. & Tyystjarvi, E. 2012, "Use of near-infrared radiation for oxygenic photosynthesis via photon up-conversion", *International Journal of Hydrogen Energy,* vol. 37, no. 10, pp. 8859-8863.

Antal, T. & Lindblad, P. 2005, "Production of H-2 by sulphur-deprived cells of the unicellular cyanobacteria Gloeocapsa alpicola and Synechocystis sp PCC 6803 during dark incubation with methane or at various extracellular pH", *Journal of Applied Microbiology,* vol. 98, no. 1, pp. 114-120.

Appel, J., Phunpruch, S., Steinmuller, K. & Schulz, R. 2000, "The bidirectional hydrogenase of Synechocystis sp PCC 6803 works as an electron valve during photosynthesis", *Archives of Microbiology,* vol. 173, no. 5-6, pp. 333-338.

Appel, J. & Schulz, R. 1998, "Hydrogen metabolism in organisms with oxygenic photosynthesis: hydrogenases as important regulatory devices for a proper redox poising?", *Journal of Photochemistry and Photobiology B: Biology,* vol. 47, no. 1, pp. 1-11.

Atsumi, S., Higashide, W. & Liao, J.C. 2009, "Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde", *Nature Biotechnology,* vol. 27, no. 12, pp. 1177-U142.

Aubert-Jousset, E., Cano, M., Guedeney, G., Richaud, P. & Cournac, L. 2011, "Role of HoxE subunit in Synechocystis PCC6803 hydrogenase", *Febs Journal,* vol. 278, no. 21, pp. 4035-4043.

Axelsson, R. & Lindblad, P. 2002, "Transcriptional regulation of Nostoc hydrogenases: Effects of oxygen, hydrogen, and nickel", *Applied and Environmental Microbiology,* vol. 68, no. 1, pp. 444-447.

Azbar, N. & Kapdan, I. 2012, *Use of immobilized cell systems in biohydrogen production, State of the Art and Progress in Production of Biohydrogen.* Bentham Science Publishers, *pp. 228-250*.

Azwar, M.Y., Hussain, M.A. & Abdul-Wahab, A.K. 2014, "Development of biohydrogen production by photobiological, fermentation and electrochemical processes: A review", *Renewable & Sustainable Energy Reviews,* vol. 31, pp. 158-173.

Baebprasert, W., Jantaro, S., Khetkorn, W., Lindblad, P. & Incharoensakdi, A. 2011, "Increased H-2 production in the cyanobacterium Synechocystis sp strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway", *Metabolic Engineering,* vol. 13, no. 5, pp. 610-616.

Bagai, R. & Madamwar, D. 1998, "Prolonged evolution of photohydrogen by intermittent supply of nitrogen using a combined system of Phormidium valderianum, Halobacterium halobium, and Escherichia coli", *International Journal of Hydrogen Energy,* vol. 23, no. 7, pp. 545-550.

Bandyopadhyay, A., Elvitigala, T., Liberton, M. & Pakrasi, H.B. 2013, "Variations in the Rhythms of Respiration and Nitrogen Fixation in Members of the Unicellular Diazotrophic Cyanobacterial Genus Cyanothece", *Plant Physiology,* vol. 161, no. 3, pp. 1334-1346.

Bandyopadhyay, A., Elvitigala, T., Welsh, E., Stoeckel, J., Liberton, M., Min, H., Sherman, L.A. & Pakrasi, H.B. 2011, "Novel Metabolic Attributes of the Genus Cyanothece, Comprising a Group of Unicellular Nitrogen-Fixing Cyanobacteria", *Mbio,* vol. 2, no. 5, pp. e00214.

Bandyopadhyay, A., Stoeckel, J., Min, H., Sherman, L.A. & Pakrasi, H.B. 2010, "High rates of photobiological H-2 production by a cyanobacterium under aerobic conditions", *Nature Communications,* vol. 1, pp. 139.

Barz, M., Beimgraben, C., Staller, T., Germer, F., Opitz, F., Marquardt, C., Schwarz, C., Gutekunst, K., Vanselow, K.H., Schmitz, R., LaRoche, J., Schulz, R. & Appel, J. 2010, "Distribution Analysis of Hydrogenases in Surface Waters of Marine and Freshwater Environments", *Plos One,* vol. 5, no. 11, pp. e13846.

Battchikova, N., Eisenhut, M. & Aro, E. 2011, "Cyanobacterial NDH-1 complexes: Novel insights and remaining puzzles", *Biochimica Et Biophysica Acta-Bioenergetics,* vol. 1807, no. 8, pp. 935-944.

Beckmann, J., Lehr, F., Finazzi, G., Hankamer, B., Posten, C., Wobbe, L. & Kruse, O. 2009, "Improvement of light to biomass conversion by de-regulation of light-harvesting protein translation in Chlamydomonas reinhardtii", *Journal of Biotechnology,* vol. 142, no. 1, pp. 70-77.

Benemann, J. & Weare, N. 1974, "Hydrogen Evolution by Nitrogen-Fixing Anabaena Cylindrical Cultures", *Science,* vol. 184, no. 4133, pp. 174-175.

Bentley, F.K., Garcia-Cerdan, J.G., Chen, H. & Melis, A. 2013, "Paradigm of Monoterpene (beta-phellandrene) Hydrocarbons Production via Photosynthesis in Cyanobacteria", *Bioenergy Research,* vol. 6, no. 3, pp. 917-929.

Bentley, F.K. & Melis, A. 2012, "Diffusion-based process for carbon dioxide uptake and isoprene emission in gaseous/aqueous two-phase photobioreactors by photosynthetic microorganisms", *Biotechnology and Bioengineering,* vol. 109, no. 1, pp. 100-109.

Berberoglu, H., Jay, J. & Pilon, L. 2008, "Effect of nutrient media on photobiological hydrogen production by Anabaena variabilis ATCC 29413", *International Journal of Hydrogen Energy,* vol. 33, no. 4, pp. 1172-1184.

Bernat, G., Waschewski, N. & Roegner, M. 2009, "Towards efficient hydrogen production: the impact of antenna size and external factors on electron transport dynamics in Synechocystis PCC 6803", *Photosynthesis Research,* vol. 99, no. 3, pp. 205-216.

Berto, P., D'Adamo, S., Bergantino, E., Vallese, F., Giacometti, G.M. & Costantini, P. 2011, "The cyanobacterium Synechocystis sp PCC 6803 is able to express an active [FeFe]-hydrogenase without additional maturation proteins", *Biochemical and Biophysical Research Communications,* vol. 405, no. 4, pp. 678-683.

Bjorn, L.O., Papageorgiou, G.C., Blankenship, R.E. & Govindjee 2009, "A viewpoint: Why chlorophyll a?", *Photosynthesis Research,* vol. 99, no. 2, pp. 85-98.

Boeck, A., King, P.W., Blokesch, M. & Posewitz, M.C. 2006, "Maturation of hydrogenases", *Advances in Microbial Physiology, Vol 51,* vol. 51, pp. 1-71.

Bothe, H. 1970, "Photosynthetic Nitrogen Fixation with Cell-Free Extract from Blue Algae Anabaena-Cylindrica", *Berichte Der Deutschen Botanischen Gesellschaft,* vol. 83, no. 9-10, pp. 421-&.

Bothe, H. & Eisbrenner, G. 1977, "Effect of 7-Azatryptophan on Nitrogen-Fixation and Heterocyst Formation in Blue-Green-Alga Anabaena-Cylindrica", *Biochemie und Physiologie der Pflanzen,* vol. 171, no. 4, pp. 323-332.

Bothe, H., Schmitz, O., Yates, M.G. & Newton, W.E. 2010, "Nitrogen Fixation and Hydrogen Metabolism in Cyanobacteria", *Microbiology and Molecular Biology Reviews,* vol. 74, no. 4, pp. 529-551.

Boyle, P.M. & Silver, P.A. 2009, "Harnessing nature's toolbox: regulatory elements for synthetic biology", *Journal of the Royal Society Interface,* vol. 6.

Bradford, M. 1976, "Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding", *Analytical Biochemistry,* vol. 72, no. 1-2, pp. 248-254.

Brouers, M. & Hall, D. 1986, "Ammonia and Hydrogen-Production by Immobilized Cyanobacteria", *Journal of Biotechnology,* vol. 3, no. 5-6, pp. 307-321.

Brown, K.A., Wilker, M.B., Boehm, M., Dukovic, G. & King, P.W. 2012, "Characterization of Photochemical Processes for H-2 Production by CdS Nanorod-[FeFe] Hydrogenase Complexes", *Journal of the American Chemical Society,* vol. 134, no. 12, pp. 5627-5636.

Buhrke, T., Lenz, O., Krauss, N. & Friedrich, B. 2005, "Oxygen tolerance of the H-2-sensing [NiFe] hydrogenase from Ralstonia eutropha H16 is based on limited access of oxygen to the active site", *Journal of Biological Chemistry,* vol. 280, no. 25, pp. 23791-23796.

Buikema, W. & Haselkorn, R. 2001, "Expression of the Anabaena hetR gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 98, no. 5, pp. 2729-2734.

Burgess, B. & Lowe, D. 1996, "Mechanism of molybdenum nitrogenase", *Chemical Reviews,* vol. 96, no. 7, pp. 2983-3011.

Burnat, M., Herrero, A. & Flores, E. 2014, "Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocystforming cyanobacterium", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 111, no. 10, pp. 3823-3828.

Burrows, E.H., Chaplen, F.W.R. & Ely, R.L. 2008, "Optimization of media nutrient composition for increased photofermentative hydrogen production by Synechocystis sp PCC 6803", *International Journal of Hydrogen Energy,* vol. 33, no. 21, pp. 6092-6099.

Burrows, E.H., Wong, W., Fern, X., Chaplen, F.W.R. & Ely, R.L. 2009, "Optimization of pH and Nitrogen for Enhanced Hydrogen Production by Synechocystis sp PCC 6803 via Statistical and Machine Learning Methods", *Biotechnology Progress,* vol. 25, no. 4, pp. 1009-1017.

Cai, Y. & Wolk, C. 1997, "Nitrogen deprivation of Anabaena sp strain PCC 7120 elicits rapid activation of a gene cluster that is essential for uptake and utilization of nitrate", *Journal of Bacteriology,* vol. 179, no. 1, pp. 258-266.

Calkins, J.O., Umasankar, Y., O'Neill, H. & Ramasamy, R.P. 2013, "High photo-electrochemical activity of thylakoid-carbon nanotube composites for photosynthetic energy conversion", *Energy & Environmental Science,* vol. 6, no. 6, pp. 1891-1900.

Callahan, S. & Buikema, W. 2001, "The role of HetN in maintenance of the heterocyst pattern in Anabaena sp PCC 7120", *Molecular Microbiology,* vol. 40, no. 4, pp. 941-950.

Camsund, D., Devine, E., Holmqvist, M., Yohanoun, P., Lindblad, P. & Stensjo, K. 2011, "A HupS-GFP fusion protein demonstrates a heterocyst-specific localization of the uptake hydrogenase in Nostoc punctiforme", *FEMS Microbiology Letters,* vol. 316, no. 2, pp. 152-159.

Carrasco, C., Holliday, S., Hansel, A., Lindblad, P. & Golden, J. 2005, "Heterocyst-specific excision of the Anabaena sp strain PCC 7120 hupL element requires xisC", *Journal of Bacteriology,* vol. 187, no. 17, pp. 6031-6038.

Carrieri, D., Ananyev, G., Costas, A.M.G., Bryant, D.A. & Dismukes, G.C. 2008, "Renewable hydrogen production by cyanobacteria: Nickel requirements for optimal hydrogenase activity", *International Journal of Hydrogen Energy,* vol. 33, no. 8, pp. 2014-2022.

Carrieri, D., Wawrousek, K., Eckert, C., Yu, J. & Maness, P. 2011, "The role of the bidirectional hydrogenase in cyanobacteria", *Bioresource Technology,* vol. 102, no. 18, pp. 8368-8377.

Carvalho, M.F., Jorge, R.F., Pacheco, C.C., De Marco, P., Henriques, I.S., Correia, A. & Castro, P.M.L. 2006, "Long-term performance and microbial dynamics of an up-flow fixed bed reactor established for the biodegradation of fluorobenzene", *Applied Microbiology and Biotechnology,* vol. 71, no. 4, pp. 555-562.

Chen, P., Fan, S., Chiang, C. & Lee, C. 2008, "Effect of growth conditions on the hydrogen production with cyanobacterium Anabaena sp strain CH3", *International Journal of Hydrogen Energy,* vol. 33, no. 5, pp. 1460-1464.

Cherubini, F. 2010, "The biorefinery concept: Using biomass instead of oil for producing energy and chemicals", *Energy Conversion and Management,* vol. 51, no. 7, pp. 1412-1421.

Clarens, A.F., Resurreccion, E.P., White, M.A. & Colosi, L.M. 2010, "Environmental Life Cycle Comparison of Algae to Other Bioenergy Feedstocks", *Environmental science & technology,* vol. 44, no. 5, pp. 1813-1819.

Compaore, J. & Stal, L.J. 2010, "Effect of Temperature on the Sensitivity of Nitrogenase to Oxygen in Two Heterocystous Cyanobacteria", *Journal of Phycology,* vol. 46, no. 6, pp. 1172-1179.

Convention on Biological Diversity , *The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (2011)*. Available: http://www.cbd.int/countries/?country=fi [2014, 8/10].

Converti, A., Oliveira, R.P.S., Torres, B.R., Lodi, A. & Zilli, M. 2009, "Biogas production and valorization by means of a two-step biological process", *Bioresource Technology,* vol. 100, no. 23, pp. 5771-5776.

Cournac, L., Guedeney, G., Peltier, G. & Vignais, P. 2004, "Sustained photoevolution of molecular hydrogen in a mutant of Synechocystis sp strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex", *Journal of Bacteriology,* vol. 186, no. 6, pp. 1737-1746.

Cournac, L., Mus, F., Bernard, L., Guedeney, G., Vignais, P. & Peltier, G. 2002, "Limiting steps of hydrogen production in Chlamydomonas reinhardtii and Synechocystis PCC 6803 as analysed by light-induced gas exchange transients", *International Journal of Hydrogen Energy,* vol. 27, no. 11-12, pp. 1229-1237.

Dagan, T., Roettger, M., Stucken, K., Landan, G., Koch, R., Major, P., Gould, S.B., Goremykin, V.V., Rippka, R., de Marsac, N.T., Gugger, M., Lockhart, P.J., Allen, J.F., Brune, I., Maus, I., Puehler, A. & Martin, W.F. 2013, "Genomes of Stigonematalean Cyanobacteria (Subsection V) and the Evolution of Oxygenic Photosynthesis from Prokaryotes to Plastids", *Genome Biology and Evolution,* vol. 5, no. 1, pp. 31-44.

Das, D. & Veziroglu, T. 2001, "Hydrogen production by biological processes: a survey of literature", *International Journal of Hydrogen Energy,* vol. 26, no. 1, pp. 13-28.

Das, D. & Veziroglu, T.N. 2008, "Advances in biological hydrogen production processes", *International Journal of Hydrogen Energy,* vol. 33, no. 21, pp. 6046-6057.

Dawes, E.A. 1992, "Storage polymers in prokaryotes", *Symposia-Society for General Microbiology, Prokaryotic Structure and Function: A New Perspective.* Cambridge University Press, pp. 81.

De Philippis, R., Sili, C. & Vincenzini, M. 1992, "Glycogen and poly-β-hydroxybutyrate synthesis in Spirulina maxima", *Journal of General Microbiology,* vol. 138, no. 8, pp. 1623-1628.

de-Bashan, L.E. & Bashan, Y. 2010, "Immobilized microalgae for removing pollutants: Review of practical aspects", *Bioresource Technology,* vol. 101, no. 6, pp. 1611-1627.

DeRuyter, Y.S. & Fromme, P. 2008, "Molecular structure of the photosynthetic apparatus", *The Cyanobacteria: Molecular Biology, Genomics and Evolution,* , pp. 217-269.

Dexter, J. & Fu, P. 2009, "Metabolic engineering of cyanobacteria for ethanol production", *Energy & Environmental Science,* vol. 2, no. 8, pp. 857-864.

Dickson, D.J. & Ely, R.L. 2013, "Silica sol-gel encapsulation of cyanobacteria: lessons for academic and applied research", *Applied Microbiology and Biotechnology,* vol. 97, no. 5, pp. 1809-1819.

Dickson, D.J., Luterra, M.D. & Ely, R.L. 2012, "Transcriptomic responses of Synechocystis sp PCC 6803 encapsulated in silica gel", *Applied Microbiology and Biotechnology,* vol. 96, no. 1, pp. 183-196.

Dickson, D.J., Page, C.J. & Ely, R.L. 2009, "Photobiological hydrogen production from Synechocystis sp PCC 6803 encapsulated in silica sol-gel", *International Journal of Hydrogen Energy,* vol. 34, no. 1, pp. 204-215.

Dilworth, M. 1966, "Acetylene Reduction by Nitrogen-Fixing Preparations from Clostridium Pasteurianum", *Biochimica et Biophysica Acta,* vol. 127, no. 2, pp. 285-&.

Ducat, D.C., Sachdeva, G. & Silver, P.A. 2011, "Rewiring hydrogenase-dependent redox circuits in cyanobacteria", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 108, no. 10, pp. 3941-3946.

Dutheil, J., Saenkham, P., Sakr, S., Leplat, C., Ortega-Ramos, M., Bottin, H., Cournac, L., Cassier-Chauvat, C. & Chauvat, F. 2012, "The AbrB2 Autorepressor, Expressed from an Atypical Promoter, Represses the Hydrogenase Operon To Regulate Hydrogen Production in Synechocystis Strain PCC6803", *Journal of Bacteriology,* vol. 194, no. 19, pp. 5423-5433.

Dutta, D., De, D., Chaudhuri, S. & Bhattacharya, S. 2005, "Hydrogen production by Cyanobacteria", *Microbial Cell Factories,* vol. 4, pp. 36.

Eckert, C., Boehm, M., Carrieri, D., Yu, J., Dubini, A., Nixon, P.J. & Maness, P. 2012, "Genetic Analysis of the Hox Hydrogenase in the Cyanobacterium Synechocystis sp PCC 6803 Reveals Subunit Roles in Association, Assembly, Maturation, and Function", *Journal of Biological Chemistry,* vol. 287, no. 52, pp. 43502-43515.

Ehira, S. & Ohmori, M. 2006, "NrrA, a nitrogen-responsive response regulator facilitates heterocyst development in the cyanobacterium Anabaena sp

strain PCC 7120", *Molecular Microbiology,* vol. 59, no. 6, pp. 1692-1703.

Ehira, S., Ohmori, M. & Sato, N. 2003, "Genome-wide expression analysis of the responses to nitrogen deprivation in the heterocyst-forming cyanobacterium Anabacna sp strain PCC 7120", *Dna Research,* vol. 10, no. 3, pp. 97-113.

Ehira, S. & Ohmori, M. 2014, "NrrA directly regulates expression of the fraF gene and antisense RNAs for fraE in the heterocyst-forming cyanobacterium Anabaena sp strain PCC 7120", *Microbiology,* vol. 160, pp. 844-850.

Eisenhut, M., von Wobeser, E.A., Jonas, L., Schubert, H., Ibelings, B.W., Bauwe, H., Matthijs, H.C.P. & Hagemann, M. 2007, "Long-term response toward inorganic carbon limitation in wild type and glycolate turnover mutants of the cyanobacterium Synechocystis sp strain PCC 6803(1[W])", *Plant Physiology,* vol. 144, no. 4, pp. 1946-1959.

Ekman, M., Ow, S.Y., Holmqvist, M., Zhang, X., van Wagenen, J., Wright, P.C. & Stensjo, K. 2011, "Metabolic Adaptations in a H-2 Producing Heterocyst-Forming Cyanobacterium: Potentials and Implications for Biological Engineering", *Journal of Proteome Research,* vol. 10, no. 4, pp. 1772-1784.

Ermakova, M., Battchikova, N., Richaud, P., Leino, H., Kosourov, S., Isojarvi, J., Peltier, G., Flores, E., Cournac, L., Allahverdiyeva, Y. & Aro, E. 2014, "Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic growth of the filamentous cyanobacterium Anabaena sp. PCC 7120", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 111, no. 30, pp. 11205-11210.

Ernst, A., Reich, S. & Boger, P. 1990, "Modification of Dinitrogenase Reductase in the Cyanobacterium Anabaena-Variabilis due to C-Starvation and Ammonia", *Journal of Bacteriology,* vol. 172, no. 2, pp. 748-755.

Eser, B.E., Das, D., Han, J., Jones, P.R. & Marsh, E.N.G. 2011, "Oxygen-Independent Alkane Formation by Non-Heme Iron-Dependent Cyanobacterial Aldehyde Decarbonylase: Investigation of Kinetics and Requirement for an External Electron Donor", *Biochemistry,* vol. 50, no. 49, pp. 10743-10750.

European Renewable Energy Council , *EU Roadmap - Mapping Renewable Energy Pathways towards 2020 (2010)*. Available: http://www.erec.org/media/publications/eu-roadmap.html [2014, 8/10].

European Renewable Energy Council , *Future Energy Policy 2050 (2009)*. Available: http://www.erec.org/policy/eu-policies/future-energy-policy-2050.html [2014, 8/10].

Fay, P. 1992, "Oxygen Relations of Nitrogen-Fixation in Cyanobacteria", *Microbiological Reviews,* vol. 56, no. 2, pp. 340-373.

Federico Ortiz-Marquez, J.C., Do Nascimento, M., Zehr, J.P. & Curatti, L. 2013, "Genetic engineering of multispecies microbial cell factories as an alternative for bioenergy production", *Trends in Biotechnology,* vol. 31, no. 9, pp. 521-529.

Ferreira, A.F., Marques, A.C., Batista, A.P., Marques, P.A.S.S., Gouveia, L. & Silva, C.M. 2012, "Biological hydrogen production by Anabaena sp - Yield, energy and CO2 analysis including fermentative biomass recovery", *International Journal of Hydrogen Energy,* vol. 37, no. 1, pp. 179-190.

Flickinger, M.C., Schottel, J.L., Bond, D.R., Aksan, A. & Scriven, L.E. 2007, "Painting and printing living bacteria: Engineering nanoporous biocatalytic coatings to preserve microbial viability and intensify reactivity", *Biotechnology Progress,* vol. 23, no. 1, pp. 2-17.

Flores, E. & Herrero, A. 2010, "Compartmentalized function through cell differentiation in filamentous cyanobacteria", *Nature Reviews Microbiology,* vol. 8, no. 1, pp. 39-50.

Floudas, C.A., Elia, J.A. & Baliban, R.C. 2012, "Hybrid and single feedstock energy processes for liquid transportation fuels: A critical review", *Computers & Chemical Engineering,* vol. 41, pp. 24-51.

Forzi, L. & Sawers, R.G. 2007, "Maturation of [NiFe]-hydrogenases in Escherichia coli", *Biometals,* vol. 20, no. 3-4, pp. 565-578.

Foster, R.A., Kuypers, M.M.M., Vagner, T., Paerl, R.W., Musat, N. & Zehr, J.P. 2011, "Nitrogen fixation and transfer in open ocean diatom-cyanobacterial symbioses", *Isme Journal,* vol. 5, no. 9, pp. 1484-1493.

Franche, C., Lindstrom, K. & Elmerich, C. 2009, "Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants", *Plant and Soil,* vol. 321, no. 1-2, pp. 35-59.

Fredriksson, C. & Bergman, B. 1997, "Ultrastructural characterisation of cells specialised for nitrogen fixation in a non-heterocystous cyanobacterium,

Trichodesmium spp.", *Protoplasma,* vol. 197, no. 1-2, pp. 76-85.

Fu, F., Warner, M.E., Zhang, Y., Feng, Y. & Hutchins, D.A. 2007, "Effects of increased temperature and CO2 on photosynthesis, growth, and elemental ratios in marine Synechococcus and Prochlorococcus (Cyanobacteria)", *Journal of Phycology,* vol. 43, no. 3, pp. 485-496.

Fu, P. 2009, "Genome-scale modeling of Synechocystis sp PCC 6803 and prediction of pathway insertion", *Journal of Chemical Technology and Biotechnology,* vol. 84, no. 4, pp. 473-483.

Gaertner, K., Lechno-Yossef, S., Cornish, A.J., Wolk, C.P. & Hegg, E.L. 2012, "Expression of Shewanella oneidensis MR-1 [FeFe]-Hydrogenase Genes in Anabaena sp Strain PCC 7120", *Applied and Environmental Microbiology,* vol. 78, no. 24, pp. 8579-8586.

Gaffron, H. & Rubin, J. 1942, "Fermentative and photochemical production of hydrogen in algae", *The Journal of General Physiology,* vol. 26, no. (2), pp. 219-240.

Galdzicki, M., Rodriguez, C., Chandran, D., Sauro, H.M. & Gennari, J.H. 2011, "Standard Biological Parts Knowledgebase", *Plos One,* vol. 6, no. 2, pp. e17005.

Gao, Z., Zhao, H., Li, Z., Tan, X. & Lu, X. 2012, "Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria", *Energy & Environmental Science,* vol. 5, no. 12, pp. 9857-9865.

Germer, F., Zebger, I., Saggu, M., Lendzian, F., Schulz, R. & Appel, J. 2009, "Overexpression, Isolation, and Spectroscopic Characterization of the Bidirectional [NiFe] Hydrogenase from Synechocystis sp PCC 6803", *Journal of Biological Chemistry,* vol. 284, no. 52, pp. 36462-36472.

Ghirardi, M.L., Dubini, A., Yu, J. & Maness, P. 2009, "Photobiological hydrogen-producing systems", *Chemical Society Reviews,* vol. 38, no. 1, pp. 52-61.

Giordano, M., Beardall, J. & Raven, J. 2005, "CO2 concentrating mechanisms in algae: Mechanisms, environmental modulation, and evolution", *Annual Review of Plant Biology,* vol. 56, pp. 99-131.

Giraldo, J.P., Landry, M.P., Faltermeier, S.M., McNicholas, T.P., Iverson, N.M., Boghossian, A.A., Reuel, N.F., Hilmer, A.J., Sen, F., Brew, J.A. & Strano, M.S. 2014, "Plant nanobionics approach to augment photosynthesis and biochemical sensing", *Nature Materials,* vol. 13, no. 4, pp. 400-408.

Golden, J., Robinson, S. & Haselkorn, R. 1985, "Rearrangement of Nitrogen-Fixation Genes during Heterocyst Differentiation in the Cyanobacterium Anabaena", *Nature,* vol. 314, no. 6010, pp. 419-423.

Gosse, J.L., Chinn, M.S., Grunden, A.M., Bernal, O.I., Jenkins, J.S., Yeager, C., Kosourov, S., Seibert, M. & Flickinger, M.C. 2012, "A versatile method for preparation of hydrated microbial-latex biocatalytic coatings for gas absorption and gas evolution", *Journal of Industrial Microbiology & Biotechnology,* vol. 39, no. 9, pp. 1269-1278.

Graetzel, M., Janssen, R.A.J., Mitzi, D.B. & Sargent, E.H. 2012, "Materials interface engineering for solution-processed photovoltaics", *Nature,* vol. 488, no. 7411, pp. 304-312.

Gruendel, M., Scheunemann, R., Lockau, W. & Zilliges, Y. 2012, "Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium Synechocystis sp PCC 6803", *Microbiology,* vol. 158, pp. 3032-3043.

Guerrero, F., Carbonell, V., Cossu, M., Correddu, D. & Jones, P.R. 2012, "Ethylene Synthesis and Regulated Expression of Recombinant Protein in Synechocystis sp PCC 6803", *Plos One,* vol. 7, no. 11, pp. e50470.

Guo, C., Zhu, X., Liao, Q., Wang, Y., Chen, R. & Lee, D. 2011, "Enhancement of photo-hydrogen production in a biofilm photobioreactor using optical fiber with additional rough surface", *Bioresource Technology,* vol. 102, no. 18, pp. 8507-8513.

Gupta, M. & Carr, N. 1981, "Enzyme-Activities Related to Cyanophycin Metabolism in Heterocysts and Vegetative Cells of Anabaena Spp", *Journal of General Microbiology,* vol. 125, no. JUL, pp. 17-23.

Gutekunst, K., Phunpruch, S., Schwarz, C., Schuchardt, S., Schulz-Friedrich, R. & Appel, J. 2005, "LexA regulates the bidirectional hydrogenase in the cyanobacterium Synechocystis sp PCC 6803 as a transcription activator", *Molecular Microbiology,* vol. 58, no. 3, pp. 810-823.

Gutekunst, K., Chen, X., Schreiber, K., Kaspar, U., Makam, S. & Appel, J. 2014, "The Bidirectional NiFe-hydrogenase in Synechocystis sp PCC 6803 Is Reduced by Flavodoxin and Ferredoxin and Is Essential under Mixotrophic, Nitrate-limiting Conditions", *Journal of Biological Chemistry,* vol. 289, no. 4, pp. 1930-1937.

Gutthann, F., Egert, M., Marques, A. & Appel, J. 2007, "Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in Syneehocystis sp PCC 6803", *Biochimica Et Biophysica Acta-Bioenergetics,* vol. 1767, no. 2, pp. 161-169.

Halinen, K., Fewer, D.P., Sihvonen, L.M., Lyra, C., Eronen, E. & Sivonen, K. 2008, "Genetic diversity in strains of the genus Anabaena isolated from planktonic and benthic habitats of the Gulf of Finland (Baltic Sea)", *FEMS Microbiology Ecology,* vol. 64, no. 2, pp. 199-208.

Hallenbeck, P.C. 2011, "Microbial paths to renewable hydrogen production", *Biofuels,* vol. 2, no. 3, pp. 285-302.

Hallenbeck, P.C. 2012, "Hydrogen Production by Cyanobacteria" in *Microbial Technologies in Advanced Biofuels Production.* Springer US, pp. 15-28.

Hallenbeck, P. & Benemann, J. 2002, "Biological hydrogen production; fundamentals and limiting processes", *International Journal of Hydrogen Energy,* vol. 27, no. 11-12, pp. 1185-1193.

Hallenbeck, P. & Benemann, J. 1978, "Characterization and Partial-Purification of Reversible Hydrogenase of Anabaena-Cylindrica", *FEBS letters,* vol. 94, no. 2, pp. 261-264.

Hallenbeck, P., Kostel, P. & Benemann, J. 1979, "Purification and Properties of Nitrogenase from the Cyanobacterium, Anabaena-Cylindrica", *European Journal of Biochemistry,* vol. 98, no. 1, pp. 275-284.

Hammarstrom, L. & Wasielewski, M.R. 2011, "Biomimetic approaches to artificial photosynthesis", *Energy & Environmental Science,* vol. 4, no. 7, pp. 2339-2339.

Hanlon, J.M., Reardon, H., Tapia-Ruiz, N. & Gregory, D.H. 2012, "The Challenge of Storage in the Hydrogen Energy Cycle: Nanostructured Hydrides as a Potential Solution", *Australian Journal of Chemistry,* vol. 65, no. 6, pp. 656-671.

Happe, T. & Naber, J. 1993, "Isolation, Characterization and N-Terminal Amino-Acid-Sequence of Hydrogenase from the Green-Alga Chlamydomonas-Reinhardtii", *European Journal of Biochemistry,* vol. 214, no. 2, pp. 475-481.

Happe, T., Schutz, K. & Bohme, H. 2000, "Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium Anabaena variabilis ATCC 29413", *Journal of Bacteriology,* vol. 182, no. 6, pp. 1624-1631.

Helm, M.L., Stewart, M.P., Bullock, R.M., DuBois, M.R. & DuBois, D.L. 2011, "A Synthetic Nickel Electrocatalyst with a Turnover Frequency Above 100,000 s(-1) for H-2 Production", *Science,* vol. 333, no. 6044, pp. 863-866.

Henson, B.J., Hartman, L., Watson, L.E. & Barnum, S.R. 2011, "Evolution and variation of the nifD and hupL elements in the heterocystous cyanobacteria", *International Journal of Systematic and Evolutionary Microbiology,* vol. 61, pp. 2938-2949.

Herrero, A., Muro-Pastor, A. & Flores, E. 2001, "Nitrogen control in cyanobacteria", *Journal of Bacteriology,* vol. 183, no. 2, pp. 411-425.

Herrero, C., Quaranta, A., Leibl, W., Rutherford, A.W. & Aukauloo, A. 2011, "Artificial photosynthetic systems. Using light and water to provide electrons and protons for the synthesis of a fuel", *Energy & Environmental Science,* vol. 4, no. 7, pp. 2353-2365.

Hoffmann, D., Gutekunst, K., Klissenbauer, M., Schulz-Friedrich, R. & Appel, J. 2006, "Mutagenesis of hydrogenase accessory genes of Synechocystis sp PCC 6803 - Additional homologues of hypA and hypB are not active in hydrogenase maturation", *Febs Journal,* vol. 273, no. 19, pp. 4516-4527.

Houchins, J. 1984, "The Physiology and Biochemistry of Hydrogen Metabolism in Cyanobacteria", *Biochimica et Biophysica Acta,* vol. 768, no. 3-4, pp. 227-255.

Houchins, J. & Burris, R. 1981, "Comparative Characterization of 2 Distinct Hydrogenases from Anabaena Sp Strain-7120", *Journal of Bacteriology,* vol. 146, no. 1, pp. 215-221.

Houchins, J. & Burris, R. 1981, "Occurrence and Localization of 2 Distinct Hydrogenases in the Heterocystous Cyanobacterium Anabaena Sp Strain-7120", *Journal of Bacteriology,* vol. 146, no. 1, pp. 209-214.

Howard, J. & Rees, D. 1996, "Structural basis of biological nitrogen fixation", *Chemical Reviews,* vol. 96, no. 7, pp. 2965-2982.

Howarth, D. & Codd, G. 1985, "The Uptake and Production of Molecular-Hydrogen by Unicellular Cyanobacteria", *Journal of General Microbiology,* vol. 131, no. JUL, pp. 1561-1569.

Huang, H., Camsund, D., Lindblad, P. & Heidorn, T. 2010, "Design and characterization of molecular

tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology", *Nucleic Acids Research,* vol. 38, no. 8, pp. 2577-2593.

Huang, X., Dong, Y. & Zhao, J. 2004, "HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 101, no. 14, pp. 4848-4853.

HyWays , *European Hydrogen Energy Roadmap (2008)*. Available: http://www.hyways.de/ [2014, 8/10].

Intergovernmental Panel on Climate Change , *Fifth Assessment Report (2013)*. Available: http://www.ipcc.ch/report/ar5/index.shtml [2014, 10/8].

Jallet, D., Thurotte, A., Leverenz, R.L., Perreau, F., Kerfeld, C.A. & Kirilovsky, D. 2014, "Specificity of the Cyanobacterial Orange Carotenoid Protein: Influences of Orange Carotenoid Protein and Phycobilisome Structures", *Plant Physiology,* vol. 164, no. 2, pp. 790-804.

Jansson, C. & Northen, T. 2010, "Calcifying cyanobacteria-the potential of biomineralization for carbon capture and storage", *Current Opinion in Biotechnology,* vol. 21, no. 3, pp. 365-371.

Jenkins, J.S., Flickinger, M.C. & Velev, O.D. 2013, "Engineering Cellular Photocomposite Materials Using Convective Assembly", *Materials,* vol. 6, no. 5, pp. 1803-1825.

Kaczmarzyk, D. & Fulda, M. 2010, "Fatty Acid Activation in Cyanobacteria Mediated by Acyl-Acyl Carrier Protein Synthetase Enables Fatty Acid Recycling", *Plant Physiology,* vol. 152, no. 3, pp. 1598-1610.

Kaneko, T., Nakamura, Y., Wolk, C., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Muraki, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takazawa, M., Yamada, M., Yasuda, M. & Tabata, S. 2001, "Complete genomic sequence of the filamentous nitrogen-fixing Cyanobacterium anabaena sp strain PCC 7120", *Dna Research,* vol. 8, no. 5, pp. 205-213.

Karradt, A., Sobanski, J., Mattow, J., Lockau, W. & Baier, K. 2008, "NblA, a Key Protein of Phycobilisome Degradation, Interacts with ClpC, a HSP100 Chaperone Partner of a Cyanobacterial Clp Protease", *Journal of Biological Chemistry,* vol. 283, no. 47, pp. 32394-32403.

Kaushik, A., Mona, S. & Kaushik, C.P. 2011, "Integrating photobiological hydrogen production with dye-metal bioremoval from simulated textile wastewater", *Bioresource Technology,* vol. 102, no. 21, pp. 9957-9964.

Kaya, V. & Picard, G. 1995, "The Viability of Scenedesmus-Bicellularis Cells Immobilized on Alginate Screens Following Nutrient Starvation in Air at 100-Percent Relative-Humidity", *Biotechnology and Bioengineering,* vol. 46, no. 5, pp. 459-464.

Kenney, M.J., Gong, M., Li, Y., Wu, J.Z., Feng, J., Lanza, M. & Dai, H. 2013, "High-Performance Silicon Photoanodes Passivated with Ultrathin Nickel Films for Water Oxidation", *Science,* vol. 342, no. 6160, pp. 836-840.

Kerby, R., Ludden, P. & Roberts, G. 1995, "Carbon Monoxide-Dependent Growth of Rhodospirillum-Rubrum", *Journal of Bacteriology,* vol. 177, no. 8, pp. 2241-2244.

Khetkorn, W., Baebprasert, W., Lindblad, P. & Incharoensakdi, A. 2012, "Redirecting the electron flow towards the nitrogenase and bidirectional Hox-hydrogenase by using specific inhibitors results in enhanced H-2 production in the cyanobacterium Anabaena siamensis TISTR 8012", *Bioresource Technology,* vol. 118, pp. 265-271.

Khetkorn, W., Lindblad, P. & Incharoensakdi, A. 2012, "Inactivation of uptake hydrogenase leads to enhanced and sustained hydrogen production with high nitrogenase activity under high light exposure in the cyanobacterium Anabaena siamensis TISTR 8012", *Journal of Biological Engineering,* vol. 6, no. 1, pp. 19.

Kibsgaard, J., Jaramillo, T.F. & Besenbacher, F. 2014, "Building an appropriate active-site motif into a hydrogen-evolution catalyst with thiomolybdate [Mo3S13](2-) clusters", *Nature Chemistry,* vol. 6, no. 3, pp. 248-253.

Kim, T.W. & Choi, K. 2014, "Nanoporous BiVO4 Photoanodes with Dual-Layer Oxygen Evolution Catalysts for Solar Water Splitting", *Science,* vol. 343, no. 6174, pp. 990-994.

Kiss, E., Kos, P.B. & Vass, I. 2009, "Transcriptional regulation of the bidirectional hydrogenase in the cyanobacterium Synechocystis 6803", *Journal of Biotechnology,* vol. 142, no. 1, pp. 31-37.

Kitashima, M., Masukawa, H., Sakurai, H. & Inoue, K. 2012, "Flexible Plastic Bioreactors for Photobiological Hydrogen Production by Hydrogenase-Deficient Cyanobacteria", *Bioscience Biotechnology and Biochemistry,* vol. 76, no. 4, pp. 831-833.

Knoll, A.H. 2008, "Cyanobacteria and earth history", *The Cyanobacteria: Molecular Biology, Genomics, and Evolution.*Caister Academic Press, pp. 1-20.

Kondo, T., Mori, T., Lebedeva, N., Aoki, S., Ishiura, M. & Golden, S. 1997, "Circadian rhythms in rapidly dividing cyanobacteria", *Science,* vol. 275, no. 5297, pp. 224-227.

Kosourov, S.N., Ghirardi, M.L. & Seibert, M. 2011, "A truncated antenna mutant of Chlamydomonas reinhardtii can produce more hydrogen than the parental strain", *International Journal of Hydrogen Energy,* vol. 36, no. 3, pp. 2044-2048.

Kosourov, S.N. & Seibert, M. 2009, "Hydrogen Photoproduction by Nutrient-Deprived Chlamydomonas reinhardtii Cells Immobilized Within Thin Alginate Films Under Aerobic and Anaerobic Conditions", *Biotechnology and Bioengineering,* vol. 102, no. 1, pp. 50-58.

Kotai, J. 1972, "Instructions for preparation of modified, nutrient solution Z8 for algae. Norwegian institute for water research", *Blindem.Oslo.B-ll/69,* vol. 5.

Kothari, A., Potrafka, R. & Garcia-Pichel, F. 2012, "Diversity in hydrogen evolution from bidirectional hydrogenases in cyanobacteria from terrestrial, freshwater and marine intertidal environments", *Journal of Biotechnology,* vol. 162, no. 1, pp. 105-114.

Krasikov, V., von Wobeser, E.A., Dekker, H.L., Huisman, J. & Matthijs, H.C.P. 2012, "Time-series resolution of gradual nitrogen starvation and its impact on photosynthesis in the cyanobacterium Synechocystis PCC 6803", *Physiologia Plantarum,* vol. 145, no. 3, pp. 426-439.

Kruse, O., Rupprecht, J., Bader, K., Thomas-Hall, S., Schenk, P., Finazzi, G. & Hankamer, B. 2005, "Improved photobiological H-2 production in engineered green algal cells", *Journal of Biological Chemistry,* vol. 280, no. 40, pp. 34170-34177.

Kucho, K., Okamoto, K., Tsuchiya, Y., Nomura, S., Nango, M., Kanehisa, M. & Ishiura, M. 2005, "Global analysis of circadian expression in the cyanobacterium Synechocystis sp strain PCC 6803", *Journal of Bacteriology,* vol. 187, no. 6, pp. 2190-2199.

Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F.X. & van Langenhove, H. 2010, "Enhanced CO2 fixation and biofuel production via microalgae: recent developments and future directions", *Trends in biotechnology,* vol. 28, no. 7, pp. 371-380.

Kumar, D. & Kumar, H. 1992, "Hydrogen-Production by several Cyanobacteria", *International Journal of Hydrogen Energy,* vol. 17, no. 11, pp. 847-852.

Kumar, K., Dasgupta, C.N., Nayak, B., Lindblad, P. & Das, D. 2011, "Development of suitable photobioreactors for CO2 sequestration addressing global warming using green algae and cyanobacteria", *Bioresource Technology,* vol. 102, no. 8, pp. 4945-4953.

Kuwada, Y. & Ohta, Y. 1987, "Hydrogen-Production by an Immobilized Cyanobacterium, Lyngbya Sp", *Journal of Fermentation Technology,* vol. 65, no. 5, pp. 597-602.

Lambert, G. & Smith, G. 1977, "Hydrogen Formation by Marine Blue-Green-Algae", *FEBS letters,* vol. 83, no. 1, pp. 159-162.

Lan, E.I. & Liao, J.C. 2012, "ATP drives direct photosynthetic production of 1-butanol in cyanobacteria", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 109, no. 16, pp. 6018-6023.

Lan, E.I. & Liao, J.C. 2011, "Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide", *Metabolic Engineering,* vol. 13, no. 4, pp. 353-363.

Lardon, L., Helias, A., Sialve, B., Stayer, J. & Bernard, O. 2009, "Life-Cycle Assessment of Biodiesel Production from Microalgae", *Environmental Science & Technology,* vol. 43, no. 17, pp. 6475-6481.

Levin, D., Pitt, L. & Love, M. 2004, "Biohydrogen production: prospects and limitations to practical application", *International Journal of Hydrogen Energy,* vol. 29, no. 2, pp. 173-185.

Li, Z., Wakao, S., Fischer, B.B. & Niyogi, K.K. 2009, "Sensing and Responding to Excess Light", *Annual Review of Plant Biology,* vol. 60, pp. 239-260.

Liberton, M., Pakrasi, H., Herrero, A. & Flores, E. 2008, "Membrane systems in cyanobacteria", *The Cyanobacteria: Molecular Biology, Genomics and Evolution.* Caister Academic Press, pp. 271-289.

Lindberg, P., Schutz, K., Happe, T. & Lindblad, P. 2002, "A hydrogen-producing, hydrogenase-free

mutant strain of Nostoc punctiforme ATCC 29133", *International Journal of Hydrogen Energy,* vol. 27, no. 11-12, pp. 1291-1296.

Lindberg, P., Devine, E., Stensjo, K. & Lindblad, P. 2012, "HupW Protease Specifically Required for Processing of the Catalytic Subunit of the Uptake Hydrogenase in the Cyanobacterium Nostoc sp Strain PCC 7120", *Applied and Environmental Microbiology,* vol. 78, no. 1, pp. 273-276.

Lindberg, P., Park, S. & Melis, A. 2010, "Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism", *Metabolic Engineering,* vol. 12, no. 1, pp. 70-79.

Liu, X., Sheng, J. & Curtiss,Roy,,III 2011, "Fatty acid production in genetically modified cyanobacteria", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 108, no. 17, pp. 6899-6904.

Livingstone, D. & Whitton, B. 1983, "Influence of Phosphorus on Morphology of Calothrix-Parietina (Cyanophyta) in Culture", *British Phycological Journal,* vol. 18, no. 1, pp. 29-38.

Lopez-Igual, R., Flores, E. & Herrero, A. 2010, "Inactivation of a Heterocyst-Specific Invertase Indicates a Principal Role of Sucrose Catabolism in Heterocysts of Anabaena sp.", *Journal of Bacteriology,* vol. 192, no. 20, pp. 5526-5533.

Los, D.A., Suzuki, I., Zinchenko, V.V. & Murata, N. 2008, *Stress responses in Synechocystis: regulated genes and regulatory systems,* Caister Academic Press: Norfolk, UK.

Lu, X. 2010, "A perspective: Photosynthetic production of fatty acid-based biofuels in genetically engineered cyanobacteria", *Biotechnology Advances,* vol. 28, no. 6, pp. 742-746.

Lubner, C.E., Applegate, A.M., Knoerzer, P., Ganago, A., Bryant, D.A., Happe, T. & Golbeck, J.H. 2011, "Solar hydrogen-producing bionanodevice outperforms natural photosynthesis", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 108, no. 52, pp. 20988-20991.

Lyon, E., Shima, S., Boecher, R., Thauer, R., Grevels, F., Bill, E., Roseboom, W. & Albracht, S. 2004, "Carbon monoxide as an intrinsic ligand to iron in the active site of the iron-sulfur-cluster-free hydrogenase H-2-Forming methylenetetrahydromethanopterin dehydrogenase as revealed by infrared spectroscopy", *Journal of the American Chemical Society,* vol. 126, no. 43, pp. 14239-14248.

Madigan, M.T., Martinko, J.M., Dunlap, P.V. & Clark, D.P. 2008, "Brock Biology of microorganisms 12th edn", *International Microbiology,* vol. 11, pp. 65-73.

Magnuson, A., Anderlund, M., Johansson, O., Lindblad, P., Lomoth, R., Polivka, T., Ott, S., Stensjo, K., Styring, S., Sundstrom, V. & Hammarstrom, L. 2009, "Biomimetic and Microbial Approaches to Solar Fuel Generation", *Accounts of Chemical Research,* vol. 42, no. 12, pp. 1899-1909.

Mallick, N. 2002, "Biotechnological potential of immobilized algae for wastewater N, P and metal removal: A review", *Biometals,* vol. 15, no. 4, pp. 377-390.

Markov, S., Bazin, M. & Hall, D. 1995, "Hydrogen Photoproduction and Carbon-Dioxide Uptake by Immobilized Anabaena-Variabilis in a Hollow-Fiber Photobioreactor", *Enzyme and Microbial Technology,* vol. 17, no. 4, pp. 306-310.

Markov, S., Lichtl, R., Rao, K. & Hall, D. 1993, "A Hollow-Fiber Photobioreactor for Continuous Production of Hydrogen by Immobilized Cyanobacteria Under Partial Vacuum", *International Journal of Hydrogen Energy,* vol. 18, no. 11, pp. 901-906.

Markowitz, V.M., Mavromatis, K., Ivanova, N.N., Chen, I.A., Chu, K. & Kyrpides, N.C. 2009, "IMG ER: a system for microbial genome annotation expert review and curation", *Bioinformatics,* vol. 25, no. 17, pp. 2271-2278.

Marques, A.E., Barbosa, A.T., Jotta, J., Coelho, M.C., Tamagnini, P. & Gouveia, L. 2011, "Biohydrogen production by Anabaena sp PCC 7120 wild-type and mutants under different conditions: Light, nickel, propane, carbon dioxide and nitrogen", *Biomass & Bioenergy,* vol. 35, no. 10, pp. 4426-4434.

Martin-Figueroa, E., Navarro, F. & Florencio, F. 2000, "The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of glsF gene from the cyanobacterium Anabaena sp PCC 7120", *FEBS letters,* vol. 476, no. 3, pp. 282-286.

Masepohl, B., Gorlitz, K., Monnerjahn, U., Mosler, B. & Bohme, H. 1997, "The ferredoxin-encoding fdxN gene of the filamentous cyanobacterium Anabaena variabilis ATCC 29413 is not essential for nitrogen fixation", *New Phytologist,* vol. 136, no. 3, pp. 419-423.

Masukawa, H., Mochimaru, M. & Sakurai, H. 2002, "Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium Anabaena sp PCC 7120", *Applied Microbiology and Biotechnology,* vol. 58, no. 5, pp. 618-624.

Masukawa, H., Inoue, K. & Sakurai, H. 2007, "Effects of disruption of homocitrate synthase genes on Nostoc sp strain PCC 7120 photobiological hydrogen production and nitrogenasev", *Applied and Environmental Microbiology,* vol. 73, no. 23, pp. 7562-7570.

Masukawa, H., Inoue, K., Sakurai, H., Wolk, C.P. & Hausinger, R.P. 2010, "Site-Directed Mutagenesis of the Anabaena sp. Strain PCC 7120 Nitrogenase Active Site To Increase Photobiological Hydrogen Production", *Applied and Environmental Microbiology,* vol. 76, no. 20, pp. 6741-6750.

Masukawa, H., Zhang, X., Yamazaki, E., Iwata, S., Nakamura, K., Mochimaru, M., Inoue, K. & Sakurai, H. 2009, "Survey of the Distribution of Different Types of Nitrogenases and Hydrogenases in Heterocyst-Forming Cyanobactera", *Marine Biotechnology,* vol. 11, no. 3, pp. 397-409.

McCormick, A.J., Bombelli, P., Lea-Smith, D.J., Bradley, R.W., Scott, A.M., Fisher, A.C., Smith, A.G. & Howe, C.J. 2013, "Hydrogen production through oxygenic photosynthesis using the cyanobacterium Synechocystis sp PCC 6803 in a bio-photoelectrolysis cell (BPE) system", *Energy & Environmental Science,* vol. 6, no. 9, pp. 2682-2690.

Meeks, J., Campbell, E., Summers, M. & Wong, F. 2002, "Cellular differentiation in the cyanobacterium Nostoc punctiforme", *Archives of Microbiology,* vol. 178, no. 6, pp. 395-403.

Meeks, J. & Castenholz, R. 1971, "Growth and Photosynthesis in an Extreme Thermophile, Synechococcus-Lividus (Cyanophyta)", *Archiv fur Mikrobiologie,* vol. 78, no. 1, pp. 25-&.

Meeks, J. & Elhai, J. 2002, "Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states", *Microbiology and Molecular Biology Reviews,* vol. 66, no. 1, pp. 94-121.

Meeks, J., Wolk, C., Lockau, W., Schilling, N., Shaffer, P. & Chien, W. 1978, "Pathways of Assimilation of [N-13]n2 and (Nh4)-N+13 by Cyanobacteria with and without Heterocysts", *Journal of Bacteriology,* vol. 134, no. 1, pp. 125-130.

Meeks, J., Wolk, C., Thomas, J., Lockau, W., Shaffer, P., Austin, S., Chien, W. & Galonsky, A. 1977, "Pathways of Assimilation of (Nh4+)-N-13 by Cyanobacterium, Anabaena-Cylindrica", *Journal of Biological Chemistry,* vol. 252, no. 21, pp. 7894-7900.

Megiatto Jr, J.D., Méndez-Hernández, D.D., Tejeda-Ferrari, M.E., Teillout, A., Llansola-Portolés, M.J., Kodis, G., Poluektov, O.G., Rajh, T., Mujica, V. & Groy, T.L. 2014, "A bioinspired redox relay that mimics radical interactions of the Tyr–His pairs of photosystem II", *Nature Chemistry,* .

Melis, A., Zhang, L., Forestier, M., Ghirardi, M. & Seibert, M. 2000, "Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii", *Plant Physiology,* vol. 122, no. 1, pp. 127-135.

Mella-Herrera, R.A., Neunuebel, M.R., Kumar, K., Saha, S.K. & Golden, J.W. 2011, "The sigE Gene Is Required for Normal Expression of Heterocyst-Specific Genes in Anabaena sp Strain PCC 7120", *Journal of Bacteriology,* vol. 193, no. 8, pp. 1823-1832.

Melnicki, M.R., Pinchuk, G.E., Hill, E.A., Kucek, L.A., Fredrickson, J.K., Konopka, A. & Beliaev, A.S. 2012, "Sustained H-2 Production Driven by Photosynthetic Water Splitting in a Unicellular Cyanobacterium", *Mbio,* vol. 3, no. 4, pp. e00197.

Meunier, C.F., Yang, X., Rooke, J.C. & Su, B. 2011, "Biofuel cells Based on the Immobilization of Photosynthetically Active Bioentities", *Chemcatchem,* vol. 3, no. 3, pp. 476-488.

Mikheeva, L., Schmitz, O., Shestakov, S. & Bothe, H. 1995, "Mutants of the Cyanobacterium Anabaena-Variabilis Altered in Hydrogenase Activities", *Zeitschrift Fur Naturforschung C-a Journal of Biosciences,* vol. 50, no. 7-8, pp. 505-510.

Min, H. & Sherman, L.A. 2010, "Hydrogen Production by the Unicellular, Diazotrophic Cyanobacterium Cyanothece sp Strain ATCC 51142 under Conditions of Continuous Light", *Applied and Environmental Microbiology,* vol. 76, no. 13, pp. 4293-4301.

Mitra, M. & Melis, A. 2010, "Genetic and biochemical analysis of the TLA1 gene in Chlamydomonas reinhardtii", *Planta,* vol. 231, no. 3, pp. 729-740.

Mona, S., Kaushik, A. & Kaushik, C.P. 2013, "Prolonged hydrogen production by Nostoc in photobioreactor and multi-stage use of the biological waste

for column biosorption of some dyes and metals", *Biomass & Bioenergy,* vol. 54, pp. 27-35.

Montagud, A., Navarro, E., Fernandez de Cordoba, P., Urchueguia, J.F. & Patil, K.R. 2010, "Reconstruction and analysis of genome-scale metabolic model of a photosynthetic bacterium", *Bmc Systems Biology,* vol. 4, pp. 156.

Moreno-Garrido, I. 2008, "Microalgae immobilization: Current techniques and uses", *Bioresource Technology,* vol. 99, no. 10, pp. 3949-3964.

Mori, T., Binder, B. & Johnson, C. 1996, "Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 19, pp. 10183-10188.

Mulkidjanian, A.Y., Koonin, E.V., Makarova, K.S., Mekhedov, S.L., Sorokin, A., Wolf, Y.I., Dufresne, A., Partensky, F., Burd, H., Kaznadzey, D., Haselkorn, R. & Galperin, M.Y. 2006, "The cyanobacterial genome core and the origin of photosynthesis", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 35, pp. 13126-13131.

Mulligan, M. & Haselkorn, R. 1989, "Nitrogen-Fixation (Nif) Genes of the Cyanobacterium Anabaena Species Strain Pcc 7120", *Journal of Biological Chemistry,* vol. 264, no. 32, pp. 19200-19207.

Muro-Pastor, M., Reyes, J. & Florencio, F. 2001, "Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels", *Journal of Biological Chemistry,* vol. 276, no. 41, pp. 38320-38328.

Mussgnug, J.H., Thomas-Hall, S., Rupprecht, J., Foo, A., Klassen, V., McDowall, A., Schenk, P.M., Kruse, O. & Hankamer, B. 2007, "Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion", *Plant Biotechnology Journal,* vol. 5, no. 6, pp. 802-814.

Nadeau, T. & Castenholz, R. 2000, "Characterization of psychrophilic oscillatorians (cyanobacteria) from Antarctic meltwater ponds", *Journal of Phycology,* vol. 36, no. 5, pp. 914-923.

Navarro, E., Montagud, A., Fernandez de Cordoba, P. & Urchueguia, J.F. 2009, "Metabolic flux analysis of the hydrogen production potential in Synechocystis sp PCC6803", *International Journal of Hydrogen Energy,* vol. 34, no. 21, pp. 8828-8838.

Neilan, B., Jacobs, D. & Goodman, A. 1995, "Genetic Diversity and Phylogeny of Toxic Cyanobacteria Determined by Dna Polymorphisms within the Phycocyanin Locus", *Applied and Environmental Microbiology,* vol. 61, no. 11, pp. 3875-3883.

Niederholtmeyer, H., Wolfstaedter, B.T., Savage, D.F., Silver, P.A. & Way, J.C. 2010, "Engineering Cyanobacteria To Synthesize and Export Hydrophilic Products", *Applied and Environmental Microbiology,* vol. 76, no. 11, pp. 3462-3466.

Nocera, D.G. 2012, "The Artificial Leaf", *Accounts of Chemical Research,* vol. 45, no. 5, pp. 767-776.

Oey, M., Ross, I.L., Stephens, E., Steinbeck, J., Wolf, J., Radzun, K.A., Kuegler, J., Ringsmuth, A.K., Kruse, O. & Hankamer, B. 2013, "RNAi Knock-Down of LHCBM1, 2 and 3 Increases Photosynthetic H-2 Production Efficiency of the Green Alga Chlamydomonas reinhardtii", *Plos One,* vol. 8, no. 4, pp. e61375.

Olguin, E. 2003, "Phycoremediation: key issues for cost-effective nutrient removal processes", *Biotechnology Advances,* vol. 22, no. 1-2, pp. 81-91.

Oliveira, P. & Lindblad, P. 2009, "Transcriptional regulation of the cyanobacterial bidirectional Hox-hydrogenase", *Dalton Transactions,* , no. 45, pp. 9990-9996.

Ort, D.R., Zhu, X. & Melis, A. 2011, "Optimizing Antenna Size to Maximize Photosynthetic Efficiency", *Plant Physiology,* vol. 155, no. 1, pp. 79-85.

Osanai, T., Kuwahara, A., Iijima, H., Toyooka, K., Sato, M., Tanaka, K., Ikeuchi, M., Saito, K. & Hirai, M.Y. 2013, "Pleiotropic effect of sigE over-expression on cell morphology, photosynthesis and hydrogen production in Synechocystis sp PCC 6803", *Plant Journal,* vol. 76, no. 3, pp. 456-465.

Otero, A. & Vincenzini, M. 2004, "Nostoc (Cyanophyceae) goes nude: Extracellular polysaccharides serve as a sink for reducing power under unbalanced C/N metabolism", *Journal of Phycology,* vol. 40, no. 1, pp. 74-81.

Ott, S., Kritikos, M., Akermark, B., Sun, L. & Lomoth, R. 2004, "A biomimetic pathway for hydrogen evolution from a model of the iron hydrogenase active site", *Angewandte Chemie-International Edition,* vol. 43, no. 8, pp. 1006-1009.

Ow, S.Y., Cardona, T., Taton, A., Magnuson, A., Lindblad, P., Stensjo, K. & Wright, P.C. 2008, "Quantitative shotgun proteomics of enriched heterocysts

from Nostoc sp. PCC 7120 using 8-plex isobaric peptide tags", *Journal of Proteome Research,* vol. 7, no. 4, pp. 1615-1628.

Oxelfelt, F., Tamagnini, P., Salema, R. & Lindblad, P. 1995, "Hydrogen Uptake in Nostoc Strain Pcc-73102 - Effects of Nickel, Hydrogen, Carbon and Nitrogen", *Plant Physiology and Biochemistry,* vol. 33, no. 6, pp. 617-623.

Page, L.E., Liberton, M. & Pakrasi, H.B. 2012, "Reduction of Photoautotrophic Productivity in the Cyanobacterium Synechocystis sp Strain PCC 6803 by Phycobilisome Antenna Truncation", *Applied and Environmental Microbiology,* vol. 78, no. 17, pp. 6349-6351.

Pernil, R., Herrero, A. & Flores, E. 2010, "Catabolic Function of Compartmentalized Alanine Dehydrogenase in the Heterocyst-Forming Cyanobacterium Anabaena sp. Strain PCC 7120", *Journal of Bacteriology,* vol. 192, no. 19, pp. 5165-5172.

Peters, J. 1999, "Structure and mechanism of iron-only hydrogenases", *Current Opinion in Structural Biology,* vol. 9, no. 6, pp. 670-676.

Peterson, R. & Wolk, C. 1978, "Localization of an Uptake Hydrogenase in Anabaena", *Plant Physiology,* vol. 61, no. 4, pp. 688-691.

Pfreundt, U., Stal, L.J., Voss, B. & Hess, W.R. 2012, "Dinitrogen fixation in a unicellular chlorophyll d-containing cyanobacterium", *Isme Journal,* vol. 6, no. 7, pp. 1367-1377.

Pfromm, P.H., Amanor-Boadu, V. & Nelson, R. 2011, "Sustainability of algae derived biodiesel: A mass balance approach", *Bioresource Technology,* vol. 102, no. 2, pp. 1185-1193.

Phlips, E. & Mitsui, A. 1986, "Characterization and Optimization of Hydrogen-Production by a Salt-Water Blue-Green-Alga Oscillatoria Sp Miami Bg 7 .2. use of Immobilization for Enhancement of Hydrogen-Production", *International Journal of Hydrogen Energy,* vol. 11, no. 2, pp. 83-89.

Picossi, S., Flores, E. & Herrero, A. 2014, "ChIP analysis unravels an exceptionally wide distribution of DNA binding sites for the NtcA transcription factor in a heterocyst-forming cyanobacterium", *Bmc Genomics,* vol. 15, pp. 22.

Pittman, J.K., Dean, A.P. & Osundeko, O. 2011, "The potential of sustainable algal biofuel production using wastewater resources", *Bioresource Technology,* vol. 102, no. 1, pp. 17-25.

Polle, J., Kanakagiri, S., Jin, E., Masuda, T. & Melis, A. 2002, "Truncated chlorophyll antenna size of the photosystems - a practical method to improve microalgal productivity and hydrogen production in mass culture", *International Journal of Hydrogen Energy,* vol. 27, no. 11-12, pp. 1257-1264.

Posten, C. 2009, "Design principles of photo-bioreactors for cultivation of microalgae", *Engineering in Life Sciences,* vol. 9, no. 3, pp. 165-177.

Price, G.D., Badger, M.R., Woodger, F.J. & Long, B.M. 2008, "Advances in understanding the cyanobacterial CO(2)-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants", *Journal of Experimental Botany,* vol. 59, no. 7, pp. 1441-1461.

Purnick, P.E.M. & Weiss, R. 2009, "The second wave of synthetic biology: from modules to systems", *Nature Reviews Molecular Cell Biology,* vol. 10, no. 6, pp. 410-422.

Rai, L. & Raizada, M. 1986, "Nickel Induced Stimulation of Growth, Heterocyst Differentiation, Co2-C-14 Uptake and Nitrogenase Activity in Nostoc-Muscorum", *New Phytologist,* vol. 104, no. 1, pp. 111-114.

Raleiras, P., Kellers, P., Lindblad, P., Styring, S. & Magnuson, A. 2013, "Isolation and Characterization of the Small Subunit of the Uptake Hydrogenase from the Cyanobacterium Nostoc punctiforme", *Journal of Biological Chemistry,* vol. 288, no. 25, pp. 18345-18352.

Ramasubramanian, T., Wei, T. & Golden, J. 1994, "2 Anabaena Sp Strain Pcc 7120 Dna-Binding Factors Interact with Vegetative Cell-Specific and Heterocyst-Specific Genes", *Journal of Bacteriology,* vol. 176, no. 5, pp. 1214-1223.

Ran, L., Larsson, J., Vigil-Stenman, T., Nylander, J.A.A., Ininbergs, K., Zheng, W., Lapidus, A., Lowry, S., Haselkorn, R. & Bergman, B. 2010, "Genome Erosion in a Nitrogen-Fixing Vertically Transmitted Endosymbiotic Multicellular Cyanobacterium", *Plos One,* vol. 5, no. 7, pp. e11486.

Rao, K. & Hall, D. 1996, "Hydrogen production by cyanobacteria: potential, problems and prospects", *Journal of Marine Biotechnology,* vol. 4, pp. 10-15.

Rashid, N., Choi, W. & Lee, K. 2012, "Optimization of two-staged bio-hydrogen production by immobilized Microcystis aeruginosa", *Biomass & Bioenergy,* vol. 36, pp. 241-249.

Rashid, N., Song, W., Park, J., Jin, H. & Lee, K. 2009, "Characteristics of hydrogen production by immobilized cyanobacterium Microcystis aeruginosa through cycles of photosynthesis and anaerobic incubation", *Journal of Industrial and Engineering Chemistry,* vol. 15, no. 4, pp. 498-503.

Rasmussen, U. & Nilsson, M. 2002, "Cyanobacterial diversity and specificity in plant symbioses" in *Cyanobacteria in symbiosis.* Springer, pp. 313-328.

Rastogi, R.P. & Sinha, R.P. 2009, "Biotechnological and industrial significance of cyanobacterial secondary metabolites", *Biotechnology Advances,* vol. 27, no. 4, pp. 521-539.

Raymond, J., Siefert, J., Staples, C. & Blankenship, R. 2004, "The natural history of nitrogen fixation", *Molecular Biology and Evolution,* vol. 21, no. 3, pp. 541-554.

Rippka, R., Deruelles, J., Waterbury, J., Herdman, M. & Stanier, R. 1979, "Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria", *Journal of General Microbiology,* vol. 111, no. 1. pp. 1-61.

Rokke, G., Korvald, E., Pahr, J., Oyas, O. & Lale, R. 2014, "BioBrick Assembly Standards and Techniques and Associated Software Tools", *Dna Cloning and Assembly Methods,* vol. 1116, pp. 1-24.

Romero, M., Muro-Pastor, A.M. & Otero, A. 2011, "Quorum sensing N-acylhomoserine lactone signals affect nitrogen fixation in the cyanobacterium Anabaena sp. PCC7120", *FEMS Microbiology Letters,* vol. 315, no. 2, pp. 101-108.

Rooke, J.C., Léonard, A., Meunier, C.F., Sarmento, H., Descy, J. & Su, B. 2010, "Hybrid photosynthetic materials derived from microalgae Cyanidium caldarium encapsulated within silica gel", *Journal of Colloid and Interface Science,* vol. 344, no. 2, pp. 348-352.

Rosenberg, G. & Paerl, H. 1981, "Nitrogen-Fixation by Blue-Green-Algae Associated with the Siphonous Green Seaweed Codium-Decorticatum - Effects on Ammonium Uptake", *Marine Biology,* vol. 61, no. 2-3, pp. 151-158.

Rusch, D.B., Halpern, A.L., Sutton, G., Heidelberg, K.B., Williamson, S., Yooseph, S., Wu, D., Eisen, J.A., Hoffman, J.M., Remington, K., Beeson, K., Tran, B., Smith, H., Baden-Tillson, H., Stewart, C., Thorpe, J., Freeman, J., Andrews-Pfannkoch, C., Venter, J.E., Li, K., Kravitz, S., Heidelberg, J.F., Utterback, T., Rogers, Y., Falcon, L.I., Souza, V., Bonilla-Rosso, G., Eguiarte, L.E., Karl, D.M., Sathyendranath, S., Platt, T., Bermingham, E., Gallardo, V., Tamayo-Castillo, G., Ferrari, M.R., Strausberg, R.L., Nealson, K., Friedman, R., Frazier, M. & Venter, J.C. 2007, "The Sorcerer II Global Ocean Sampling expedition: Northwest Atlantic through Eastern Tropical Pacific", *Plos Biology,* vol. 5, no. 3, pp. 398-431.

Sagan, L. 1967, "On the origin of mitosing cells", *Journal of Theoretical Biology,* vol. 14, no. 3, pp. 255-274.

Sakr, S., Dutheil, J., Saenkham, P., Bottin, H., Leplat, C., Ortega-Ramos, M., Aude, J., Chapuis, V., Guedeney, G., Decottignies, P., Lemaire, S., Cassier-Chauvat, C. & Chauvat, F. 2013, "The activity of the Synechocystis PCC6803 AbrB2 regulator of hydrogen production can be post-translationally controlled through glutathionylation", *International Journal of Hydrogen Energy,* vol. 38, no. 31, pp. 13547-13555.

Sakurai, H. & Masukawa, H. 2007, "Promoting R & D in photobiological hydrogen production utilizing mariculture-raised cyanobacteria", *Marine Biotechnology,* vol. 9, no. 2, pp. 128-145.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989, *Molecular cloning: a laboratory manual,* 2nd edn, Cold spring harbor laboratory press, New York.

Sarkar, S., Pandey, K. & Kashyap, A. 1992, "Hydrogen Photoproduction by Filamentous Nonheterocystous Cyanobacterium Plectonema-Boryanum and Simultaneous Release of Ammonia", *International Journal of Hydrogen Energy,* vol. 17, no. 9, pp. 689-694.

Savakis, P.E., Angermayr, S.A. & Hellingwerf, K.J. 2013, "Synthesis of 2,3-butanediol by Synechocystis sp PCC6803 via heterologous expression of a catabolic pathway from lactic acid- and enterobacteria", *Metabolic Engineering,* vol. 20, pp. 121-130.

Sazuka, T., Yamaguchi, M. & Ohara, O. 1999, "Cyano2Dbase updated: Linkage of 234 protein spots to corresponding genes through N-terminal microsequencing", *Electrophoresis,* vol. 20, no. 11, pp. 2160-2171.

Schmitz, O., Boison, G. & Bothe, H. 2001, "Quantitative analysis of expression of two circadian clock-controlled gene clusters coding for the bidirectional hydrogenase in the cyanobacterium Synechococcus sp PCC7942", *Molecular Microbiology,* vol. 41, no. 6, pp. 1409-1417.

Schmitz, O., Boison, G., Hilscher, R., Hundeshagen, B., Zimmer, W., Lottspeich, F. & Bothe, H. 1995, "Molecular Biological Analysis of a Bidirectional Hydrogenase from Cyanobacteria", *European Journal of Biochemistry,* vol. 233, no. 1, pp. 266-276.

Schmitz, O., Boison, G., Salzmann, H., Bothe, H., Schutz, K., Wang, S. & Happe, T. 2002, "HoxE - a subunit specific for the pentameric bidirectional hydrogenase complex (HoxEFUYH) of cyanobacteria", *Biochimica Et Biophysica Acta-Bioenergetics,* vol. 1554, no. 1-2, pp. 66-74.

Schopf, J.W. 2012, "The Fossil Record of Cyanobacteria" in *Ecology of Cyanobacteria II.* Springer, pp. 15-36.

Schutz, K., Happe, T., Troshina, O., Lindblad, P., Leitao, E., Oliveira, P. & Tamagnini, P. 2004, "Cyanobacterial H-2 production - a comparative analysis", *Planta,* vol. 218, no. 3, pp. 350-359.

Seabra, R., Santos, A., Pereira, S., Moradas-Ferreira, P. & Tamagnini, P. 2009, "Immunolocalization of the uptake hydrogenase in the marine cyanobacterium Lyngbya majuscula CCAP 1446/4 and two Nostoc strains", *FEMS Microbiology Letters,* vol. 292, no. 1, pp. 57-62.

Sekar, R., Venugopalan, V., Nandakumar, K., Nair, K. & Rao, V. 2004, "Early stages of biofilm succession in a lentic freshwater environment", *Hydrobiologia,* vol. 512, no. 1-3, pp. 97-108.

Serebryakova, L.T. & Sheremetieva, M.E. 2006, "Characterization of catalytic properties of hydrogenase isolated from the unicellular cyanobacterium Gloeocapsa alpicola CALU 743", *Biochemistry-Moscow,* vol. 71, no. 12, pp. 1370-1376.

Serebryakova, L.T. & Tsygankov, A.A. 2007, "Two-stage system for hydrogen production by immobilized cyanobacterium Gloeocapsa alpicola CALU 743", *Biotechnology Progress,* vol. 23, no. 5, pp. 1106-1110.

Shah, V., Garg, N. & Madamwar, D. 2003, "Ultrastructure of the cyanobacterium Nostoc muscorum and exploitation of the culture for hydrogen production", *Folia Microbiologica,* vol. 48, no. 1, pp. 65-70.

Shah, V., Garg, N. & Madamwar, D. 2001, "An integrated process of textile dye removal and hydrogen evolution using cyanobacterium, Phormidium valderianum", *World Journal of Microbiology & Biotechnology,* vol. 17, no. 5, pp. 499-504.

Shah, V., Garg, N. & Madamwar, D. 2001, "Ultrastructure of the fresh water cyanobacterium Anabaena variabilis SPU 003 and its application for oxygen-free hydrogen production", *FEMS Microbiology Letters,* vol. 194, no. 1, pp. 71-75.

Sheremetieva, M., Troshina, O., Serebryakova, L. & Lindblad, P. 2002, "Identification of hox genes and analysis of their transcription in the unicellular cyanobacterium Gloeocapsa alpicola CALU 743 growing under nitrate-limiting conditions", *FEMS Microbiology Letters,* vol. 214, no. 2, pp. 229-233.

Shirvani, T., Yan, X., Inderwildi, O.R., Edwards, P.P. & King, D.A. 2011, "Life cycle energy and greenhouse gas analysis for algae-derived biodiesel", *Energy & Environmental Science,* vol. 4, no. 10, pp. 3773-3778.

Shomura, Y. & Higuchi, Y. 2012, "Structural Basis for the Reaction Mechanism of S-Carbamoylation of HypE by HypF in the Maturation of [NiFe]-Hydrogenases", *Journal of Biological Chemistry,* vol. 287, no. 34, pp. 28409-28419.

Show, K., Lee, D. & Chang, J. 2011, "Bioreactor and process design for biohydrogen production", *Bioresource Technology,* vol. 102, no. 18, pp. 8524-8533.

Sicard, C., Perullini, M., Spedalieri, C., Coradin, T., Brayner, R., Livage, J., Jobbagy, M. & Bilmes, S.A. 2011, "CeO2 Nanoparticles for the Protection of Photosynthetic Organisms Immobilized in Silica Gels", *Chemistry of Materials,* vol. 23, no. 6, pp. 1374-1378.

Sihvonen, L.M., Lyra, C., Fewer, D.P., Rajaniemi-Wacklin, P., Lehtimaki, J.M., Wahlsten, M. & Sivonen, K. 2007, "Strains of the cyanobacterial genera Calothrix and Rivularia isolated from the Baltic Sea display cryptic diversity and are distantly related to Gloeotrichia and Tolypothrix", *FEMS Microbiology Ecology,* vol. 61, no. 1, pp. 74-84.

Sinclair, C. & Whitton, B. 1977, "Influence of Nutrient Deficiency on Hair Formation in Rivulariaceae", *British Phycological Journal,* vol. 12, no. 3, pp. 297-313.

Sjoholm, J., Oliveira, P. & Lindblad, P. 2007, "Transcription and regulation of the bidirectional hydrogenase in the cyanobacterium Nostoc sp. strain PCC 7120", *Applied and Environmental Microbiology,* vol. 73, no. 17, pp. 5435-5446.

Skizim, N.J., Ananyev, G.M., Krishnan, A. & Dismukes, G.C. 2012, "Metabolic Pathways for Photobiological Hydrogen Production by Nitrogenase-

and Hydrogenase-containing Unicellular Cyanobacteria Cyanothece", *Journal of Biological Chemistry,* vol. 287, no. 4, pp. 2777-2786.

Smith, R., Noy, R. & Evans, M. 1971, "Physiological Electron Donor Systems to Nitrogenase of Blue-Green Alga Anabaena-Cylindrica", *Biochimica et Biophysica Acta,* vol. 253, no. 1, pp. 104-119.

Stal, L. 1992, "Poly(hydroxyalkanoate) in Cyanobacteria - an Overview", *FEMS Microbiology Letters,* vol. 103, no. 2-4, pp. 169-180.

Stensjo, K., Ow, S.Y., Barrios-Llerena, M.E., Lindblad, P. & Wright, P.C. 2007, "An iTRAQ-based quantitative analysis to elaborate the proteomic response of Nostoc sp PCC 7120 under N-2 fixing conditions", *Journal of Proteome Research,* vol. 6, no. 2, pp. 621-635.

Steunou, A., Jensen, S.I., Brecht, E., Becraft, E.D., Bateson, M.M., Kilian, O., Bhaya, D., Ward, D.M., Peters, J.W., Grossman, A.R. & Kuhl, M. 2008, "Regulation of nif gene expression and the energetics of N-2 fixation over the diel cycle in a hot spring microbial mat", *Isme Journal,* vol. 2, no. 4, pp. 364-378.

Stucken, K., John, U., Cembella, A., Murillo, A.A., Soto-Liebe, K., Fuentes-Valdes, J.J., Friedel, M., Plominsky, A.M., Vasquez, M. & Gloeckner, G. 2010, "The Smallest Known Genomes of Multicellular and Toxic Cyanobacteria: Comparison, Minimal Gene Sets for Linked Traits and the Evolutionary Implications", *Plos One,* vol. 5, no. 2, pp. e9235.

Subhadra, B.G. 2010, "Sustainability of algal biofuel production using integrated renewable energy park (IREP) and algal biorefinery approach", *Energy Policy,* vol. 38, no. 10, pp. 5892-5901.

Summers, M., Wallis, J., Campbell, E. & Meeks, J. 1995, "Genetic-Evidence of a Major Role for Glucose-6-Phosphate-Dehydrogenase in Nitrogen-Fixation and Dark Growth of the Cyanobacterium Nostoc Sp Strain Atcc-29133", *Journal of Bacteriology,* vol. 177, no. 21, pp. 6184-6194.

Sveshnikov, D., Sveshnikova, N., Rao, K. & Hall, D. 1997, "Hydrogen metabolism of mutant forms of Anabaena variabilis in continuous cultures and under nutritional stress", *FEMS Microbiology Letters,* vol. 147, no. 2, pp. 297-301.

Swingley, W.D., Chen, M., Cheung, P.C., Conrad, A.L., Dejesa, L.C., Hao, J., Honchak, B.M., Karbach, L.E., Kurdoglu, A., Lahiri, S., Mastrian, S.D., Miyashita, H., Page, L., Ramakrishna, P., Satoh, S., Sattley, W.M., Shimada, Y., Taylor, H.L., Tomo, T., Tsuchiya, T., Wang, Z.T., Raymond, J., Mimuro, M., Blankenship, R.E. & Touchman, J.W. 2008, "Niche adaptation and genome expansion in the chlorophyll d-producing cyanobacterium Acaryochloris marina", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 105, no. 6, pp. 2005-2010.

Takahama, K., Matsuoka, M., Nagahama, K. & Ogawa, T. 2003, "Construction and analysis of a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the psbAI locus", *Journal of Bioscience and Bioengineering,* vol. 95, no. 3, pp. 302-305.

Tamagnini, P., Leitao, E., Oliveira, P., Ferreira, D., Pinto, F., Harris, D.J., Heidorn, T. & Lindblad, P. 2007, "Cyanobacterial hydrogenases: diversity, regulation and applications", *FEMS Microbiology Reviews,* vol. 31, no. 6, pp. 692-720.

Tan, X., Yao, L., Gao, Q., Wang, W., Qi, F. & Lu, X. 2011, "Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria", *Metabolic Engineering,* vol. 13, no. 2, pp. 169-176.

Tchernov, D., Silverman, J., Luz, B., Reinhold, L. & Kaplan, A. 2003, "Massive light-dependent cycling of inorganic carbon between oxygenic photosynthetic microorganisms and their surroundings", *Photosynthesis Research,* vol. 77, no. 2-3, pp. 95-103.

Thiel, T. 2005, "Nitrogen fixation in heterocyst-forming cyanobacteria" in *Genetics and regulation of nitrogen fixation in free-living bacteria.* Springer, pp. 73-110.

Thiel, T. 1993, "Characterization of Genes for an Alternative Nitrogenase in the Cyanobacterium Anabaena-Variabilis", *Journal of Bacteriology,* vol. 175, no. 19, pp. 6276-6286.

Thiel, T., Lyons, E. & Erker, J. 1997, "Characterization of genes for a second Mo-dependent nitrogenase in the cyanobacterium Anabaena variabilis", *Journal of Bacteriology,* vol. 179, no. 16, pp. 5222-5225.

Thiel, T., Lyons, E., Erker, J. & Ernst, A. 1995, "A 2nd Nitrogenase in Vegetative Cells of a Heterocyst-Forming Cyanobacterium", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 20, pp. 9358-9362.

Tripp, H.J., Bench, S.R., Turk, K.A., Foster, R.A., Desany, B.A., Niazi, F., Affourtit, J.P. & Zehr, J.P. 2010, "Metabolic streamlining in an open-ocean ni-

trogen-fixing cyanobacterium", *Nature,* vol. 464, no. 7285, pp. 90-94.

Troshina, O., Serebryakova, L., Sheremetieva, M. & Lindblad, P. 2002, "Production of H-2 by the unicellular cyanobacterium Gloeocapsa alpicola CALU 743 during fermentation", *International Journal of Hydrogen Energy,* vol. 27, no. 11-12, pp. 1283-1289.

Tsygankov, A.A. 2007, "Nitrogen-fixing cyanobacteria: A review", *Applied Biochemistry and Microbiology,* vol. 43, no. 3, pp. 250-259.

Tsygankov, A., Borodin, V., Rao, K. & Hall, D. 1999, "H-2 photoproduction by batch culture of Anabaena variabilis ATCC 29413 and its mutant PK84 in a photobioreactor", *Biotechnology and Bioengineering,* vol. 64, no. 6, pp. 709-715.

Tsygankov, A., Fedorov, A., Kosourov, S. & Rao, K. 2002, "Hydrogen production by cyanobacteria in an automated outdoor photobioreactor under aerobic conditions", *Biotechnology and Bioengineering,* vol. 80, no. 7, pp. 777-783.

Ungerer, J., Tao, L., Davis, M., Ghirardi, M., Maness, P. & Yu, J. 2012, "Sustained photosynthetic conversion of CO2 to ethylene in recombinant cyanobacterium Synechocystis 6803", *Energy & Environmental Science,* vol. 5, no. 10, pp. 8998-9006.

van der Zwaan, B., Keppo, I. & Johnsson, F. 2013, "How to decarbonize the transport sector?", *Energy Policy,* vol. 61, pp. 562-573.

Venter, J., Remington, K., Heidelberg, J., Halpern, A., Rusch, D., Eisen, J., Wu, D., Paulsen, I., Nelson, K., Nelson, W., Fouts, D., Levy, S., Knap, A., Lomas, M., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y. & Smith, H. 2004, "Environmental genome shotgun sequencing of the Sargasso Sea", *Science,* vol. 304, no. 5667, pp. 66-74.

Vignais, P.M. & Billoud, B. 2007, "Occurrence, classification, and biological function of hydrogenases: An overview", *Chemical Reviews,* vol. 107, no. 10, pp. 4206-4272.

Vignais, P., Billoud, B. & Meyer, J. 2001, "Classification and phylogeny of hydrogenases", *FEMS Microbiology Reviews,* vol. 25, no. 4, pp. 455-501.

Volbeda, A., Charon, M., Piras, C., Hatchikian, E., Frey, M. & Fontecilla-Camps, J. 1995, "Crystal-Structure of the Nickel-Iron Hydrogenase from Desulfovibrio-Gigas", *Nature,* vol. 373, no. 6515, pp. 580-587.

Vyas, D. & Kumar, H. 1995, "Nitrogen-Fixation and Hydrogen Uptake in 4 Cyanobacteria", *International Journal of Hydrogen Energy,* vol. 20, no. 2, pp. 163-168.

Walsby, A.E. 2007, "Cyanobacterial heterocysts: terminal pores proposed as sites of gas exchange", *Trends in Microbiology,* vol. 15, no. 8, pp. 340-349.

Wang, F., Wang, W., Wang, X., Wang, H., Tung, C. & Wu, L. 2011, "A Highly Efficient Photocatalytic System for Hydrogen Production by a Robust Hydrogenase Mimic in an Aqueous Solution", *Angewandte Chemie-International Edition,* vol. 50, no. 14, pp. 3193-3197.

Wang, H., Sivonen, K., Rouhiainen, L., Fewer, D.P., Lyra, C., Rantala-Ylinen, A., Vestola, J., Jokela, J., Rantasarkka, K., Li, Z. & Liu, B. 2012, "Genome-derived insights into the biology of the hepatotoxic bloom-forming cyanobacterium Anabaena sp strain 90", *Bmc Genomics,* vol. 13, pp. 613.

Weyman, P.D., Pratte, B. & Thiel, T. 2010, "Hydrogen production in nitrogenase mutants in Anabaena variabilis", *FEMS microbiology letters,* vol. 304, no. 1, pp. 55-61.

Weyman, P.D., Pratte, B. & Thiel, T. 2008, "Transcription of hupSL in Anabaena variabilis ATCC 29413 is regulated by NtcA and not by hydrogen", *Applied and Environmental Microbiology,* vol. 74, no. 7, pp. 2103-2110.

Weyman, P.D., Vargas, W.A., Tong, Y., Yu, J., Maness, P., Smith, H.O. & Xu, Q. 2011, "Heterologous Expression of Alteromonas macleodii and Thiocapsa roseopersicina [NiFe] Hydrogenases in Synechococcus elongatus", *Plos One,* vol. 6, no. 5, pp. e20126.

Whitton, B.A. & Potts, M. 2012, "Introduction to the cyanobacteria" in *Ecology of Cyanobacteria II.* Springer, pp. 1-13.

Wilson, S.T., Kolber, Z.S., Tozzi, S., Zehr, J.P. & Karl, D.M. 2012, "Nitrogen Fixation, Hydrogen Cycling, and Electron Transport Kinetics in Trichodesmium Erythraeum (Cyanobacteria) Strain Ims101", *Journal of Phycology,* vol. 48, no. 3, pp. 595-606.

Wilson, W.A., Roach, P.J., Montero, M., Baroja-Fernandez, E., Jose Munoz, F., Eydallin, G., Viale, A.M. & Pozueta-Romero, J. 2010, "Regulation of glycogen metabolism in yeast and bacteria", *FEMS Microbiology Reviews,* vol. 34, no. 6, pp. 952-985.

Wolk, C., Thomas, J., Shaffer, P., Austin, S. & Galonsky, A. 1976, "Pathway of Nitrogen-Metabolism After Fixation of N-13-Labeled Nitrogen Gas by Cyanobacterium, Anabaena-Cylindrica", *Journal of Biological Chemistry,* vol. 251, no. 16, pp. 5027-5034.

Wong, F. & Meeks, J. 2001, "The hetF gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium Nostoc punctiforme", *Journal of Bacteriology,* vol. 183, no. 8, pp. 2654-2661.

Wu, J., Zhao, F., Wang, S., Deng, G., Wang, J., Bai, J., Lu, J., Qu, J. & Bao, Q. 2007, "cTFbase: a database for comparative genomics of transcription factors in cyanobacteria", *Bmc Genomics,* vol. 8, pp. 104.

Yeager, C.M., Milliken, C.E., Bagwell, C.E., Staples, L., Berseth, P.A. & Sessions, H.T. 2011, "Evaluation of experimental conditions that influence hydrogen production among heterocystous Cyanobacteria", *International Journal of Hydrogen Energy,* vol. 36, no. 13, pp. 7487-7499.

Yoo, S., Keppel, C., Spalding, M. & Jane, J. 2007, "Effects of growth condition on the structure of glycogen produced in cyanobacterium Synechocystis sp PCC6803", *International Journal of Biological Macromolecules,* vol. 40, no. 5, pp. 498-504.

Yoon, H. & Golden, J. 2001, "PatS and products of nitrogen fixation control heterocyst pattern", *Journal of Bacteriology,* vol. 183, no. 8, pp. 2605-2613.

Yoon, H. & Golden, J. 1998, "Heterocyst pattern formation controlled by a diffusible peptide", *Science,* vol. 282, no. 5390, pp. 935-938.

Yoon, J., Shin, J., Kim, M., Sim, S. & Park, T. 2006, "Evaluation of conversion efficiency of light to hydrogen energy by Anabaena variabilis", *International Journal of Hydrogen Energy,* vol. 31, no. 6, pp. 721-727.

Yoshikawa, K., Kojima, Y., Nakajima, T., Furusawa, C., Hirasawa, T. & Shimizu, H. 2011, "Reconstruction and verification of a genome-scale metabolic model for Synechocystis sp PCC6803", *Applied Microbiology and Biotechnology,* vol. 92, no. 2, pp. 347-358.

Yoshino, F., Ikeda, H., Masukawa, H. & Sakurai, H. 2007, "High photobiological hydrogen production activity of a Nostoc sp PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity", *Marine Biotechnology,* vol. 9, no. 1, pp. 101-112.

You, L., Berla, B., He, L., Pakrasi, H.B. & Tang, Y.J. 2014, "13C-MFA delineates the photomixotrophic metabolism of Synechocystis sp. PCC 6803 under light-and carbon-sufficient conditions", *Biotechnology journal,* vol. 9, no. 5, pp. 684-692.

Zhang, X., Sherman, D.M. & Sherman, L.A. 2014, "The Uptake Hydrogenase in the Unicellular Diazotrophic Cyanobacterium Cyanothece sp Strain PCC 7822 Protects Nitrogenase from Oxygen Toxicity", *Journal of Bacteriology,* vol. 196, no. 4, pp. 840-849.