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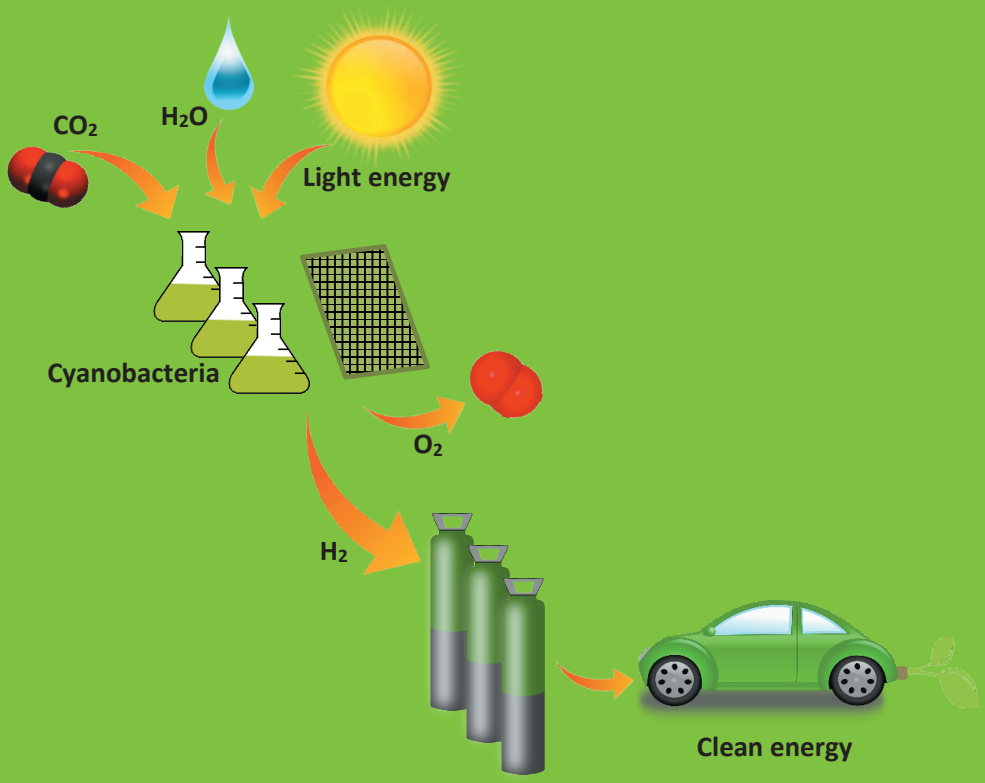


# ACCLIMATION RESPONSES OF HYDROGEN PRODUCING CYANOBACTERIA

Gayathri Murukesan

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*However difficult life may seem, there is always something you can do and succeed at.*
*It matters that you don't just give up.*

-***Stephen Hawking***

## LIST OF ORIGINAL PUBLICATIONS

This thesis is comprised of the following scientific articles:

**Paper I**. Kosourov.S, Leino.H, Murukesan.G, Lynch.F, Tsygankov.A, Aro.E, and Allahverdiyeva.Y (**2014**) Hydrogen Photoproduction by Immobilized $N_2$-fixing Cyanobacteria: Understanding the Role of Uptake Hydrogenase in the Long-term Process. *Applied and Environmental Microbiology* 80 (18): 5807-17.

**Paper II**. Kosourov.S, Murukesan.G, Jokela.J, and Allahverdiyeva.Y (**2016**) Carotenoid biosynthesis in *Calothrix* 336/3: composition of carotenoids on full medium, during diazotrophic growth and after long-term $H_2$ photoproduction. *Plant cell physiology* 57 (11): 2269-2282.

**Paper III**. Murukesan.G, Lynch.F, Allahverdiyeva.Y, and Kosourov.S (**2018**) Acclimation responses of immobilized $N_2$-fixing heterocystous cyanobacteria to long-term $H_2$ photoproduction conditions: Carbon allocation, oxidative stress and carotenoid production. *Journal of Applied Phycology.* DOI:10.1007/s10811-018-1535-x.

**Paper IV**. Murukesan.G, Leino.H, Mäenpää.P, Ståhle.K, Raksajit.W, Lehto.H, Allahverdiyeva.Y, and Lehto.K (**2015**) Pressurized Martian-like pure $CO_2$ atmosphere supports strong growth of cyanobacteria, and causes significant changes in their metabolism. *Origin of Life and Evolution of Biospheres* 46 (1): 119-31.



**Other publications from PhD project**

Kosourov.S, Murukesan.G, Seibert.M, and Allahverdiyeva.Y (**2017**) Evaluation of light energy to $H_2$ energy conversion efficiency in thin films of cyanobacteria and green alga under photoautotrophic conditions. *Algal research* 28: 253-263.

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## ABBREVIATIONS

| | |
|---|---|
| $^1O_2$ | singlet oxygen |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| BG11/BG11$_0$ | growth medium for cyanobacteria with/without combined N |
| BLSS | biological life support system |
| Chl *a* | chlorophyll *a* |
| CCM | carbon concentrating mechanism |
| C/N | carbon/nitrogen ratio |
| CM | cell membrane |
| Crt / *crt* | carotenoid biosynthesis enzyme / gene of the first group |
| Cru / *cru* | carotenoid biosynthesis enzyme / gene of the second group |
| Cyt *b6f* | cytochrome *b6f* complex |
| D1 | protein coding for PsbA gene |
| DTT | Dithiothreitol |
| EDTA | ethylene di-amine tetra acetic acid |
| EPS | extracellular polymeric substances |
| ETC | electron transport chain |
| Fd | ferredoxin |
| $F_v$ | variable fluorescence, ($F_m$ - $F_0$) |
| $F_v$' | variable fluorescence in light, (Fm'–Fo') |
| $F_m$ | the maximum fluorescence in the dark |
| $F_m$' | the maximum level of fluorescence under the light |
| GC | gas chromatography |

| | |
|---|---|
| $H_2$-ase | hydrogenase |
| HEP | heterocyst envelope polysaccharide |
| HGL | heterocyst-specific glycolipids |
| Hox / *hox* | hydrogen oxidation enzyme / gene |
| HPLC | high performance liquid chromatography |
| HRP | horseradish peroxidase |
| Hup / *hup* | hydrogen uptake enzyme / gene |
| IgY | Immunoglobulin Y |
| ISRU | in situ resource utilization |
| LC-MS | liquid chromatograph mass spectrometer |
| Mo-Fe | molybdenum-iron |
| MQ | ultrapure water of type 1 |
| $N_2$-ase | nitrogenase |
| $NADP^+$ | oxidized nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| Nif / *nif* | nitrogen fixation enzyme / gene |
| NtcA | Global nitrogen regulator |
| OCP | orange carotenoid protein |
| OM | outer membrane |
| PAR | photosynthetically active radiation |
| PBS | phycobilisomes |
| PCC | Pasteur culture collection |
| *pmf* | proton motive force |
| pH | negative logarithm of the proton concentration |

| | |
|---|---|
| PHA | polyhydroxyalkanoates |
| Pi | inorganic phosphate |
| PSI | photosystem 1 |
| PSII | photosystem 2 |
| PQ | plastoquinone |
| PTFE | polytetrafluoroethylene |
| PVDF | poly vinylidene fluoride |
| ROS | reactive oxygen species |
| Rubisco | ribulose bisphosphate carboxylase/oxygenase |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sp. | species |
| Tris-HCl | Tris hydrochloride |
| TW | Tera Watts |
| UV | ultra violet |
| WT | wild type |
| Y (II) | the yield of photosystem 2 |
| Z8/Z8x | growth medium for cyanobacteria with/without combined nitrogen |

# ABSTRACT

During recent years, cyanobacteria have been gaining popularity rapidly as a platform for $CO_2$ sequestration and production of a wide range of industrially attractive products. Factors such as simple nutritional requirements, flexibility to genetic manipulations and ability to adapt to fluctuating environmental conditions make these organisms suitable for bioindustrial processes that could be leveraged to address earthbound challenges like climate change and extraterrestrial ambitions like long-term manned missions to space. The main objective of this thesis was to improve our understanding of the growth and cellular acclimation of cyanobacteria in response to specific environmental conditions such as N deficiency leading to the improved $H_2$ photoproduction yield and simulated Martian atmosphere. The research activities extended from cyanobacterial cultivation for biomass accumulation, $CO_2$ sequestration and production of some valuable metabolites, such as carbohydrates and carotenoids to conversion of solar energy into energy of hydrogen biofuel by alginate-entrapped cultures.

Major part of my research was dedicated to optimization of a biohydrogen production platform using heterocystous cyanobacteria. I evaluated possible routes to prolong $H_2$ photoproduction in native (*Calothrix* 336/3) and model (*Anabaena* PCC 7120, Δ*hupL*) strains of cyanobacteria entrapped in $Ca^{2+}$-alginate films. Periodic supplementation of nitrogen through addition of air, or air + 6% $CO_2$ was shown to restore the photosynthetic activity of the entrapped cells and increased the $H_2$ production yields in *Calothrix* 336/3 and Δ*hupL* cells (excluding air + 6% $CO_2$). Despite obvious recovery of the photosynthetic activity, the $H_2$ photoproduction yields did not alter post air-treatments of the wild-type *Anabaena* PCC 7120, which could be linked to the presence of active uptake hydrogenase recycling $H_2$. In general, *Calothrix* showed a more stable photosynthetic apparatus and resilience to $H_2$ photoproducing conditions. Such robustness is most probably determined by an efficient reactive oxygen species scavenging network. Indeed, characterization of carotenogenesis pathway in *Calothrix* 336/3 showed high content of hydroxycarotenoids that are efficient antioxidants. Research revealed that alginate-entrapped cyanobacteria under $H_2$ photoproducing conditions tend to employ strain-specific strategies to counteract the C/N imbalance and oxidative stress, especially when exposed over extended periods. Further, my research proposed the prominent role of

the uptake hydrogenase enzyme in photoprotection of the filaments during stress conditions such as the long-term N-deprivation. Part of my research was focused on the growth and acclimation of unicellular and heterocystous filamentous cyanobacteria under a low-pressure atmosphere simulating Martian (< 1 atm, N-limitation, high $CO_2$) conditions. Here, the availability of $CO_2$ and $N_2$, and the presence or absence of $O_2$ showed an effect on the growth and heterocyst formation in cyanobacteria, in an interdependent manner. The tested strains were able to tolerate 100% $CO_2$ in atmospheric pressures as low as 100 mbars.

To summarize, my results show that the acclimation responses of $H_2$ producing cyanobacteria to various stress-inducing conditions are indeed strain specific. In depth understanding of such behavior is especially important to consider when designing a commercially inclined platform incorporating cyanobacteria.

# TIIVISTELMÄ

Nykyisin hiilidioksidin sitominen ilmakehästä sekä monien erilaisten kaupallisesti kiinnostavien yhdisteiden tuotto tapahtuu yhä useammin syanobakteereiden avulla. Syanobakteerien ravinnevaatimukset ovat yksinkertaiset, niitä on mahdollista muunnella geneettisesti ja ne kykenevät sopeutumaan vaihteleviin ympäristöolosuhteisiin. Nämä ominaisuudet tekevät tästä organismista sopivan erilaisiin bioteollisuuden sovelluksiin, jotka pyrkivät vastaamaan sellaisiin haasteisiin kuten ilmastonmuutos tai pitkäkestoiset miehitetyt avaruuslennot. Väitöstutkimukseni päätavoite oli lisätä ymmärrystämme syanobakteerien kasvusta ja sopeutumisesta tiettyihin ympäristöoloihin kuten typenpuutteeseen, jolloin monien syanobakteerien vedyntuotto valossa lisääntyy, sekä Marsin ilmakehää muistuttaviin oloihin. Kokeet käsittivät syanobakteerien kasvatusta biomassan tuotantoa, hiilidioksidin sitomista ilmakehästä ja erinäisten arvokkaiden lopputuotteiden kuten hiilihydraattien ja karotenoidien tuottoa varten sekä lisäksi vedyntuottoa aurinkoenergian avulla alginaatille kiinnitetyissä syanobakteereissa.

Merkittävä osa tutkimuksestani keskittyi heterokystejä muodostavien syanobakteerien vedyntuoton optimointiin. Arvioin erilaisia keinoja pidentää $Ca^{2+}$-alginaattifilmien sisällä kasvavien syanobakteerien vedyntuottoa käyttäen kotoperäistä syanobakteeria (*Calothrix* 336/3) sekä malliorganismia (*Anabaena* PCC 7120, Δ*hupL*). Jaksottainen typenlisäys, altistamalla kasvatus ilmalle tai ilmalle jossa on 6 % hiilidioksidia, palautti solujen fotosynteesiaktiivisuuden ja paransi vedyntuottoa *Calothrix* 336/3 sekä Δ*hupL* -kannoissa (lukuunottamatta käsittelyä ilma + 6 % $CO_2$). Huolimatta fotosynteesiaktiivisuuden palautumisesta *Anabaena* PCC 7120 -villityypin vedyntuotto ei lisääntynyt, mikä johtunee vetyä kuluttavan hydrogenaasin (engl. uptake hydrogenase) aktiivisuudesta. *Calothrix*-syanobakteerin yhteytyskoneisto kesti muita testattuja kantoja paremmin olosuhteita, joissa vedyntuotto lisääntyi, mikä todennäköisesti johtui tehokkaasta reaktiivisten happiyhdisteiden vaimentumisesta. *Calothrix* 336/3 -syanobakteerin karoteenisynteesin tutkiminen paljastikin suuren määrän hydroksikarotenoideja, jotka ovat tehokkaita antioksidantteja. Tutkimukseni paljasti että olosuhteissa, jotka johtavat tehokkaaseen vedyntuotantoon, alginaatissa kasvavat syanobakteerit käyttävät organismista riippuen erilaisia strategioita selvitäkseen epäoptimaalisesta C/N-suhteesta sekä hapettavasta stressistä, erityisesti kun olosuhteet jatkuvat pidemmän aikaa. Lisäksi

tutkimusteni perusteella vetyä kuluttavalla hydrogenaasilla on tärkeä merkitys filamenttien suojautumisessa valolta stressioloissa kuten pitkäaikaisessa typenpuutteessa. Osa tutkimuksestani keskittyi yksisoluisten tai heterokystejä ja filamentteja muodostavien syanobakteerien kasvuun ja sopeutumiseen olosuhteissa, jotka jäljittelivät Marsin ilmakehää (>yhden ilmakehän paine, vähäinen typen määrä, suuri hiilidioksidipitoisuus). Hiilidioksidin ja typpikaasun saatavuus sekä hapen saatavuus vaikuttivat syanobakteerien kasvuun ja heterokystien muodostumiseen. Testatut kannat pystyivät sietämään 100 % hiilidioksidia niinkin alhaisessa paineessa kuin 100 mbar.

Kaiken kaikkiaan tulokseni osoittavat, että vetyä tuottavien syanobakteerien sopeutuminen erilaisiin stressioloihin vaihtelee kannasta riippuen. Syvällinen ymmärrys syanobakteerien stressivasteista on erityisen tärkeää, kun suunnitellaan teolliseen tuotantoon tähtääviä syanobakteerisovelluksia.

# 1. BACKGROUND

## 1.1. Global energy challenge

Our current global economy is heavily dependent on fossil fuels like natural gas, petroleum and coal. These fuels are non-renewable and have a severe impact on the environment. The annual energy consumption of the world population has been at a steady exponential rise, especially over the past few decades, and this trend is extrapolated to continue with the simultaneous increase of the world population as projected in Stephens *et al*., 2010. Previous studies have shown that we are currently at the verge of depletion of the natural oil reserves and finding alternative fuel sources to combat fuel shortage has become a pressing concern. It is therefore important to steer our focus towards the global energy consumption, since energy is one of the main factors that influence the economic growth of all nations. On a global level, about 80% of the energy that we consume today originates from fossil fuels (International Energy Outlook, 2016). With the use of combustion fuels, there is an inevitable increase in Carbon dioxide ($CO_2$) gas emission (**Figure 1**). Gas emissions are notorious for their contribution to environmental changes, such as global warming and the greenhouse effect, which could pose a serious threat to life on Earth (Stern, 2006). $CO_2$ produced as a result of anthropogenic activities is released into the atmosphere at a rate of about 49 Gt $yr^{-1}$ (IPCC, 2014). Yet, natural processes can only remove about 12 Gt $CO_2$ $yr^{-1}$ (Bilanovic *et al*., 2009). Consequently, a significant amount of $CO_2$ is trapped within the Earth's atmosphere. Presently, the atmospheric concentration of $CO_2$ is approximately 390 parts per million (ppm), which is significantly higher than the pre-industrial (historic) level of 280 ppm (Dlugokencky, 2016).

Several efforts have been made worldwide to mitigate these environmental issues through the search for alternative forms of energy that could at least partially replace fossil fuels in the future. Today, the United States (940,000 barrels a day), Brazil (449,000 barrels a day) and Germany (68,000 barrels a day) are the world's largest producers of biofuels. Recently, Nordic countries have been climbing to the top three positions within Europe in terms of extensive implementation of biofuels in their public transport networks. In addition, according to the European Commission's reports and plans, 10% of the

transport fuel used in each EU country should be of renewable origin by 2020 (European commission renewable energy directive, 2015).

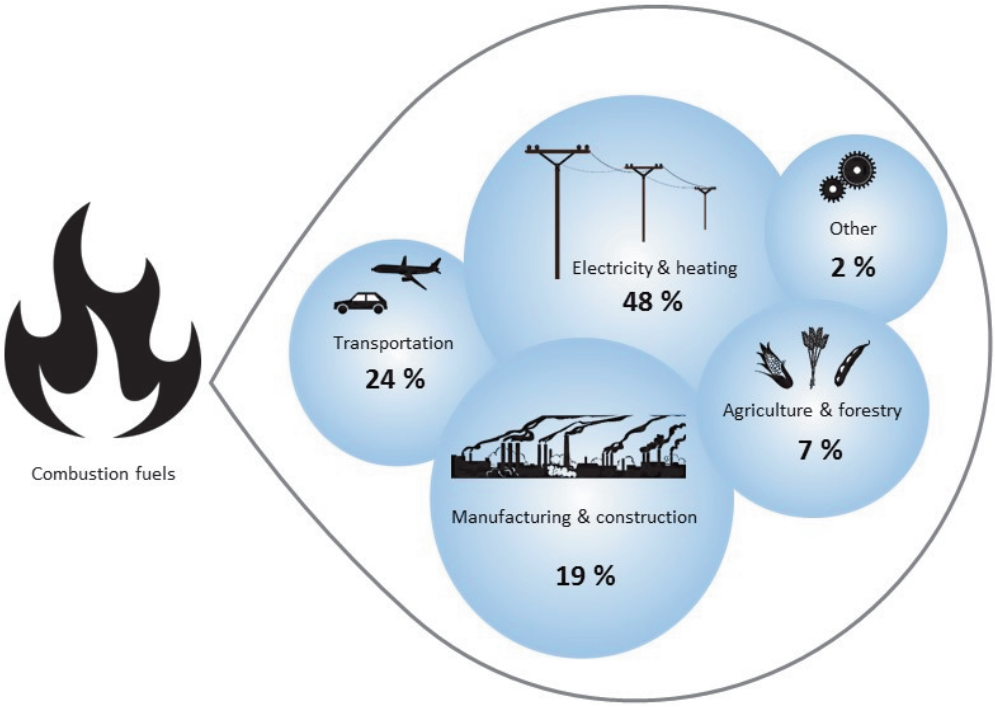


**Figure 1:** Infographics of global $CO_2$ emissions from fuel combustion by sector, 2015; data source: $CO_2$ Emissions from Fuel Combustion 2017 Highlights, International Energy Agency (IEA).

Bearing in mind all these goals different countries have set in order to tackle the huge global energy demand, the mitigation of global warming calls for sustainable and locally available energy sources with minimal carbon footprints. The Paris Agreement (2016) is a very recent example of a worldwide initiative with participation from 195 countries, including China and India, the two population giants that joined recently (2017). The initiative aims to mitigate global warming by setting out an action plan to limit temperature rise globally to under 2°C above the pre-industrial level (Paris agreement, 2016). Therefore, rediscovering and developing multiple energy sources that are storable, renewable, highly energy dense and environmentally friendly in the long run has become a global need for our future. Extensive scientific and technological research has revealed several alternative energy sources, including wind, geothermal, hydropower and solar, which in comparison to fossil fuels are

relatively clean and sustainable (Jafari *et al*., 2016). Importantly, sunlight is considered a very promising energy source in comparison to other renewable sources due to its relatively lesser location dependence on a global scale. Additionally, solar energy is by far the most abundant source of free energy that is available to us year-round. Earth receives approximately 120,000 terawatts (1 TW = $10^{12}$ W) of solar energy, which is about 8,000 times more than the current global energy consumption of 15 TW (Blankenship *et al.,* 2011; Hoffert *et al.,* 2002). This vast energy source could be harnessed and converted to forms that can be easily utilized by existing technologies. With the abundance of water available on our planet, sunlight can be harnessed to create high energy density molecules, such as hydrogen, through solar water splitting (Jafari *et al*., 2016).

### 1.2. Hydrogen as a sustainable fuel

Hydrogen gas is a valuable energy carrier. During combustion, $H_2$ produces heat energy and water. Therefore, $H_2$ is considered the cleanest and most carbon-neutral fuel (Momirlan and Veziroğlu, 1999). In addition, $H_2$ has other attractive features like high energy density (142 MJ/kg of compressed gas), low solubility in water and ease of harvest. $H_2$ gas can be utilized directly in fuel cells to produce electricity. The electricity can then be used to run an engine. Cars driven by fuel cell engines are already in the market, and this technology is expected to replace combustion engines in the future. The current global market for $H_2$ (over 53 million metric tons estimated in 2010) is primarily for commercial purposes, such as petroleum refineries, the manufacturing of ammonium-based fertilizers and petrochemicals (Singh and Rathore, 2016). Today, the majority of industrially used $H_2$ is generated through thermochemical steam reforming of methane at high temperatures (700–1000 $^{o}$C). Methane reforming is a cost-effective process, costing approximately $3 per kg $H_2$ (Bartels *et al*., 2010). On the other hand, methane reforming contributes significantly to $CO_2$ emissions and is not an energy efficient process (Christopher and Dimitrios, 2012). The market value of global $H_2$ production is estimated to be $82.6 billion, with an annual growth rate of 5.6%. Currently, $H_2$ is neither renewable nor carbon-neutral, because the industrial-scale manufacturing of $H_2$ leaves behind large greenhouse-gas footprint due to its dependence on fossil fuels (Lee *et al*., 2010). Therefore, several ecologically sustainable methods have been tested with the aim of producing $H_2$ with a smaller carbon footprint. Listed below are the most researched and applied approaches:

(i) Electrolysis
(ii) Fermentation
(iii) Biological water splitting

Among these, biological water splitting through photosynthesis would be one of the most sustainable options for capturing and converting solar energy into chemical energy (Stephens *et al*., 2010b). Biological water splitting using photosynthetic organisms for the photoproduction of $H_2$ has been known since the early 40's and has been studied extensively for the past two decades due to its sustainable and eco-friendly nature (Homann, 2003; Prince and Kheshqi, 2005; Ghirardi *et al*., 2009; Khanna and Lindblad, 2015; Sakurai *et al*., 2015). The primary reason for its rising popularity is that photo-biological production technologies have the potential to enable the economical production of $H_2$ directly from sunlight with low to nearly net-zero carbon emissions.

### 1.2.1. Photosynthesis

Photosynthesis refers to processes by which phototrophic organisms convert solar energy to chemical energy, forming organic molecules. These processes are broadly classified into the oxygenic and anoxygenic categories. In oxygenic photosynthesis, which is common for cyanobacteria, algae and plants, water acts as the initial electron donor; molecular oxygen is evolved as a result of oxidation of water (Eaton-Rye *et al*., 2012). Photosynthetic light reactions occur in protein complexes embedded into the thylakoid membrane, namely photosystem II (PS II) and photosystem I (PS I). PSII is a massive pigment-protein complex composed of more than 20 polypeptides and more than 90 different cofactors (Suga *et al*., 2015). P680, also known as a primary electron donor of PSII, consist of two chlorophyll dimers, which absorb photons of light to energize electrons and initiate charge separation. PSII drives two chemical reactions, namely (i) the oxidation of water and (ii) the reduction of plastoquinone (PQ). Electrons reaching the PQ pool are transported first to the cytochrome $b_6f$ complex, followed by transfer to a soluble electron carrier (plastocyanin and cytochrome $c_{553}$). This soluble one-electron carrier then reduces the oxidized P700. This oxidized state of the P700 is a result of the light-activated relocation of an electron from PS I to ferredoxin (Fd) and later to $NADP^+$ (Govindjee *et al.,* 2017). The proton motive force (*pmf*) generated across the thylakoid membrane during photosynthesis is the driving force for ATP production.

Oxygenic photosynthesis may be summarized as follows:

$6\ CO_2 + 6\ H_2O + h\nu \rightarrow C_6H_{12}O_6 + 6\ O_2$

Several groups of the domain Bacteria capture light energy through anoxygenic photosynthesis, with the exception of cyanobacteria: purple bacteria, green sulfur bacteria (GSB), heliobacteria, red and green filamentous phototrophs, and acidobacteria (Hanada, 2003; 2016). Instead of water, they use organic compounds such as succinate, malate or inorganic compounds such as hydrogen sulfide ($H_2S$), $H_2$ as electron donors (Hunter *et al*., 2009; Blankenship *et al*., 1995). As a result, photosynthesis by these bacteria does not evolve $O_2$ (Hanada, 2016). In addition, anoxygenic phototrophic bacteria have bacteriochlorophyll(s) instead of chlorophyll and contain only one reaction center i.e. either type I or type II. The various anoxygenic photosynthesis reactions can be represented in the form of a generalized formula as given below:

$CO_2 + 2\ H_2A + h\nu \rightarrow [CH_2O] + 2A + H_2O$

The letter 'A' is a variable, '*hv*' is light energy and '$H_2A$' represents the electron donor (Whitmarsh and Govindjee, 1999).

### 1.3. Cyanobacteria: significance and features

Cyanobacteria are a versatile group of ancient, Gram-negative, ecologically important and ubiquitously found photosynthetic prokaryotes (Rippka *et al*., 1979). Cyanobacteria are unique in nature among prokaryotes due to their complex photosynthetic pigment system and their capacity to perform oxygenic photosynthesis (Stanier and Cohen-Bazire, 1977; Mimuro *et al.,* 2008). They are common in aquatic environments and play an important role in the nitrogen, oxygen and carbon cycles of our planet. These microbes are also major contributors to the primary biomass production in the oceans. Cyanobacteria are morphologically and physiologically diverse organisms. They are widely distributed in terrestrial and aquatic environments but also in extreme habitats such as hot springs, volcanoes, hypersaline locations, sub-zero terrains with high UV radiation and arid deserts (Steunou *et al*., 2006; Cockell and Stokes, 2004; Fogg *et al*., 1973) (**Figure 2**). In nature, these organisms are often found to live in symbiotic association with other microbes, forming microbial mats and benthic communities (Kulasooriya, 2011). Fossilized evidence from rocks shows

that cyanobacteria are approximately 2.5 to 3.5 billion years old (Lopez-Garcia *et al*., 2006; Schirrmeister *et al*., 2015). Morphological forms of cyanobacteria are found in stromatolites dating back as far as 0.5 to 3.5 billion years, making them the oldest known oxygenic phototrophs to have thrived on Earth (Allwood *et al.,* 2006; Bosak *et al.,* 2009; Rishworth *et al*., 2016). Geological evidence indicates that around 3.5 billion years ago, free molecular oxygen began to gather in the atmosphere (Bekker *et al*., 2004). Hence, cyanobacterial photosynthesis could be a link to the early evolution of Earth's oxygen-rich atmosphere.

**Figure 2:** Diverse habitats of cyanobacteria.

(Source: Free images labeled for reuse, Pixabay)

Along with their adaptability to a wide range of habitats, cyanobacteria also have a variety of cellular organization options that enable them to survive in a dynamic ecosystem. They can range from unicellular spherical and cylindrical morphologies such as *Synechocystis* and *Synechococcus* to multicellular (filamentous, filamentous branched, colonial) forms such as *Arthrospira, Nostoc* and *Stigonema* (Castenholz, 2001; Knoll 2008). Morphological groups include filamentous non-heterocystous, heterocystous genera and coccoid forms. In some multicellular cyanobacterial strains, there are four different cell types: vegetative cells, heterocysts, spore-like akinetes and motile hormogonia, as summarized in Table 1 (Meeks and Elhai, 2002; Flores and Herrero, 2010). Under favorable growth conditions, cyanobacteria mainly form vegetative cells, which

are responsible for carbon fixation during light exposure. The vegetative cells differentiate into akinetes when the organism is exposed to extreme unfavorable conditions, such as drought or exposure to high levels of UV radiation. Vegetative cells differentiate into hormogonia as a method for dispersal of the strain, and structurally they differ from the mature trichomes. They are smaller in cell size, motile and heterocysts are absent (Stal, 2015). Cyanobacteria have special environmental adaptations for survival under various nutrient deprived conditions. When combined-N is absent from the growth substrate, a small percentage (depending on the species) of the vegetative cells differentiate into heterocysts. In contrast to heterocysts, akinetes and hormogonia are able to convert back to vegetative cells under favorable conditions and restart cell division (Kaplan-Levy *et al*., 2010). Heterocysts and vegetative cells are interdependent for their survival. In low $CO_2$ concentrations, the conserved carbon concentrating mechanism (CCM) in cyanobacterial vegetative cells gets enhanced. CCM actively transports and gathers $HCO_3^-$ and $CO_2$ (inorganic carbon) inside the cell. This creates a spike in the $CO_2$ concentration pool in carboxysomes around the $CO_2$-fixing enzyme, Rubisco (ribulose bisphosphate carboxylase-oxygenase). The enzyme, Rubisco, mediates the conversion of $CO_2$ to sugars in all oxygenic photosynthetic organisms, including cyanobacteria (Badger *et al*., 1998). Both CCM and Rubisco are absent in the heterocysts, and therefore the carbon compounds are provided by the neighboring vegetative cells (Lopez-Igual *et al*., 2010).

**Table 1:** Summary of different cell types, their morphological traits and known functions in filamentous cyanobacteria.

| Cell Types | Morphological Traits | Known Functions |
|---|---|---|
| Vegetative cells | small, circular photoautotrophic cells, typically blue-green in colour | oxygenic photosynthesis |
| Heterocysts | larger and rounder shape, diminished pigmentation, thicker cell envelopes, and contain cyanophycin granules | nitrogen fixation |
| Akinetes | enveloped, thick-walled, non-motile, dormant cells, larger than the vegetative cells | survival under unfavorable conditions, such as cold and desiccation |
| Hormogonia | motile filaments containing cells that are smaller than the vegetative cells, tapered at the ends | serves as dispersal agent |

### 1.3.1. $N_2$-fixing cyanobacteria

Nitrogen is a constituent of several bio-molecules that are essential to life, such as amino acids, nucleic acids and ATP, making it an element that is essential for the sustenance of life on Earth (Rees *et al*., 2005). Although there is an enormous reservoir of dinitrogen in the atmosphere, this abundant resource is not directly reactive due to the triple bond between nitrogen atoms (Stal, 2015). Therefore, most organisms are unable to directly capture this element (Burgess and Lowe, 1996). Synthetically, $N_2$ is captured by chemically reducing it to $NH_3$ with the application of very high temperature and pressure. Industrially, this process is termed the Haber–Bosch process, and currently it is the most widely used method for artificial $N_2$ fixation in fertilizer manufacturing (Ritter, 2008). However, this process is expensive, energy intensive, causes imbalance to the natural $N_2$ cycle and contributes heavily to pollution; therefore it is an unsustainable method. This creates a constant need to develop alternative sustainable methods for fixing $N_2$. Biological nitrogen fixation (BNF) is a potential approach for sustainable $N_2$-fixation. There are a minor group of prokaryotic microbes in nature that have the ability to fix atmospheric $N_2$ to $NH_3$. These organisms play an essential role in maintaining the stability the level of $N_2$ in the Earth's biosphere (Howard and Rees, 1996). In general, atmospheric $N_2$-fixation is a key step for maintaining the global nitrogen cycle in balance (Hue and Ribbe, 2016).

$N_2$-fixing cyanobacteria are among the most ubiquitous and major $N_2$ fixers on Earth, making them important for the global nitrogen cycle (Rodrigo and Novelo, 2007; Peter *et al*., 2002). In cyanobacteria, biological $N_2$-fixation is catalyzed by the $O_2$ sensitive nitrogenase enzyme. Ammonia and molecular hydrogen are released as products (Bothe *et al*., 2010). This is a highly energy-intensive reaction that uses at least 16 ATP to fix a single molecule of atmospheric $N_2$. The ability to fix atmospheric $N_2$ is found in heterocyst-forming filamentous cyanobacteria (e.g. *Anabaena*, *Calothrix*, *Nodularia*, etc.), some non-heterocyst-forming filamentous cyanobacteria (e.g. *Trichodesmium*, *Oscillatoria*, *Symploca*) and some unicellular strains of cyanobacteria (e.g. *Gloeothece*, *Cyanothece* (Bergman *et al*., 1997).

**Unicellular and non-heterocystous $N_2$-fixing cyanobacteria.** Non-heterocystous filamentous cyanobacteria use temporal separation strategy for fixation of $N_2$ and photosynthetic evolution of $O_2$ (Berman-Frank *et al*., 2001).

When combined N is absent in the growth substrate under diurnal cycle of light-dark, these cyanobacteria fix $N_2$ in the dark period of the cycle (Stal and Krumbein, 1987; Waterbury *et al*., 1988). However, there are exceptions such as the filamentous non-heterocystous cyanobacteria *Trichodesmium* and unicellular cyanobacteria UCYN-A (Montoya *et al*., 2004). These two cyanobacteria are the primary biological $N_2$ fixers of the oceans. *Trichodesmium* uses a combination of the temporal and spatial strategies for $N_2$ fixation. It fixes $N_2$ under aerobic conditions in the light (Capone and Carpenter, 1982; Zehr *et al*., 1999). In a study published by Fredriksson and Bergmann in 1997, it was revealed that in *Trichodesmium*, a small group of cells in the trichome contained nitrogenase and these cells were termed "diazocytes". This study also speculates that diazocytes are temporary $N_2$ fixing cells, unlike heterocysts, which are irreversibly differentiated. In *Trichodesmium*, $N_2$ fixation begins at the end of the night cycle and ends towards the middle of the light cycle. The distribution of nitrogenase within the trichomes also varied depending on the diurnal changes (Bergman and Carpenter, 1991). In addition, the $N_2$ fixing cells are maintained in a state of anoxia by high respiration rates. UCYN-A (*Candidatus Atelocyanobacterium thalassa*), which is a symbiotic cyanobacterium in association with a haptophytic picoplankton alga, fixes $N_2$ during the day (Zehr *et al*., 2016; Montoya *et al*., 2004). Cells of this cyanobacterium are incapable of oxygenic photosynthesis due to lack of PS II (although the PS I apparatus is intact). In-depth genome studies also showed that this organism lacks the enzymes required by the Calvin cycle, the biosynthesis of some amino acids and the TCA cycle (Tripp *et al*., 2010). Therefore, UCYN-A is categorized as a photo-heterotrophic symbiont (Martinez-Perez *et al*., 2016). Cyanobacteria belonging to the genus *Cyanothece* are capable of aerobic $N_2$-fixation (Bandyopadhyay *et al*., 2011). Photoautotrophically grown *Cyanothece* 51142 under a light-dark diurnal cycle exhibit high rates (152 µmol $H_2$ mg $Chl^{-1}$ $h^{-1}$) of nitrogenase-mediated $H_2$ production under aerobic incubation conditions in continuous light, which is an unusual trait for a wild-type $N_2$-fixing cyanobacteria (Bandyopadhyay *et al*., 2010). Here, *Cyanothece* 51142 develops an intracellular environment that is suitable for the function of the nitrogenase enzyme. Under anaerobic incubation, the rate of $H_2$ production was further enhanced (373 µmol $H_2$ mg $Chl^{-1}$ $h^{-1}$) in this strain (Bandyopadhyay *et al*., 2010). This unicellular cyanobacterial genus plays important roles in in both the terrestrial and aquatic nitrogen cycles. Interestingly, UCYN-A's genome is closely related to those of

Cyanothece species (Zehr *et al*., 2008). It has been hypothesized by Kneip and colleagues (2008) that these organisms may have evolved from a Cyanothece-like ancestor as a result of targeted (photosynthesis related) gene loss while maintaining an elaborate gene cluster responsible for $N_2$-fixation.

**Heterocystous cyanobacteria**. These cyanobacteria use the spatial separation of photosynthetic $O_2$ evolution as a strategy for $N_2$ fixation. When combined N is absent, about 5–15% vegetative cells differentiate into heterocysts (**Figure 3**). HetR and NtcA genes are the major players in regulating this cellular differentiation (Huang *et al*., 2004; Herrero *et al*., 2013). The former is responsible for controlling the heterocyst differentiation and the later is a global regulator for nitrogen assimilation and metabolism. In some cyanobacteria, the expression of these two genes may be activated in a mutually dependent style during the heterocyst differentiation phase (Muro-Pastor *et al*., 2002). NtcA commences the transcription of the HetR gene, which in response enhances the transcription of NtcA (Frias *et al*., 1994).

Typically, heterocysts can be easily distinguished from their vegetative counterparts due to their larger size, lighter pigmentation, presence of cyanophycin granules at the poles and thicker cell envelope (Kumar *et al*., 2010). The thick cell envelope of the heterocysts contains long chain glycolipids and acts as a barrier for gas exchange (Awai and Wolk, 2007). In addition, the heterocysts also have terminal pores or septa that connect the heterocysts with vegetative cells (Giddings and Staehelin, 1978). The pores in the septa connecting heterocysts and vegetative cells are much smaller than the pores between two vegetative cells, and it is speculated that these pores might be the main diffusion pathway for $O_2$ and $N_2$. Other morphological changes taking place in the proheterocysts during the maturation stage are the disappearance of carboxysomes and the addition of two layers of envelope around the heterocyst, which could be categorized as the inner laminated layer and the outer polysaccharide layer (Nicolaisen *et al*., 2009). The inner laminated layer consists of heterocyst-specific glycolipids (HGL) and the outer polysaccharide layer consists of heterocyst envelope polysaccharide (HEP). A continuous periplasm is contained between the cytoplasmic membrane (CM) and the outer membrane (OM) that keeps the heterocysts and vegetative cells connected (Flores and Herrero, 2010). The heterocysts then appear at non-random intervals along the filaments containing strings of vegetative cells (Maldener and Muro-Pastor, 2010). The frequency and location of the heterocysts are

often species specific. For example, a native strain (isolated from the Baltic region) *Calothrix* sp. 336/3 has terminal heterocysts at the basal end of the filaments whereas in the model strain *Anabaena* sp. PCC 7120 the heterocysts are both intercalary and terminal (Leino *et al*., 2014). Heterocyst frequency is also dependent on the light intensity.

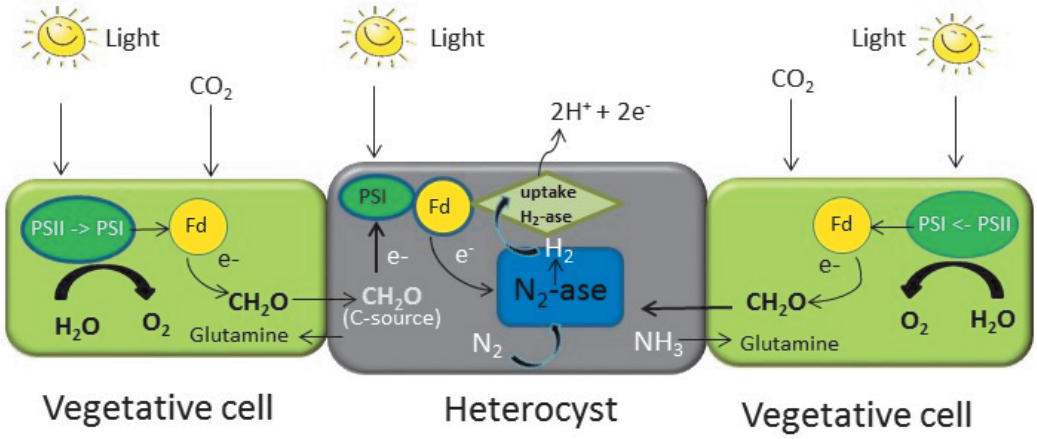


**Figure 3:** Schematic diagram of hydrogen metabolism in heterocystous filamentous cyanobacteria.

Functionally, prospective heterocysts have enhanced respiration rates (Wolk *et al*., 1994). It has been accepted that the different respiratory terminal oxidases and flavodiiron 3B protein (Flv3B) consume the residual $O_2$, creating a micro-oxic environment favorable for the activity of the $O_2$ sensitive enzyme nitrogenase (Valladares *et al*., 2007; Ermakova *et al*., 2014; Maldener and Muro-Pastor, 2010). Mature heterocysts are not capable of fixing atmospheric carbon, therefore fixed carbon is provided by the adjoining vegetative cells to the heterocysts in the form of sucrose (Wolk, 1968; Currati *et al*., 2002). In response, mature heterocysts provide the vegetative cells with fixed $N_2$ in the form of amino acids (Wolk *et al*., 1974). Therefore, it is clear that there is an intercellular exchange of metabolites taking place between the heterocysts and the vegetative cells. Previously, it has been suggested that some metabolite and signal molecule exchange could take place through the periplasm, because the cells are connected by a continuous periplasm located between the outer membrane and cytoplasmic membrane (Flores *et al*., 2006). The heterocysts possess a specific ferredoxin (Fd) and the energy (ATP) required for $N_2$-fixation

is generated mostly by the PS I-dependent cyclic photophosphorylation (Bothe *et al*., 2010).

### 1.4. Cyanobacterial $H_2$ photoproduction

Photobiological $H_2$ production was first reported in green alga (*Scenedesmus obliquus*) by Hans Gaffron and Jack Rubin in 1942. Evolution of $H_2$ by $N_2$-fixing cyanobacterium *Anabaena cylindrica* under an Argon (Ar) atmosphere was first reported in the work performed by Benemann and Weare in 1974. Since this early work, a total of 14 Cyanobacteria genera have been documented for $H_2$ production ability under a wide range of cultivation regimes (Lopes *et al*., 2002). These genera include the following cyanobacteria: *Oscillatoria*, *Anabaena*, *Cyanothece*, *Calothrix*, *Nostoc*, *Synechococcus*, *Mycrocystis*, *Anabaenopsis*, *Gloebacter*, *Aphanocapsa*, *Gleocapsa*, *Synechocystis*, *Chroococcidiopsis* and *Microcoelus* (Quintana *et al*., 2011).

Under certain conditions, some cyanobacteria can use water-splitting photosynthetic processes to produce molecular $H_2$. They utilize light as an energy source to split water into $H_2$. Biophotolysis is of two types: direct biophotolysis and indirect biophotolysis (Benemann, 1997). In direct biophotolysis, $H_2$ production is a single stage process where $H_2$ is derived directly from water and light energy using the hydrogenase enzyme (Rahman *et al*., 2015). This process does, however, have a drawback of simultaneous production of $O_2$. Indirect biophotolysis addresses the problem of $O_2$ inhibition of $H_2$ production, here $H_2$ production may occur in single-stage or two stages. Heterocystous cyanobacteria are an example for single-stage indirect biophotolysis. Here, $CO_2$ is fixed to make sugars through photosynthesis in the vegetative cells and the $O_2$ evolved as a result is separated spatially from the highly $O_2$-sensitive enzymes responsible nitrogen fixation, through specialized heterocyst cells. During nitrogen fixation process, $H_2$ is released as a by-product. In the two-stage indirect biophotolysis, first-stage is $CO_2$ fixation to make sugars through photosynthesis and the concomitant evolution of $O_2$ is separated temporally from the highly $O_2$-sensitive enzymes. In the second-stage, the stored sugars undergo fermentative degradation and subsequently releasing $H_2$ in dark anaerobic conditions (Hallenbeck, 2012; Kaushik and Sharma, 2017). Cyanobacteria use two sets of enzymes to produce $H_2$, nitrogenase and hydrogenase (**Figure 4**).

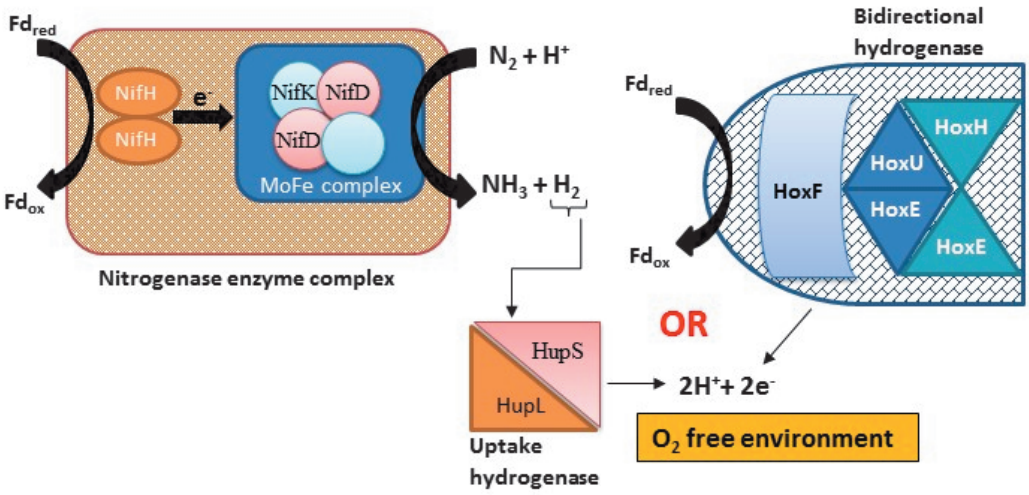


**Figure 4:** Schematics of role of nitrogenase and hydrogenase in $N_2$-fixing heterocystous filamentous cyanobacteria. (Source: adapted from Tiwari and Pandey, 2012)

### 1.4.1. Enzymes involved

***Nitrogenases***: Nitrogenases are present in all the $N_2$-fixing cyanobacteria and are known to be absent in all $H_2$ producing eukaryotes. This enzyme evolves $H_2$ under two different conditions. Under the first condition, nitrogenase catalyzes ammonia synthesis with the evolution of $H_2$ as a byproduct, according to the reaction scheme given below:

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

This is a reaction with high energy consumption, using 16 ATP per fixed nitrogen molecule. Therefore, the maximum theoretical energy conversion efficiency from light to $H_2$ driven by nitrogenase is about 6–7% (Sakurai *et al*., 2015). The ammonia synthesized as a result of $N_2$-fixation is transformed into proteins and nucleic acids (Meeks and Elhai, 2002). Under the second condition, when $N_2$ or any other compatible substrate is absent, nitrogenase catalyzes the reduction of protons by allocating all electrons to $H_2$ production (Hinnemann and Norskov, 2006). The reaction is as follows:

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

This reaction is more energy efficient, consuming only 4 ATP and requiring only two photosynthetic electrons per $H_2$ molecule evolved. $H_2$ formation by

nitrogenases is also a unidirectional and irreversible process (Yoshino *et al*., 2007). Other possible substrates other than $N_2$ and protons include acetylene ($C_2H_2$), cyanide (CN) and sulfur-containing molecules (Hinnemann and Norskov, 2006). The enzyme complex consists of two components. One component is a Mo-Fe protein, called dinitrogenase, and the other is a Fe-containing protein, called dinitrogenase reductase (Fay 1992; Rubio and Ludden 2008; Seefeldt *et al*., 2009). The structural gene *nifHDK1* encodes the conventional Mo-nitrogenase (Bothe *et al*., 2010). In addition, there is an alternative Mo-Fe nitrogenase encoded by the gene cluster *nifHDK2*. It is expressed in both heterocysts and vegetative cells of *Anabaena variabilis* under anaerobic conditions (Bothe *et al*., 2010). The most widely distributed and best-studied group consists of molybdenum-iron (Mo-Fe) nitrogenases, while the other forms may contain Vanadium (V) and Ferrum (Fe) in place of Mo. In some cyanobacteria like *Anabaena variabilis* under conditions of N and Mo depletion, an alternative nitrogenase system kick starts (Masukawa *et al*. 2009; Thiel and Pratte, 2013).

***Hydrogenases***: Some microbes are capable of metabolizing molecular $H_2$ and the enzyme hydrogenases have a very important role in this function. Hydrogenases are distributed in many micro-organisms such as algae, trichomonads and anaerobic ciliates (Tamagnini *et al*., 2002). Hydrogenases can be branched into three distinct classes depending on the metal composition of the active site: [Fe-Fe]-hydrogenases, [Ni-Fe]-hydrogenases and [Fe]-hydrogenases (Tamagnini *et al*., 2007). All cyanobacterial hydrogenases belong to the class of [Ni-Fe]-hydrogenases (Tamagnini *et al*., 2002). The [Fe-Fe]-hydrogenases are present in prokaryotes and some algal species (Vignais and Billoud, 2007). This enzyme requires anaerobic induction for its expression. Irreversible inhibition of this enzyme by $O_2$ is a major limiting factor and affects sustainable and efficient $H_2$ production in algae (Ghirardi *et al*., 2007). Compared to [Fe-Fe]-hydrogenases, the [Ni-Fe]-hydrogenases are constitutively expressed in cyanobacteria and, in spite of their $O_2$ sensitivity, can be re-activated upon the removal of $O_2$. These hydrogenases catalyze a simple reversible reductive reaction leading to the formation of molecular hydrogen from protons and electrons:

$2H^+ + 2e^- <-> H_2$

These enzymes can be further classified into two functionally different types: uptake hydrogenase and bidirectional hydrogenase (Tamagnini *et al*., 2002; 2007). These two hydrogenase enzymes can be distinguished by their physiological roles as either an uptake or a bidirectional (reversible) enzyme. Uptake hydrogenase is encoded by *hupSL*. It consists of two subunits: a large subunit (HupL) of 60 kDa and a smaller subunit (HupS) of 35 kDa. In cyanobacteria, the uptake hydrogenase is found to be present in all $N_2$-fixing species except *Synechococcus* BG04351 and some *Chroococcidiopsis* isolates (Ludwig *et al*., 2006). This enzyme catalytically aids the utilization of $H_2$ produced by the nitrogenase enzyme. The HupL subunit harbors the active site and uptakes $H_2$, whilst the smaller subunit HupS mediates electron transport to and from the active site to the redox partners (Tiwari and Pandey, 2012; Vignais and Billoud, 2007). It has been suggested as a cellular mechanism for recycling electrons that were lost during nitrogenase-mediated $H_2$ evolution (Tamagnini *et al*., 2002). This recycling strategy is hypothesized to provide several benefits to the organism such as the provision of ATP via oxyhydrogen reactions, the removal of $O_2$ from nitrogenase and the supply of electrons to aid several other cellular functions (Tamagnini *et al*., 2007). Previous studies have revealed that this enzyme is activated by the photosynthetically reduced thioredoxin and is thus tightly connected to the electron transport chain (ETC) (Papen *et al*., 1986). A recent study has also shown that the uptake hydrogenase in the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133 can be modified with a single point mutation to convert the cyanobacterium into a sustained hydrogen producer under $N_2$-fixing conditions in light (Raleiras *et al*., 2015).

In the case of bidirectional hydrogenase, it can both evolve and consume $H_2$ as demonstrated in **Figure 4** (Houchins, 1984; Carrieri *et al*., 2011). It is generally accepted that NAD(P)H acts as the electron donor for this enzyme, however recent research shows that under *in vitro* conditions flavodoxin and ferredoxin reduce the bidirectional hydrogenase (Gutekunst *et al*., 2014). Bidirectional hydrogenase is distributed widely in unicellular, filamentous non-heterocystous and heterocystous strains of cyanobacteria (Tamagnini *et al*., 2000; 2002; Zhang *et al*., 2005; Troshina *et al*., 2002). Yet a recent study showed that, at least in $N_2$-fixing strains, this enzyme is not universal unlike the uptake hydrogenase (Tamagnini *et al*., 2000). Bidirectional hydrogenases present in cyanobacteria are classified by their sensitivity to $O_2$, tolerance to high temperature, and high affinity to $H_2$ (Houchins, 1984; Eisbrenner, 1981). The bidirectional hydrogenase

enzyme is considered to be a heteropentameric enzyme encoded by *hoxEFUYH* (Schmitz *et al*., 2002). It comprises of a hydrogenase moiety (HoxYH) and a diaphorase moiety (HoxFUE). The locality of this enzyme is also quite diverse. It can be found in both the heterocysts and the vegetative cells (Tamagnini *et al*., 2007). Previous studies have suggested that bidirectional hydrogenase is membrane-associated (Houchins and Burris, 1981; Kentemich *et al*., 1991). Bidirectional hydrogenase is expressed in both aerobic and anaerobic conditions, however it is active only during dark anoxic conditions or when a transition from dark anoxic conditions to light takes place (Carrieri *et al*., 2011). It is also understood that the hydrogenase additionally helps in balancing redox potential under fermentative conditions in nature, for instance in microbial mats during the night cycle, when the cells consume photosynthates that were accumulated during the day cycle (Stal and Moezelaar, 1997). In *Synechocystis* sp. PCC 6803, bidirectional hydrogenase has been proposed to work as an electron valve during light to dark transition phases (Appel *et al*., 2000). To elaborate, when excess electrons are formed during photosynthesis, the Hox hydrogenase uses these electrons to produce $H_2$ as an alternative electron sink. In the paper by Schmitz *et al*., 1995, it was suggested that the Hox hydrogenase may function in the oxidation of $H_2$ at the periplasmic side. The studies that followed hypothesize that under specific stress conditions, Hox hydrogenase may exhibit functional relationship to the photosynthetic and respiratory electron transport pathways in *Synechocystis* sp. PCC 6803 (De Rosa *et al*., 2015). This enzyme is not a universal in cyanobacteria (absent in marine strains), it is unlikely to be responsible for a central role. In conclusion, the exact physiological role of the bidirectional hydrogenases is still vaguely understood and debated.

Several filamentous $N_2$-fixing cyanobacteria harbour either bidirectional or uptake hydrogenase and, in some cases, even both enzymes. Bidirectional hydrogenase is present in *Anabaena* sp. PCC 7120 and absent in *Nostoc punctiforme* (model organism) and *Calothrix* sp. 336/3 (Leino *et al*., 2014).

### 1.4.2. Challenges for $H_2$ photoproduction in filamentous cyanobacteria

Many factors may challenge photoproduction of $H_2$ by heterocystous $N_2$-fixing cyanobacteria, such as: (a) interference by competing electron transport pathways, (b) active consumption of $H_2$ by uptake hydrogenase, (c) low light utilization efficiency, (d) low cell fitness due to nutrient deprivation,

photoinhibition and oxidative damage, etc. (Pinto *et al*., 2002). Engineering nitrogenase enzymes to overcome competition with $N_2$, using mutants lacking uptake hydrogenase activity, identifying and eliminating competing electron transport pathways and improving light energy utilization by employing superior immobilization techniques are possible solutions to the above-mentioned challenges (Happe *et al*., 2000; Masukawa *et al.,* 2002). The maximum theoretical efficiency of nitrogenase driven $H_2$ production from solar energy with water as the electron donor is about 6% (Ghirardi *et al*., 2009; Sakurai *et al*., 2015). This is calculated based on the amount of light reaching the surface of the photosynthetic organism (algae and cyanobacteria) and with the assumption that the photon has energy at 550 nm as the average energy. The energy level of the photon and the energy conversion efficiency can vary slightly depending on the assumed wavelength (Prince and Kheshgi, 2005). However, the conversion efficiencies under natural conditions in practice can be as low as 0.1% (Tsygankov, 2007; Sakurai *et al*., 2015). The currently known photon conversion efficiency of cyanobacterial $H_2$ production in suspension cultures is not sufficient for commercial applications. In general, close to 6% photon conversion efficiency should be achieved before considering the creation of an industrial-scale production system (Rupprecht *et al*., 2006).

### 1.4.3. Immobilization of cyanobacteria

The vast majority of the previous studies have employed suspension cultures for cyanobacterial $H_2$ production. The use of suspension culture does, however, have several drawbacks, especially when working with filamentous cyanobacteria: for instance, intensive mixing is required for achieving optimal light utilization by the cells; however this process causes breakage of the fragile cyanobacterial filaments (Leino *et al*., 2012). Under such circumstances, the immobilization of cyanobacteria may provide a solution (Das and Veziroglu, 2001). As compared to suspension cultures, immobilized cultures produce $H_2$ at higher volumetric production rates (Leino *et al*., 2012). In addition, ease of handling, minimum self-shading and an increasing in the surface area exposed to light are other advantages worth mentioning.

Immobilization of a cell can be simply defined as a cell that is restrained from moving independently by either artificial or natural mechanisms (Tampion and Tampion, 1987). The main objective of immobilizing cyanobacteria is to sustain and regulate the photosynthetic efficiency and biological activity of the cells,

while simultaneously boosting stability and volumetric cell density. In nature, several cyanobacterial strains such as the benthos and lithotrophs have a natural tendency to form biofilms, which provide mechanical support and a barrier against pathogens (Rossi and De Philippis, 2015). These characteristics are mimicked by artificial biofilms. The artificial biofilm matrix similarly forms a physical barrier and tightly controls the diffusion of gases, depending on the porosity of the material, as well as limits contamination to a certain extent (Moreno-Garrindo, 2008; Meunier *et al*., 2011). Immobilization techniques in general can be divided into six categories: entrapment, affinity immobilization, confinement in a liquid–liquid emulsion, capture behind a semipermeable membrane, adsorption and covalent coupling (Mallick, 2002). Entrapment is based on embedding the cells within a three-dimensional gel matrix. This technique is by far the most frequently used method for immobilizing cyanobacteria and algae (Kayano *et al*., 1981; Kosourov and Siebert, 2009; Kosourov *et al*., 2014). The binding material for immobilization can be natural or synthetic. Acrylamide, cross-linkable resins and polyurethanes are a few examples of synthetic polymers that could be employed for immobilization by entrapment. Entrapment using natural polysaccharide matrixes is the most widely used immobilization technique for cyanobacteria and algae (Meunier *et al*., 2011). Among them, seaweed derived agars, carrageenans or alginates are the most employed for entrapment due to their non-toxicity, mass production potential and biodegradable nature (Stolarzewicz *et al*., 2011). Alginates are simple linear unbranched polymers and form structural components of brown algae (Lee and Mooney, 2012). They form chains of heteropolysaccharides made up of blocks of mannuronic acid and guluronic acid. Alginate is industrially extracted from brown algae belonging to the genera *Macrocystis* and *Laminaria* (Smidsrod and Skjak-Bræk, 1990). The commercial form of alginate usually comes in the form of a Sodium salt of alginic acid which, when dissolved in water and treated with a solution enriched with a divalent cation (such as $Ca^{2+}$, $Sr^{2+}$ or $Ba^{2+}$), leads to the gelation of the hydrogel (Ignacio, 2008; Moreira *et al*., 2006).

Furthermore, alginates have been the most widely preferred natural polymers used for entrapping cyanobacteria due to their translucent nature, controllable porosity and the ease of breaking down the hydrogel to harvest the entrapped biomass (Moreno-Garrido, 2008). Additionally, it is fast, easy and cheap to use in large-scale applications. On the other hand, the low mechanical stability of the alginate matrix is a major disadvantage, especially in large-scale handling

and installations in natural water bodies. Several techniques for improving the mechanical stability have been tested, for instance: mixing alginate with sodium carboxymethyl cellulose or poly-vinyl alcohol; replacing $Ca^{2+}$ with $Ba^{2+}$ as the polymerizing agent for improved stability of the hydrogel although it compromised the porosity; and smearing alginate on supporting templates such as steel screens or insect-screen mesh (Bagai and Madamwar, 1998; Moreno-Garrindo, 2008; Kaya and Picard, 1995; Kosourov and Siebert, 2009).

### 1.5. Cyanobacterial carotenoids and tocopherols

Carotenoids (alternatively known as "tetraterpenoids") are fat-soluble naturally occurring organic pigments that are yellow, orange, or red in color (Morais *et al*., 2006). They are produced by all known photosynthetic organisms, such as plants, algae and bacteria, as well as by some non-photosynthetic bacteria and fungi. To date, over 640 carotenoids have been identified, making them one of the largest classes of naturally occurring pigments in organisms. Carotenoids are mostly composed of a $C_{40}$ backbone made up of hydrocarbons. Based on their chemical structure, carotenoids can be subdivided into two: carotenes (e.g. β-carotene, lycopene) and xanthophylls (e.g. echinenone, myxoxanthophylls, nostoxanthin, zeaxanthin) (Takaichi and Mochimaru, 2007; Domonkos *et al*., 2013; Kusama *et al*., 2015; Zakar *et al*., 2016). Carotenes lack oxygen in their hydrocarbon structure, whereas xanthophylls are oxygenated (Hirschberg and Chamovitz, 1994). To date, the known major carotenoids in cyanobacteria are echinenone, β-carotene, nostoxanthin, zeaxanthin, myxol-2'-glycosides, oscillol-2,2'-diglycosides and canthaxanthin (Zhang *et al*., 2015). In photosynthetic organisms, carotenoids can be further subdivided into two groups based on their biological role as primary and secondary carotenoids. The former are directly involved in photosynthesis, while the later are expressed by the cells in response to various environmental conditions, such as essential nutrient deprivation, exposure to high light, fluctuations in temperature and pH and osmotic and oxidative stress (Minhas *et al*., 2016). Examples of primary carotenoids are lutein, α-carotene, neoxanthin, zeaxanthin, violaxanthin, and β-carotene; examples of secondary carotenoids include canthaxanthin, echinenone and astaxanthin (Leya *et al*., 2009).

In cyanobacteria, carotenoids are generally located in the membranes and play three major roles:

1) functioning as accessory pigments for light harvesting by transferring energy to the chlorophyll and initiating electron transfer (Stamatakis *et al*., 2014),
2) regulating of membrane organization (Zakar *et al*., 2016; Varkonyi *et al*., 2002), and
3) helping protect membranes against photo-oxidative damage (Liang *et al*., 2006; Kerfeld *et al*., 2003; Kerfeld, 2004).

Carotenes and xanthophylls are integral membrane components of both the cytoplasm and thylakoid of cyanobacteria (Zakar *et al*., 2016; Zhang *et al*., 2015). Especially carotenes are indispensable, due to their various functions in light harvesting and photoprotection (Schafer *et al*., 2005; Sozer *et al*., 2010). Most carotenoids are bound to proteins, but some are also found as components of membrane lipids, where they influence viscosity and membrane dynamics (Gruszecki and Strzalka, 2005). It has been previously reported that in cyanobacteria, xanthophylls such as myxoxanthophylls and zeaxanthin provide adequate protection against photo-oxidation and lipid peroxidation under several stress conditions (Masamoto *et al*., 1999; Steiger *et al*., 1999). Additionally, zeaxanthin is known to participate in the repair cycle of the photo-damaged PS II by suppressing the level of singlet oxygen in the system (Kusama *et al*., 2015). Myxoxanthophylls can influence membrane fluidity and polarity by contributing to the stability of the membranes (Mohamed *et al*., 2005). CRT is the gene cluster that is responsible for carotenoid biosynthesis in cyanobacteria. The carotenogenesis pathways in some cyanobacteria, like the heterocyst-forming filamentous cyanobacterium *Anabaena* sp. PCC 7120, have been extensively investigated. In *Anabaena* PCC 7120, the major carotenoids are: echinenone, β-carotene, canthaxanthin, 4-ketomyxol 2'-fucoside and myxol 2'-fucoside (Takaichi *et al*., 2005; Mochimaru *et al*., 2008). In $N_2$-fixing heterocystous strains like *Anabaena* sp. PCC 7120, there is a constant battle between $O_2$ evolved by the photosynthetic apparatus and the $O_2$-sensitive nitrogenase enzyme. $O_2$ permeates into the heterocysts from the adjoining vegetative cells and creates a microoxic environment despite the lack of active PSII complexes in heterocysts, the strong respiration and the thick cell wall enveloping the heterocysts (Zhao *et al*., 2007). As a result, the nitrogenase complex is vulnerable to $O_2$ and ROS toxicity. To combat the accumulation of ROS, cyanobacteria can use non-enzymatic antioxidants, such as carotenoids, for scavenging. In a study by Staal and co-authors (2003) presented the contributions of β-carotene and echinenone to nitrogenase activity and

concluded that the above-mentioned carotenoids were present in heterocysts. Most importantly, they showed evidence that heterocysts can vary the composition of their pigments in response to N-deprivation. Under N-deprived conditions, which are favorable to $H_2$ photoproduction, the excess light energy absorbed by the photosynthetic apparatus is dissipated efficiently by the re-arrangement of phycobilisomes and the quenching property initiated by the orange carotenoid protein (OCP) (Onishi *et al*. 2015). OCPs as well as OCP-like proteins are known to function as quenchers of singlet oxygen or triplet chlorophyll and to transport other carotenoids (Lopez-Igual *et al*., 2016; Sedoud *et al*., 2014; Kerfeld, 2004; Domonkos *et al*., 2013). 3'-hydroxyechinenone (ketocarotenoid) is a major carotenoid component of the OCPs (Kerfeld *et al*., 2003).

In cyanobacteria, carotenoid composition may vary from species to species. Such variations could be the result of the presence or absence of particular carotenogenesis pathways (Takaichi and Mochimaru, 2007). The carotenoid composition may also vary depending on growth conditions such as nitrogen source, light intensity, growth stage, day/night cycle and concentration of N in the cultures (Hirschberg and Chamovitz, 1994). Therefore, it is plausible for the same strain of cyanobacteria to have variation in its carotenoid composition under different environmental conditions, making this a very dynamic system.

Many cyanobacteria are also known to produce high amounts of vitamins, which have a number of beneficial health effects. Tocopherol (also known as vitamin E) is one of these vitamins. Tocopherols are a group of lipid soluble compounds that are known to function as antioxidants (Sakuragi and Bryant, 2006). They can be divided into four types: α, β, γ and δ. This division is based on the structural differences in the number and position of methyl groups on the chromanol head. The polar chromanol head is attached to a hydrophobic phytyl tail; this combination is crucial for its function as a lipid-soluble antioxidant (Maeda *et al*., 2005). It is synthesized mainly in oxygenic phototrophs such as some cyanobacteria, all green algae and plants. In general, the production of tocopherols is primarily induced by stress conditions such as nutrient deprivation, high light intensity and drought (Krieger-Liszkay and Trebst, 2006). Tocopherols function by undergoing two oxidation reactions: (i) oxidation by ROS to a tocopheryl radical and (ii) conversion of singlet oxygen to hydroperoxide. Either reaction can be reverted by ascorbate (vitamin C), a recycling strategy for tocopherols (Latifi *et al*., 2009). Studies on the role of α-tocopherol in

*Synechocystis* sp. PCC 6803 have revealed that in addition to its already known antioxidant property, it is crucial for the normal physiology of this organism, for example in photosynthesis regulation and macronutrient homeostasis (Sakuragi *et al*., 2006). Additionally, the synthesis of this antioxidant is found to be conserved in a majority of the oxygenic photosynthetic organisms (Cheng *et al*., 2003; Maeda *et al*., 2005). It may be speculated that α-tocopherol has a role in one or more critical functions in the biological machinery of these organisms. The biological functions of α-tocopherol in oxygenic phototrophs have not yet been completely elucidated. Therefore, considering α-tocopherol's eminent role as a dietary component, most of the currently available information on its functions has been collected from animal studies (Sakuragi *et al*., 2006). However, it is projected that it is likely to perform similar functions in cyanobacteria as reported in animals, and probably also other functions that are unique to photosynthetic organisms. The known functions of tocopherols include the scavenging and quenching of reactive oxygen species (Sattler *et al*., 2003). There is not much information available on the localization of α-tocopherol in cyanobacteria; in plants, however, it has been reported to have been synthesized on the inner membranes of plastids and distributed between the chloroplast envelope and thylakoid membranes, suggesting it is of a cyanobacterial origin (Arango and Heise, 1998; Fryer, 1992).

## 1.6. Applications of cyanobacteria

### 1.6.1. Cyanobacteria in biofuel production

In general, cyanobacteria and algae are viewed as stronger candidates for industrial biofuel production than plants. Firstly, they have a very short lifecycle and can be cultivated throughout the year, unlike the seasonal crop plants that are currently in use for biofuel production, such as sugarcane, corn, soybeans, rapeseed, etc. Secondly, ease of handling and minimum nutrient requirements also make them very attractive for use in low-cost production systems. Thirdly, cyanobacteria are able to utilize non-drinkable sources like seawater and capture $CO_2$ even from flue gas for growth and use non-arable land for large-scale production (Schenk *et al*., 2008). Several strains of native cyanobacteria are capable of producing biofuel related compounds, and novel pathways could be engineered to turn them into living factories (Machado and Atsumi, 2012; Zhou *et al*., 2016). The most extensively researched biofuels produced from cyanobacteria are hydrogen (Dutta *et al*., 2005; Allahverdiyeva *et al*. 2010;

Tiwari and Pandey, 2012), diesel (Wahlen *et al*., 2011), butanol (Lan and Liao, 2012), ethanol (Dexter and Fu, 2009; Gao *et al*., 2012) and ethylene (Ungerer *et al*., 2012).

### 1.6.2. Relevance in space missions and manned settlements

The future of space exploration may rely on the development of highly efficient methods of recycling readily available (e.g. *in situ* resource utilization, ISRU) for the sustainable production of life support supplies such as clean oxygen, water and nutrition. The ability to generate basic life support components through ISRU would reduce the need for resupply missions, decrease launch weights and cut mission costs, thereby increasing the feasibility of long-distance missions and the establishment of manned settlements in faraway locations such as Mars. Different photosynthetic organisms from higher plants to microorganisms have been considered so far (Lehto *et al*., 2006). Among these candidates, cyanobacteria seem to be the most suitable choice due to their photosynthetic capacity, diverse adaptability and ability to produce several useful bio-compounds. They are fast growers (with a doubling time of a couple of hours to a few days, depending on the strain) and do not require much space and in general are easy to maintain. In addition, the self-replicative nature of cyanobacteria would allow the initial payload of the cyanobacterial samples to be very low, and once onsite, the cultures could be progressively scaled up as needed. Until now, several cyanobacterial strains have been screened for survival under extraterrestrial conditions (de Vera *et al.,* 2013; Cockell *et al.,* 2005). Some cyanobacterial species also produce edible and highly nutritious biomass, which contains compounds with antioxidative properties, such as carotenoids. This biomass could be harvested and consumed as a nutritional supplement by the crewmembers or used as bio-fertilizer for nourishing other organisms such as plants (Singh *et al*., 2016). Other potential applications include the production of drugs, biomaterials and metal leaching (Menezes *et al*., 2015).

To summarize, cyanobacteria grown on Mars could be used for resource production directly (via the production of oxygen and edible biomass) and indirectly (by feeding plants and useful bacteria), using locally available materials (Verseux *et al*., 2016). Therefore, including cyanobacteria in a BLSS system would allow onsite resource utilization and ensure sustainability in the long term.

# 2. AIMS OF THE STUDY

The aim of my thesis is to investigate the acclimation strategies of selected strains of $N_2$-fixing heterocystous cyanobacteria to conditions that are favorable to efficient $H_2$ photoproduction. The main research topics addressed here are the following:

(i) To optimize the long-term $H_2$ photoproduction by selected cyanobacterial strains entrapped in alginate films (**Paper I**).

(ii) To investigate the effects of prolonged $H_2$ photoproduction by cyanobacteria on metabolites, such as carotenoids and glycogen (**Paper II and III**).

(iii) To assess cyanobacterial growth and $H_2$ photoproduction under simulated Martian-like atmospheric conditions (**Paper IV**).

# 3. METHODOLOGY

## 3.1. Cyanobacterial strains and growth conditions

Paper I, II, III: The wildtype *Anabaena* sp. PCC 7120 also known as *Nostoc* sp. PCC 7120 (planktonic strain, hereafter referred to as *Anabaena* 7120) was acquired from the Pasteur Culture Collection (Paris, France). The native strain *Calothrix* sp. 336/3 (benthic strain) was selected from the University of Helsinki Culture Collection (UHCC) as described previously (Allahverdiyeva et al., 2010). The Δ*hupL* and Δ*hupL* Δ*hoxH* mutants of *Anabaena* PCC 7120 lacking uptake hydrogenase and both uptake and bidirectional hydrogenases, respectively, (Masukawa *et al*., 2002) were kindly provided by Prof. H. Sakurai. The stock cultures of these strains were maintained in the Z8x medium (Z8 medium deficient in combined nitrogen) at room temperature, without mixing. The medium for pre-experimental cultures of Δ*hupL* and Δ*hupL* Δ*hoxH* was supplemented with 25 µg/ml spectinomycin or 25 µg/ml spectinomycin plus 10 µg/ml neomycin, respectively. The culture flasks were illuminated from above with fluorescence lamps (cool-daylight, Lumilux T8 15W/865) at a light intensity averaging to 30 µmol photons $m^{-2}s^{-1}$ photosynthetic active radiation (PAR).

The experimental cultures were grown in 1000 ml flasks containing 500 ml of Z8x medium at pH 7.5 under continuous illumination by fluorescent lamps (Lumilux T8 15W/865) providing 50 µmol photons $m^{-2}s^{-1}$ PAR and a temperature of 22$^{o}$C. For mixing and aeration, the cell suspensions were bubbled with air that was filtered through membrane filters (0.2 µm pore-size, Acro 37TF). The cell cultures were grown until log phase before harvesting.

Paper IV: The other cyanobacterial species used were *Synechocystis* sp. PCC 6803, *Anabaena cylindrica* (PCC 6309) and *Arthrospira platensis* (PCC 8005). These strains were obtained from the Pasteur Culture Collection (Paris, France). *Synechocystis* sp. PCC 6803 and *Anabaena cylindrica* were cultivated in BG11 growth medium (Allen, 1968) and *Arthrospira platensis* in Zarrouk medium (Zarrouk 1966). The growth conditions (light and temperature conditions) for these strains were maintained at 50 µmol photons $m^{-2}$ $s^{-1}$ PAR and 32$^{o}$C, 50 µmol photons $m^{-2}$ $s^{-1}$ and 25$^{o}$C and 70 µmol photons $m^{-2}$ $s^{-1}$ and 32$^{o}$C, respectively. The three strains mentioned above were grown under agitation using a shaker (around 100 rpm).

Mimicking Martian atmospheric conditions: To test the effects of the low-pressure atmospheres with simulated Martian air composition (low N, high $CO_2$ availability, anoxic) as an environment for photosynthetic microorganisms, the experiments were conducted in vacuum-tight glass containers (summarized in **Table 2**). Here, the $CO_2$ availability was maintained at a steady level using a controlled gas flow system and continuous supply (**Figure 5**). This continuous flow of $CO_2$ through the growth system also allowed the maintenance of the photosynthetic, $O_2$-producing cultures under the presence of minimal oxygen, thus creating a Martian-like atmospheric composition (excluding $N_2$, which was provided entirely through the growth medium). Normally, the growth substrate would be depleted through evaporation under such low-pressure conditions. Here it was partially prevented by moisturizing the gas flow by saturating it with water.

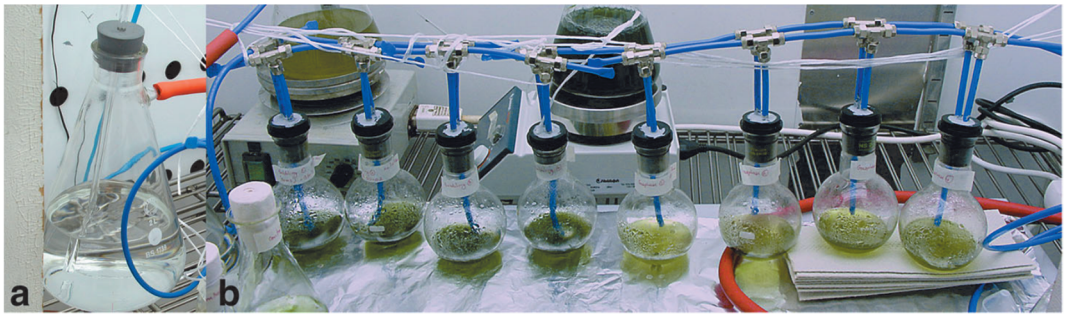


**Figure 5**: Vacuum line set-up where the gas flow is first saturated with water vapor (left) and then lead into the culture bottles. The gas flow is regulated with a rheostat and the pressure inside the closed system is controlled by a pressure gauge, and a vacuum pump

**Table 2:** Overview of the experiments conducted in Paper I, II, III and IV.

| Publication | Organisms | Experimental conditions | Variables measured |
|---|---|---|---|
| Paper I | *Calothrix* sp. 336/3, *Anabaena* sp. PCC 7120, Δ*hupL* and Δ*hupL* Δ*hoxH* mutants of *Anabaena* PCC 7120 | Control (0h), Ar + $CO_2$, Air, Air + $CO_2$, $N_2$ + $CO_2$ | $H_2$ and $O_2$ photoproduction yields, Chl *a* content and PSII yield |
| Paper II | *Calothrix* sp. 336/3, *Anabaena* sp. PCC 7120 and Δ*hupL* mutant of *Anabaena* PCC 7120 | Ar + 3% $CO_2$, Ar + 6% $CO_2$, Air, Z8 (Air), Z8x (Air) | carotenoid composition, identification of carotenoids in *Calothrix* sp. 336/3, identification of putative genes encoding enzymes |
| Paper III | *Calothrix* sp. 336/3, *Anabaena* sp. PCC 7120 and Δ*hupL* mutant of *Anabaena* PCC 7120 | Control (+$N_2$, 0h), Control (0h), Ar + $CO_2$, Air, Air + $CO_2$ | $H_2$ and $O_2$ photoproduction yields, Chl *a* content, carotenoid composition, α-tocopherol, extent of oxidative damage, glycogen, total (non-glycogen, stored) carbohydrates and expression levels of targeted proteins (D1 and NifH) |
| Paper IV | *Synechocystis* sp. PCC 6803, *Arthrospira platensis* and *Anabaena cylindrica* PCC 6309 | Control (ambient air), varying air pressure (50, 60, 100, 150, 200, 250, 300, 400, 500, 600 mbars), varying $CO_2$ concentrations (0.4, 1, 10, 20, 100 %), Stationary (ambient), Stirring (ambient), Bubbling (0.1 mbars), Gas-phase (0.1 mbars), Stationary_10% $CO_2$, Shaking_10% $CO_2$, Growth in BG-$11_0$, Growth in BG-11, Growth in BG-11+10% $CO_2$ in assay, Growth in BG-11+10% $CO_2$, Growth in BG-$11_0$+10% $CO_2$ | hydrogen and oxygen yields, Chl *a* content, heterocyst frequency, relative growth |

### 3.2. Immobilization of cyanobacterial cells

The cells were harvested by centrifugation at 3000 X g (F15-6x100, Thermo Scientific) for 5 minutes, washed once with the growth medium (Z8x) and pelleted again by centrifugation. The harvested cells were then encapsulated within $Ca^{2+}$-alginate films following the procedure described by Kosourov and Seibert (2009), with adaptation for cyanobacteria as described in Leino *et al.* (2012). The details are shown in **Figure 6**. The $Ca^{2+}$ alginate films were cut into 3x1 cm strips and washed with sterile Milli-Q water to remove excess $CaCl_2$.

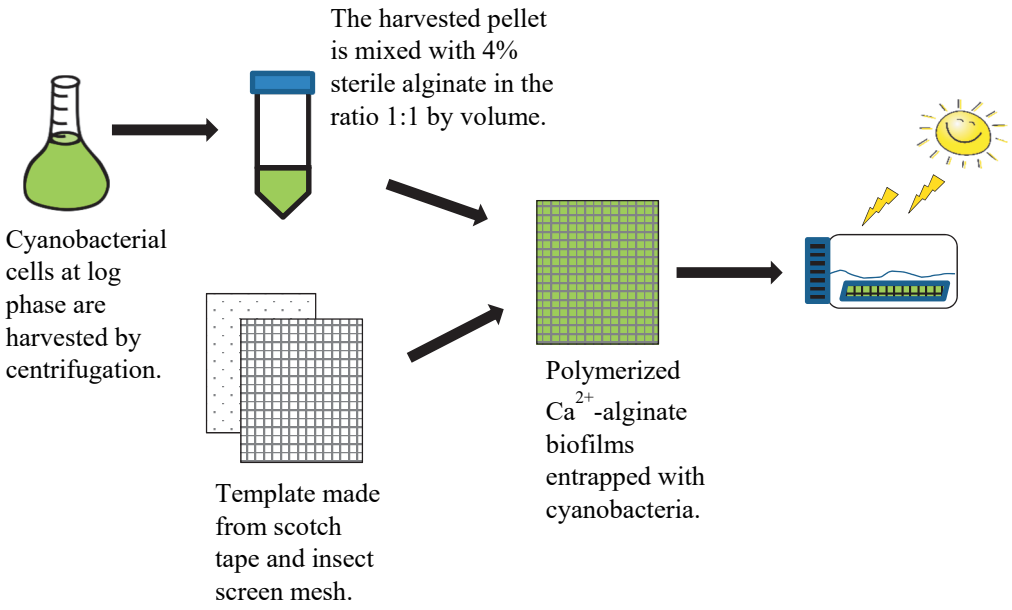


**Figure 6:** Schematic representation of immobilization of cyanobacteria with $Ca^{2+}$-alginate (Source: adapted from Kosourov and Seibert, 2009)

### 3.3. $H_2$ photoproduction assay

At the beginning of each experiment, freshly prepared alginate strips with entrapped cells were transferred to 23 ml sterile glass vials containing 5 ml of Z8x medium. The headspace of the vials was purged with argon (Ar) and then tightly sealed with caps containing PTFE-coated rubber septa and supplemented with 6% $CO_2$. This $CO_2$ concentration was selected based on a previous study (Leino *et al.*, 2012). The vials were then placed in a growth chamber and incubated at 26°C under continuous top illumination with fluorescent lamps

(cool-daylight, ~70 µmol photons $m^{-2}s^{-1}$, Philips Master TL-D T8 15W/865). Depending on the approach (summarized in **Table 2**), the headspace of the vials was periodically replenished (at the start of each incubation cycle) with the following gas compositions: (i) Ar containing 6% $CO_2$, (ii) ambient air and (iii) ambient air containing 6% $CO_2$ or (iv) $N_2$ supplemented with 6% $CO_2$. In the case of air-treatments (conditions ii and iii), the headspace in the vials was re-flushed with Ar supplemented with 6% $CO_2$ after 16 to 20 hours of incubation (**Paper I**). This additional step was applied to recover efficient $H_2$ photoproduction by the alginate-entrapped cyanobacteria. The $H_2$ and $O_2$ contents in the headspace were routinely monitored (once per day) with a GC (Clarus 500, PerkinElmer, Inc.). The GC was set up with a thermal conductivity detector and a molecular sieve 5A column (60/80 mesh) using Ar as the carrier gas.

### 3.4. Nitrogenase activity assay

The activity of nitrogenase enzyme was determined by acetylene reduction assay, as previously described (Dilworth, 1966). Freshly prepared alginate films entrapped with cyanobacteria were placed into vials (23 ml) containing Z8x medium (5 ml). These vials were then flushed with Ar and supplemented with 10% acetylene, followed by incubation in a growth chamber for 18 h at 26°C under continuous overhead light using fluorescent lamps (cool-white light, ~150 µmol photons $m^{-2}s^{-1}$, Philips Master TL-D T8 15W/840). Using a syringe, 10 µl of the gas samples were collected from the headspace of the vials and injected into a GC (PerkinElmer Autosystem) set up with a flame ionization detector (FID) and a CP-CarboBond column (Varian) using helium as the carrier gas. The system was calibrated using 1% ethylene. Nitrogenase activity was measured based on the Chl *a* content of the cells and per film area.

### 3.5. Analytical techniques

#### 3.5.1. Determination of $H_2$ photoproduction yields and rates

For measuring the $H_2$ production yields, 150 µl of samples from the gas-phase of the vials were drawn using a gas-tight syringe (Hamilton Co.) and measured with a gas chromatography machine (Clarus 500, Perkin-Elmer). The GC was set up with a thermal conductivity detector and a Molecular Sieve 5A column (60/80 mesh) using Ar as the carrier gas. The rate of hydrogen production was calculated based on the Chl *a* content of the cells.

### 3.5.2. HPLC analysis of pigments and α-tocopherol

Samples for HPLC analysis were collected and treated with 50 mM EDTA (pH = 7.0) to break down the $Ca^{2+}$-alginate hydrogel matrix and release the cells. The obtained mixture was pelleted and washed once with the Z8x medium to remove any remnants of EDTA. The harvested cell pellets of *Anabaena* 7120, *Calothrix* sp. 336/3 and Δ*hupL* were quickly frozen in liquid nitrogen and stored at -80°C until processed. Pigments soluble in organic solvents were extracted from the pellets using 100% methanol in the dark at 10°C overnight. This process was repeated until the extraction of the pigments was complete. The extracts were centrifuged and filtered through a 0.2 µm filter unit (Millipore) to remove any remaining debris. The filtered samples were analyzed by high-performance liquid chromatography (HPLC) using the C18 encapped column (LiChroCART 125-4, Merck KGaA, Darmstadt, Germany). The pigments were eluted with two solvents: solvent A consisted of acetonitrile/methanol/0.1 M Tris-HCl buffer adjusted to pH 8.0 (72:8:3, v/v) and solvent B consisted of methanol/hexane (4:1, v/v). These solvents were applied consecutively at a constant flow rate of 0.5 ml $min^{-1}$. The separation began with an initial isocratic run for 4 min and was followed by a linear gradient from 0 to 100% for 15 min. Next, an isocratic run of solvent B was applied for another 26 min. Standards for the following pigments were run prior to running the samples: chlorophyll *a*, β-carotene, zeaxanthin, echinenone, canthaxanthin, myxoxanthophyll and α-tocopherol (DHI LAB Products, Hørsholm, Denmark). The rest of the pigments were identified by LC-MS based on ion masses and spectral properties.

### 3.5.3. LC-MS analysis of carotenoids

High-performance liquid chromatograph mass spectrometer (Agilent 1100 Series LC/MSD Trap XCT Plus, Agilent Technologies, Palo Alto, CA) was used for separating the pigments. The samples in the form of methanol extracts were fed into the column (Luna C8) and eluted at a rate of 0.15 ml $min^{-1}$ with different gradients of isopropanol + 0.1% formic acid in water at 40°C. The mass spectra were obtained using electrospray ionization in positive mode.

### 3.5.4. Spectrophotometer analysis of pigments

In order to obtain *in situ* suspension absorbance spectra (370–750 nm) of the samples, 8 ml of the sample was transferred to the integrating cavity of the OLIS

CLARiTY 17 UV/VIS/NIR spectrophotometer (On Line Instrument Systems, Inc., Bogart, GA, USA) and run. The resulting data was obtained in the form of raw absorbance data and it was converted to absorbance values using the Fry's method for interpretation (Fry *et al*., 1992).

The Chl *a* content in the alginate films was assayed spectrophotometrically after the solubilization of randomly chosen alginate strips in 50 mM Na-EDTA solution (pH = 7.0). The cells were washed once with fresh medium (Z8x) to remove any residues of Na-EDTA. The cell pellets were then mixed with 90% methanol and the Chl *a* was extracted and quantified spectrophotometrically at 665 nm.

## 3.6. Analysis of proteins

### 3.6.1. Isolation of total protein fraction

Cells were harvested in the beginning (at 0 hours), in the middle (250 h) and at the end of the experiment (450 h) by treating the alginate entrapped cells with 50 mM EDTA, followed by washing of the pellets in Z8x media. Total protein samples were isolated as previously described (Pollari *et al*., 2011), with minor modifications. The cell pellets were washed with ice-cold STNE buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 20 mM Na-EDTA, and 50mM DTT) and this step was repeated after transferring the pellet to a beater tube (Polypropylene Micro vials). One-third volume of acid-washed glass beads was added, and the cells were broken by a Mini-BeadBeater-24 under darkness in cold room facility. The tubes were placed in the beater device and beat for 1 min, followed by immediate cooling on ice for 1 min. This cycle of beating and cooling was repeated six times. The supernatants were collected to Eppendorf tubes and centrifuged at 1500x g for 5 min at 40$^{o}$C to remove any remaining unbroken cells and the collected supernatant was used as a total-protein sample.

### 3.6.2. SDS-PAGE, immunodetection of targeted proteins

Samples for protein oxidation were prepared according to the instructions of the Oxyblot™ Protein Oxidation Detection Kit (Millipore). Total proteins were separated using 12% SDS-PAGE (mini gel) and the separated proteins were transferred to Immobilon-PVDF membranes (Millipore) by electroblotting. A chemiluminescent HRP substrate was used for the detection of carbonylated proteins. The membranes were later stained with Coomassie brilliant blue R-

250 (Bio-Rad) to check uniform loading and even transfer of the protein samples.

The cells harvested for detection of targeted proteins were broken down mechanically using glass beads (acid washed, 150-212 µm) in the Mini bead beater (Biospec products) and total protein was extracted. We followed the standard procedure of separating proteins with SDS-PAGE and protein transfer to PVDF membranes by electroblotting. The antibodies used were D1 N-terminal specific and NifH reductase specific (AS01021A, Agrisera). The rabbit anti-hen IgY (H&L), the CDP star chemiluminescence kit (New England Biolabs) and alkaline phosphatase conjugate were used for detection in Western blotting. The membranes were stained with the Coomassie stain to verify the uniform loading and even transfer of the samples.

### 3.7. Glycogen and sugar estimation

The samples collected for glycogen assay were treated with 50 mM EDTA (KOH, adjusted to pH = 7.0) to break down the alginate hydrogel matrix. The cells were spun down by centrifugation (6000 rpm) for 5 minutes. The cell pellet thus obtained was collected and resuspended in 1 ml of methanol (HPLC grade). This mixture was again centrifuged and the methanol extract containing pigments was removed. The remaining pellet was dried using speed-vac for 10 minutes at 43°C (chamber heated). The dried pellet was mixed gently in 2 ml of 100 mM Na-acetate buffer (pH = 4.8) with a pipette. This mixture was transferred to sealed Hungate tubes and autoclaved for 20 minutes at 120$^{o}$C for solubilization. After cooling, 1 ml of acetate buffer (pH = 4.8) containing 4 units of freshly prepared amyloglucosidase (1011-51G-F, Sigma) was added and mixed well. The sealed tubes were then placed in a hot water bath (55$^{o}$C) and incubated overnight. The following day, 100 µl of the supernatant (the pellets settled to the bottom of the tube were later used for sugar determination) from the tubes was subjected to enzymatic treatment with hexokinase and tested at 340 nm for the quantitative determination of glucose (according to the instructions specified by the glucose hexokinase kit (DiaSys)). The pellets collected after the amyloglucosidase reaction in the glycogen assay were resuspended in 1 ml of 3% $H_2SO_4$ (v/v). This mixture was autoclaved in tightly sealed tubes at 120$^{o}$C for 40 minutes. The pH was adjusted to neutral with the addition of 2 M NaOH. This step was followed by assay with glucose hexokinase kit (as described in the glycogen assay).

### 3.8. Photochemical activity

Dual-PAM 100 system (Walz, Effelrich, Germany) was used to evaluate the photochemical performance of alginate encapsulated cyanobacterial films. The films were positioned at the center of the leaf holder module (Dual-BA; Walz). Actinic red light with an intensity of ~50 or 150 µmol photons $m^{-2}$ $s^{-1}$ was applied to the films for 5 min to determine the steady-state Chl *a* fluorescence level ($F_t$). The maximum fluorescence level under light ($F_m'$) was determined by applying saturating light pulses with duration of 300 ms and light intensity of 3000 µmol photons $m^{-2}$ $s^{-1}$. The effective photosystem (PS II) yield, Y(II), was calculated as ($F_m' – F_t/F_m'$).

### 3.9. Microscopy

The sample slides for light microscopy visualization were prepared without stain as wet mounts. This approach was used to observe the specimens in their natural condition. The light microscope (Orthoplan, Leitz) used for capturing bright-field images was set up with a digital camera (Leica DFC 420C) and a 10x objective. The heterocysts were clearly identified by their thick cell envelope and larger cell size in comparison to the vegetative cells. The heterocyst frequency was determined by counting the number of vegetative cells located between two heterocysts in each filaments. Multiple filaments from each of the three biological replicates were scored and averaged for each experimental condition.

# 4. OVERVIEW OF THE RESULTS

## 4.1. Long-term hydrogen photoproduction by immobilized cyanobacteria

In **paper I**, two approaches were tested to improve and extend $H_2$ photoproduction in filamentous heterocyst-forming cyanobacteria entrapped in $Ca^{2+}$-alginate films. In the first approach, (a) the effect of $CO_2$ supplementation cycles on long-term $H_2$ production and (b) the effect of different gas compositions in the $CO_2$ supplementation cycles on the cell fitness of cyanobacteria were investigated. Firstly, the two *Anabaena* strains (WT and Δ*hupL*) were subjected to cycles of $CO_2$ supplementation where the headspaces of the vials were periodically (every six days) flushed with Ar and 6% $CO_2$ was added. The results showed an extension of the overall $H_2$ photoproduction period in Δ*hupL* cells until about 600 h. The wildtype *Anabaena* did not show any significant changes after the first cycle (**Figure 1A in Paper I**). The maximum specific $H_2$ production rates for both strains were the highest in the first $CO_2$ supplementation cycle (**Table 1 in Paper I**). Both strains displayed a gradual decline in the $H_2$ production activity by the end of the experimental period and the 6% $CO_2$ supplementation did not result in complete recovery of production rates to the levels recorded in the first half of the experimental period (**Figure 1A in Paper I**). Secondly, both *Anabaena* strains were placed under different gas compositions (Ar, air, and $N_2$) in the 6% $CO_2$ supplementation cycles to test the effects on $H_2$ photoproduction and cell fitness.

The Δ*hupL* mutant produced $H_2$ under all the three gas compositions, but at substantially low rates when subjected to the air and $N_2$ gas compositions. In contrast, the *Anabaena* 7120 only produced $H_2$ under the Ar atmosphere (**Figure S1 in Paper I**). To test cell fitness after the treatments, the encapsulated cells were retrieved from the $Ca^{2+}$-alginate films after 12 days, suspended in Z8x medium and adjusted to the same optical density. These suspension samples were then monitored for regrowth under standard conditions for 6 days. Cells retrieved from the films immediately after immobilization were used as a control. The results showed that the recovered Δ*hupL* cells from Ar and air supplemented with 6% $CO_2$ gas compositions were adversely affected, with final OD values significantly lower than the control samples. The wildtype strain recovered considerably faster (**Figure 3 in Paper I**) than the mutant cells.

In the second approach, the potential to simultaneously prolong the photoproduction of $H_2$ and maintain cell fitness of the immobilized cells was investigated. The $Ca^{2+}$-alginate entrapped cells of *Calothrix* 336/3, *Anabaena* 7120 and its mutant *ΔhupL* were subjected to a number of periodic air-treatments (for a duration of 16 to 20 h) every three to four days with (i) ambient air (air) or (ii) air supplemented with 6% $CO_2$ (air + 6% $CO_2$) between the $CO_2$ supplementation cycles (Ar + 6% $CO_2$). Periodic exposure to air allowed the N-limited cells to restore the N/C balance. Notably, the above-mentioned periodic air-treatments significantly improved the $H_2$ photoproduction yield in *Calothrix* 336/3 when compared to the Ar + 6% $CO_2$ counterparts (**Figure 7**). In *Anabaena* 7120, as anticipated, there were no significant changes in the $H_2$ photoproduction yield under any treatments (**Figure 7**). Due to the lack of a fully functional uptake hydrogenase, *ΔhupL* displayed the highest $H_2$ production activity among the three strains investigated (**Figure 7**). The Δ*hupL* samples treated with air + 6% $CO_2$ showed excessive Chl *a* degradation and decreased photochemical activity (**Figure 5 & 6 in Paper I**). In contrast, the *Calothrix* strain demonstrated the highest yields when treated with air + 6% $CO_2$ (**Figure 7**), especially in the thin films with the low Chl *a* content.

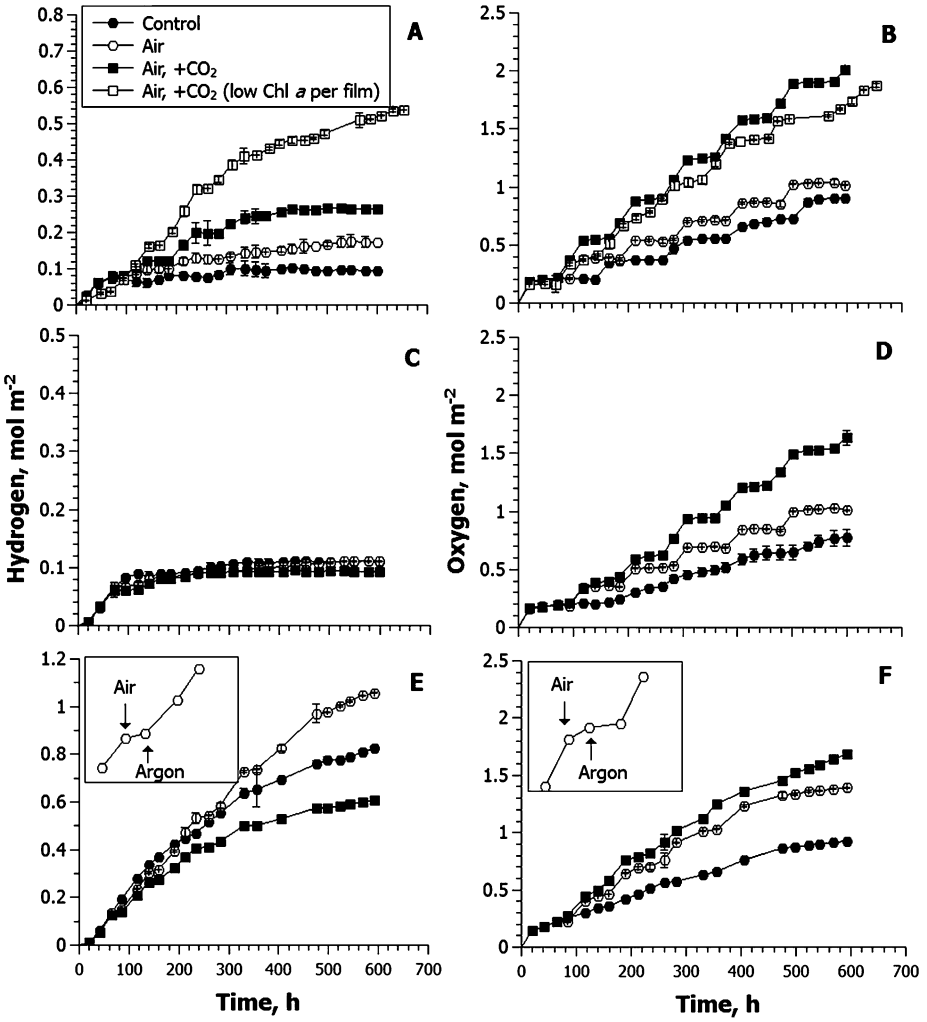


**Figure 7:** Long-term $H_2$ (A, C, and E) and $O_2$ (B, D, and F) photoproduction yields from alginate encapsulated *Calothrix* 336/3 (A and B), *Anabaena* PCC 7120 (C and D), and the Δ*hupL* (E and F).

### 4.2. Carotenogenesis pathways in *Calothrix* sp. 336/3

In **Paper II**, we characterized carotenogenesis pathways in *Calothrix* sp. 336/3 to understand the role of carotenoids in the acclimation strategies of this strain during $H_2$ photoproducing conditions. Carotenogenesis in *Anabaena* 7120 has been well characterized previously (Takaichi *et al* 2005; Graham and Bryant, 2009), but only limited information is available on the carotenoid profiles of *Calothrix* strains (Stransky and Hager, 1970).

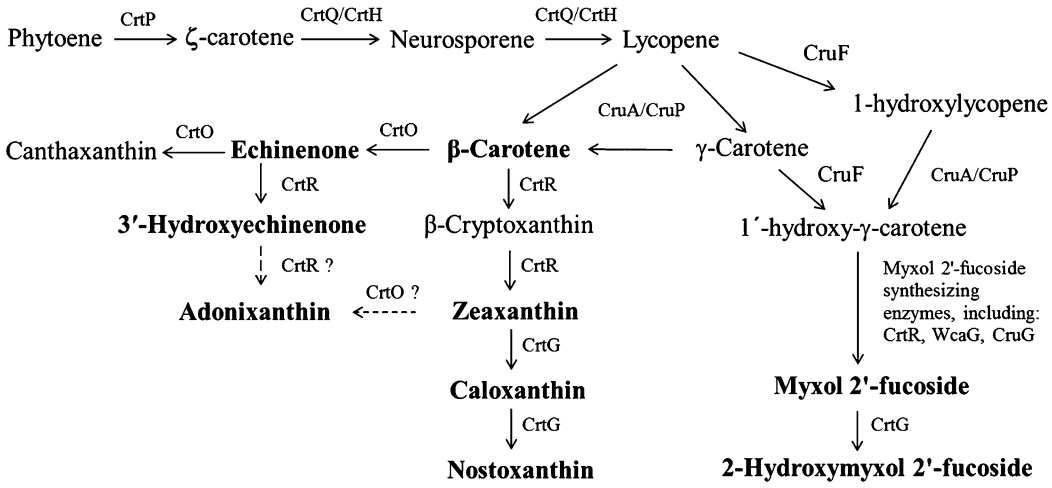


**Figure 8:** Proposed carotenogenesis pathways and responsible enzymes in *Calothrix* 336/3.

Upon separation of the extracted carotenoids with HPLC, the elution profile obtained with the solvent system 1 showed the presence of 9 major and 5 minor carotenoid products (**Figure 1 of Paper II**). The 9 major carotenoids were 2-hydroxymyxol 2′-methylpentoside, myxol 2′-methylpentoside, nostoxanthin, adonixanthin, caloxanthin, zeaxanthin, 3′-hydroxyechinenone, echinenone and β-carotene; and the 5 minor carotenoid products detected were *cis* form of myxol 2′-methylpentoside, *cis* form of nostoxanthin, *cis* form of caloxanthin, 9-*cis*-β-carotene and 13-*cis*-β-carotene. Based on the available genome sequence of *Calothrix* sp. 336/3 (Isojärvi *et al*., 2015), genes and enzymes involved in biosynthesis of carotenoids in this strain were identified and a carotenogenesis pathway was proposed in **Paper II**.

The presence of three distinguishable carotenogenesis pathways was identified in *Calothrix* sp. 336/3 (**Figure 8**) as follows: (i) first pathway resulted in the production of myxoxanthophylls, (ii) the second pathway led to accumulation of hydroxycarotenoids and (iii) the third pathway in the production of ketocarotenoids and their hydroxylated derivatives. In detail, the first pathway branches out from lycopene to form myxol and its derivatives (myxoxanthophylls), similarly as in *Anabaena* 7120 (**Figure 8**). Next, lycopene is converted to myxol by the action of CruA-type lycopene cyclase (CruA or CruP), CrtR hydroxylase and CruF hydratase. It is followed by the glycosylation step to form myxoxanthophyll. 2'-*O*-glycosyltransferase (CruG) and GDP-fucose

synthase (wcaG) are speculated to be responsible for this reaction. The second pathway branches out from β-carotene towards the production of hydroxycarotenoids such as zeaxanthin, caloxanthin, and nostoxanthin, catalyzed by the enzymes: β-carotene hydroxylase (CrtR) and 2,2'-β-hydroxylase (CrtG). The third pathway begins with the ketolation of β-carotene with β-carotene ketolase, CrtO to form echinenone. Unlike *Anabaena* 7120, *Calothrix* lacks CrtW-like β-carotene ketolase. As a result, the second ketolation is driven by the same CrtO enzyme, resulting in minor production of canthaxanthin. The pathway also leads to the biosynthesis of hydroxylated forms of ketocarotenoids (3'-hydroxyechinenone and adonixanthin) depending on the presence of hydroxylase, which is most probably driven by CrtR.

### 4.3. Changes in carotenoids and α-tocopherol contents under $H_2$ photoproducing conditions

Due to the important role of carotenoids in the cyanobacterial photosynthetic machinery's (particularly in PSII) assembly and function, it was predictable that the total carotenoid content may undergo significant changes in composition when subjected to N-limitation (Zakar *et al*., 2016). To confirm this assumption, the carotenoid content was characterized and compared between the alginate entrapped films of *Calothrix* 336/3, *Anabaena* 7120 and Δ*hupL*, harvested in the beginning of the experiment (0 h) and after long term incubation (450 h) under the following conditions: air-treated (air, air + 6% $CO_2$) and untreated (Ar + 6% $CO_2$). Varying degrees of accumulation of total carotenoids (ranging from an increase of 0.5 to 2-fold) was seen in all the studied cyanobacteria during the $H_2$ production assay as compared to samples from 0 h, except the air + 6% $CO_2$ treated samples of *Anabaena* 7120 and its mutant Δ*hupL* (**Figure 6 in Paper III**). The total carotenoid to Chl *a* ratio increased in all strains by the end of the experiment, but the most noticeable increase in the total carotenoid to Chl *a* ratio was observed in untreated (Ar + 6% $CO_2$) and air + 6% $CO_2$ treated samples of Δ*hupL*. The pigment content of the cyanobacteria entrapped in films changed after their long-term incubation in both air-treated and untreated films (**Table 2 in Paper II** and **Table 1 in Paper III**). The most prominent change in carotenoid content was the steep decline of echinenone in all the three strains tested. The most pronounced decline of echinenone was seen in films that were untreated with air (Ar + 6% $CO_2$ samples) and periodically treated with air + 6% $CO_2$. Moreover, there was a tendency for 3'-hydroxyechinenone, which is a derivative

of echinenone and a co-factor of OCP, to increase in the untreated samples of *Calothrix* 336/3 (**Table 2 in Paper II**). Concurrently, the overall content of myxoxanthophylls (2-hydroxymyxol 2'-fucoside and myxol 2'-fucoside) increased in all films of *Calothrix* 336/3 after long-term incubation. In *Anabaena* 7120 and Δ*hupL*, a similar trend was observed. Here, instead of 2-hydroxymyxol 2'-fucoside and myxol 2'-fucoside, elevated levels of 4-ketomyxol 2'-fucoside were noted in the *Anabaena* strains due to the presence of the CrtW-like ketolase.

The spike in the production of α-tocopherol in all the treatments of all the tested strains at 450h was noteworthy (**Figure 4 in Paper III**). Such an acclimation may be an indication of a protective mechanism against lipid peroxidation, as explained in the work published by Steiger and co-authors in 1999. Particularly in *Calothrix* 336/6, there was an increase in α-tocopherol in the range of ~3 to 5 times in all the periodically treated samples in comparison to samples harvested at 0 h. In contrast, in *Anabaena* strains it ranged from a ~6 to 7 times increase in wild type to a ~9 to 11 times increase in Δ*hupL*. Overall, *Calothrix* 336/6 samples showed ~5 to 6 times higher α-tocopherol content than the *Anabaena* strains. Interestingly, we noticed no significant difference in the level of α-tocopherol between the samples from the three periodic treatments, except that the untreated samples of *Calothrix* had less accumulation of α-tocopherol than the treated samples of the same strain.

### 4.4. Oxidative stress in immobilized cells

The experiments were conducted under phototrophic conditions, facilitating the evolution of oxygen as a product. Alginate hydrogel has the drawback of limited diffusion of gases due to its compromised porosity (Hasset, 1996). As a result, when these films are exposed to continuous light under $H_2$ photoproducing conditions over extended periods, accumulation of photosynthetically produced $O_2$ might occur in the immobilized cells, especially under high $CO_2$ levels. Thus, the development of oxidative stress may be anticipated. As expected, oxidative damage was very well visible in our samples, both treated and untreated. The most prominent visual changes were observed in the Δ*hupL* samples (**Figure 5A in Paper I**): there was considerable bleaching of the films. Total proteins were extracted from all the three tested cyanobacterial samples and treated to visualize the extent of protein carbonylation. In cells, the carbonylation of proteins is caused by the elevated

production of ROS due to oxidative stress and due to this reason protein carbonylation was used as a marker for oxidative damage. Results showed varying degrees of enhanced protein carbonylation in the total protein extracts from the $H_2$-producing films (**Figure 3 in Paper III**) in all strains by the end of the experiment (450 h) under all tested treatments, as compared to the corresponding samples taken at 0 h. The stability of the PS II was also seen to be affected when analyzed with immunodetection using a specific antibody for PsbA proteins (PSII reaction center protein D1). A significant decline in the level of D1 protein was noted in all periodically treated samples harvested from 250 h, apart from the untreated samples of *Calothrix* 336/3, as compared to the films collected at the 0 h time point. The most intriguing observation was made regarding the *Calothrix* samples, which showed a partial recovery of D1 protein by the end of the experiment (450 h), but only in the air and air + $CO_2$ treated samples (**Figure 5a in Paper III**). The D1 of the *Anabaena* 7120 samples remained particularly stable throughout the experiment, with a gradual decline at 450 h. Δ*hupL*, however, was more sensitive and showed a considerable decrease in D1, especially in the Ar + 6% $CO_2$ and air + 6% $CO_2$ samples.

### 4.5. Acclimation of cyanobacteria to low pressure conditions

A part of my doctoral studies has been dedicated to the investigation of the use of selected strains of cyanobacteria as candidates that could adapt to simulated Martian conditions. The experimental conditions were close to those on Mars: an anoxic, high $CO_2$ and low nitrogen atmosphere. In these experiments, selected cyanobacteria were grown in vacuum-tight containers under very high (99.9%) $CO_2$ content and varying low pressures (from 50 mbar up to 200 mbars) that kept nitrogen as a limiting factor.

Firstly, the model organism, *Synechocystis* sp. PCC 6803, was cultivated under elevated $CO_2$ concentrations and exposed to various air pressures for 3 days in liquid suspension. Here, growth was mostly influenced by the partial pressure of the $CO_2$ supply. 100% $CO_2$ provided in pressures ranging from 50 mbar to 200 mbar strongly enhanced the growth of *Synechocystis* sp. PCC 6803. Secondly, the effect of 100% $CO_2$ under different air pressures on the growth of *Arthrospira platensis* was also investigated. Here, the $CO_2$ supplied at 50 mbar pressure was shown to enhance the growth, but merely by 25%, as compared to the growth of control cultures in ambient air (**Figure 3 in Paper IV**). Interestingly, elevated supply of carbon along with a rate-limiting supply of

nitrogen strongly affected the growth and metabolic activity of the tested cyanobacteria. In addition, we tested the effect of these environmental conditions on the growth and $H_2$ photoproduction possibility of the filamentous heterocystous nitrogen fixing strain, *Anabaena cylindrica*. This strain was grown in the presence of 100% $CO_2$, low pressure (100 mbar) and nutritionally abundant BG-11 growth medium for a period of 7 days. This set-up was designed to enable the cells to enter a phase where the nitrogen availability became the limiting factor for cellular growth. The results showed a total of 3-times increase in growth when compared to the control samples grown in ambient air composition and pressure (**Figure 4 in Paper IV**). It was noteworthy that the samples mixed by shaking or by bubbling $CO_2$ into the growth medium showed slightly increased accumulation of biomass in comparison to samples that were stationary. It was found that high $CO_2$ and low $N_2$ ratio allow *Anabaena cylindrica* to produce a significant amount of $H_2$ in the light due to the enhanced differentiation of heterocysts even in the presence of combined nitrogen. Strikingly, *A. cylindrica* cultures produced 6.1 µmol $H_2$ mg Chl $a^{-1}h^{-1}$ i.e. an approximately 30 times increase as compared to the control samples grown in ambient air (**Figure 5 in Paper IV**).

# 5. DISCUSSION

## 5.1. Optimization of long-term $H_2$ photoproduction by improving cell fitness

Efficient $H_2$ photoproduction in $N_2$-fixing heterocystous cyanobacteria can be achieved by the elimination of $N_2$ from the gas-phase (headspace) above the growth medium. Under such conditions, the nitrogenase enzyme catalyzes the reduction of protons to $H_2$ (Masukawa *et al*., 2010). Although the efficiency of the reaction towards $H_2$ production improves in the absence of $N_2$, previous research has shown that the cyanobacterial samples under this condition showed a rapid halt of $H_2$ photoproduction after 5 days (Leino *et al*., 2012). Carbon limitation was spotted as a potential cause. Leino and co-authors supplemented the headspace of the sample vials with 2–10% $CO_2$ in Ar as an additional carbon source, at regular intervals. With the addition of $CO_2$, the $H_2$ production process was able to continue for about 25 days (Leino *et al*., 2012), but the $H_2$ yield declined over time. Apart from carbon limitation, there are other factors that may affect the duration of photoproduction of $H_2$ by immobilized heterocystous filamentous cyanobacteria. With the elevated availability of carbon, the photosynthetic rates also increase, creating a demand for nitrogen. Prolonged N-limitation (lack of combined N and elimination of $N_2$) can impair the efficient repair of the photosynthetic apparatus, thereby compromising the overall fitness of the cells.

To further optimize $H_2$ photoproduction, an additional phase was introduced for the recovery of cells exposed to the long-term experiments in **Paper I**. Here, the cells were periodically exposed (for about 16–20) to air-treatments such as air + 6% $CO_2$, or air with atmospheric levels of $CO_2$, every fourth day. The air-treatments supplied cells with $N_2$, thus allowing nitrogen-deficient cells to fix $N_2$, to repair the photosynthetic apparatus and other impaired metabolic activities. The results from **Paper I** clearly show that the recovery phase improved $H_2$ photoproduction in *Calothrix* 336/3 and Δ*hupL*, while it made negligible improvements in *Anabaena* 7120, although this strain showed improved $O_2$ evolution. Such difference in the wildtype *Anabaena* may be due to specific unknown features of $H_2$ metabolism in different cyanobacterial strains. Most importantly, the optimization study demonstrated that an immobilized cyanobacterial matrix is a very complex system, and several factors such as (i)

oxidative stress, (ii) nutrient limitation and (iii) C/N balance can affect efficient and prolonged $H_2$ photoproduction.

### 5.2. Response to C/N imbalance and oxidative damage

An interesting observation was made regarding the Δ*hupL* mutant of *Anabaena* 7120 entrapped in alginate films and treated with Ar + 6% $CO_2$ and Air + 6% $CO_2$ (**Paper I**). These samples showed significant degradation in their Chl *a* content and a decline in photochemical activity (**Figure 5 & 6 in Paper I**) in comparison to the wildtype *Anabaena*. Additionally, Δ*hupL* also showed reduced cell fitness as compared to its WT when exposed to prolonged incubation under Ar + 6% $CO_2$ and air + 6% $CO_2$ (**Figure 3 in Paper I**). Here, presumably (i) the lack of the oxyhydrogen reaction due to impaired uptake hydrogenase, although partially compensated by respiration, and (ii) compromised porosity of the alginate films led to elevated levels of $O_2$ that eventually seep to the heterocysts from the vegetative cells. As a result, the activity of the $O_2$ sensitive nitrogenase enzyme is impaired during the periods of air treatments, resulting in inefficient fixation of $N_2$. Consequently, the inability to efficiently fix $N_2$ leads to a failure in restoring the organism's photosynthetic apparatus (**Paper I**). The carotenoid to chlorophyll *a* ratio also plunged in the Ar + 6% $CO_2$ and air + 6% $CO_2$ samples of Δ*hupL* (**Figure 6 in Paper III**) towards the end of the experiment (450 h), showing changes in carotenoid content and a need for photoprotection. Following this chain of reactions, an imbalance in the C/N ratio would have caused metabolic imbalances in the Ar + 6% $CO_2$ and air + 6% $CO_2$ samples of Δ*hupL*. Similarly, in *Cyanothece* sp. strain PCC 7822 (unicellular diazotrophic cyanobacterium), Hup was found to protect nitrogenase from $O_2$ toxicity (Zhang *et al*., 2014).

Previously, several lines of evidence have shown that the availability of elements such as nitrogen, phosphate, sulfate and carbon can all influence the production of glycogen and extrapolymeric substances (EPS) in cyanobacteria (Sohm *et al*., 2011; Austin *et al*., 2004; Moreno *et al*., 1998); the C/N ratio is an especially important parameter (Rossi and De Philippis, 2015). A study published a few years ago on different *Nostoc* species found that a higher amount of available carbon than N triggers EPS production in cyanobacteria, in order to store the excess carbon (Otero and Vincenzini, 2014). In the tested immobilized cells, growth is limited by N-deprivation. However, the CCM is robust and the consumption of $CO_2$ takes place in spite of the inability of the filaments to grow. The resulting fixed carbon needs to find a sink for storage. Depending on the

species, cyanobacteria have different strategies for dealing with the excess fixed carbon. In the case of *Calothrix* 336/3, the EPS could be the C-sink due to its known sheath layer, which is a component of EPS. It is also visible as stored carbohydrates outside the cell, unlike in the *Anabaena* species, where roughly equal divisions of stored (glycogen and non-glycogen) carbohydrates were observed (**Figure 2 in Paper III**).

Photosynthetic organisms constantly evolve $O_2$ when exposed to light, and as a result, oxidative stress is an inevitable factor. For example, cyanobacterial films in nature are frequently battling with the accumulation of ROS due to limited diffusion of the evolved $O_2$ molecules. As a result, there is a constant challenge of toxic reactive oxygen species (ROS) in the cyanobacterial photosynthetic apparatus (Jensen *et al*., 2011). Similarly, signs of oxidative stress were evident in our films that were subjected to long-term $H_2$ photo-producing conditions; these signs included elevated levels of α-tocopherol and xanthophylls, total protein oxidation and the degradation of D1 protein (**Figure 3, 4, 5 and Table 1 in Paper III**).

### 5.3. Role of carotenoids in photosynthetic stability under $H_2$ producing conditions

In cyanobacteria, carotenoids (carotenes and xanthophylls) are essential for light harvesting, photoprotection (Schafer *et al*., 2005; Sozer *et al*., 2010) and the structural stability of a variety of pigment-protein complexes. Recent studies have demonstrated that carotenoid pigments such as zeaxanthin and echinenone have a complex protective mechanism for shielding the repair of the PSII recovery cycle from photoinhibition. These carotenoids achieve this by decreasing the level of singlet oxygen that inhibits protein synthesis (Kusama *et al*., 2015). In my study, zeaxanthin did not appear to cause major changes, though echinenone showed a tendency to decline in both the *Calothrix* and *Anabaena* species (**Paper III**) despite photo-damage being quite evident, especially in Δ*hupL*. The decreased level of echinenone could be due to this carotenoid being involved in the light harvesting mechanism for the $N_2$ fixation process under active growth, and thus not required to the same extent under $H_2$ photo-producing conditions (**Paper II**). Another possible explanation could be damaged protein biosynthesis machinery, which failed to efficiently repair the photosynthetic apparatus (**Paper I**).

It is worth mentioning that another recent study (Sedoud *et al*., 2014) has revealed the dual role of carotenoids in OCP (orange carotenoid protein): (i) protection of photosystems by quenching the excess energy, and (ii) quenching of singlet oxygen generated during the light reactions. 3'-hydroxyechinenone is an important component of the OCP (Kerfeld *et al*. 2003). A spiking tendency in the level of 3'-hydroxyechinenone under $H_2$ photo-producing conditions was noted in the *Calothrix* samples (**Paper II and IV**), which correlated well with the decline of echinenone in the tested samples. When the cells are not in a state of active growth, the excess energy reaching the reaction centers of the photosynthetic apparatus is dissipated by the rearrangement of the PBSs and by the quenching properties of the OCPs (Onishi *et al*., 2015; Punginelli *et al*., 2009). The prominent accumulation of different myxoxanthophylls in *Calothrix* samples suggests that oxidative stress is present. In general, it was observed that *Calothrix* 336/3 had a wider variety of oxygenated carotenoids (ketocarotenoids and hydroxycarotenoids) than *Anabaena* 7120 and displayed a more stable photosynthetic apparatus that is able to partially recover even under N-deprivation and high $CO_2$ levels (**Paper II and IV**). Therefore, the accumulated hydroxylated carotenoids may contribute to the stability of the *Calothrix* strain in response to photoinhibition and oxidative stress under $H_2$ photo-producing conditions.

### 5.4. α-Tocopherol and its antioxidant property in cyanobacteria

α-Tocopherol is an efficient antioxidant known to scavenge singlet oxygen in phototrophic organisms (Rastogi *et al*., 2015). In **paper III**, a significant spike in the level of α-Tocopherol was observed in all the tested samples harvested at the end of the experiment (450 h) when compared to 0 h samples. Predominantly, *Calothrix* showed a significantly high level of α-Tocopherol in all the samples in comparison to *Anabaena*. We speculate that this spike in α-Tocopherol content might be in response to the increased levels of $^1O_2$ caused by the $O_2$ evolved during long-term exposure of the films (entrapped with cyanobacteria) to continuous light. Supporting this observation, a significant accumulation of myxoxanthophylls was measured in the cells harvested at 450 h, evidently marking the presence of oxidative stress. In a work published by Trebst *et al.* (2002), it was proposed that in the model organism green alga, *Chlamydomonas reinhardtii* α-Tocopherol protects PSII from oxidative stress that is induced high light. Furthermore, Maeda *et al*., in a paper published in

2005, demonstrated poor growth in tocopherol-deficient mutants of *Synechocystis* sp. PCC 6803 when subjected to oxidative stress caused by the combined effect of polyunsaturated fatty acids and high light. These studies in oxygenic phototrophic model organisms indicate that the antioxidant role of α-tocopherol is conserved among the oxygenic phototrophs.

### 5.5. Peculiarities of the native strain *Calothrix* sp. 336/3

The native strain *Calothrix* 336/3 showed more resilience to long-term $H_2$ photo-producing conditions than the reference strains *Anabaena* and Δ*hupL* (**Paper I, IV**). I propose that the following reasons contribute to this ability: (i) the presence of a very diverse carotenoid synthesis pathway including diverse xanthophylls (**Paper II, IV**), (ii) the high biofilm-forming feature of this strain and (iii) the high level of α-tocopherol accumulation under oxidative stress conditions (**Paper III**). In the immunoblots, it was noted that D1, the PSII core protein of *Calothrix* 336/3, recovered to some extent in all three studied conditions by the end of the experiment (**Paper III**), which was not the case with the *Anabaena* strains.

### 5.6. Atmospheric composition affects growth and heterocyst formation in cyanobacteria

The effects of the changes in atmospheric gas ratio and partial pressure on the cellular growth of photosynthetic microorganisms is an area of science that is under-studied, especially considering the potential uses of cyanobacteria in space applications, for instance in bio-regenerative systems linked to local resource utilization and the procurement of biomass for nutrient retrieval, fuel and clean $O_2$ (Cockell, 2014; Verseux *et al*., 2015). The research findings from **Paper IV** are an indication of the direction of cyanobacterial cellular acclimation and response to simulated Martian-like atmospheric composition under duration of either 3 or 7 days. Changes in the atmospheric composition of the cyanobacterial cultures, such as supplementation with $CO_2$ or nitrogen and the presence or absence of $O_2$, had an impact on the growth of the tested cyanobacterial strains. As anticipated, this change in the gaseous ratio did cause fluctuations in the C/N ratio and lead to heterocyst differentiation in *Anabaena cylindrica* filaments. The highest heterocyst frequency and $H_2$ photoproduction was noted in cultures that were grown in $BG11_0$ (N-deficient) media exposed to ambient pressure and air supplemented with 10% $CO_2$ (**Figure 5a & 6d in Paper**

**IV**). In general, high $CO_2$ availability led to an inclination towards heterocyst formation and $H_2$ photoproduction in *Anabaena cylindrica.* Interestingly, all the species tested (*Synechocystis* sp. PCC 6803, *Anabaena cylindrica* and *Arthrospira platensis*) were able to tolerate 100% $CO_2$ at varying pressures. Some cases even showed statistically significant increased growth under various low-pressure conditions ranging from 50 – 250 mbars in comparison to the control cells grown under ambient pressure (1 atm). Eventually, however, the inhibitory nature of $CO_2$ was evident when applied at levels as high as 100% under 1 atm pressure (**Figure 2a in Paper IV**). The results of this work depict that modified Martian-like atmospheric composition when combined with various low-pressure conditions supported cellular growth in cyanobacteria. In addition, a high $CO_2$/low $N_2$ ratio triggered differentiation of a high amount of heterocysts, resulting in significant amount of $H_2$.

# 6. CONCLUSIONS AND FUTURE PERSPECTIVES

The major part (**Papers I, II** and **III**) of my doctoral thesis deals with the optimization of long-term $H_2$ photoproduction in the native and model N-fixing heterocystous cyanobacteria immobilized in $Ca^{2+}$-alginate films and investigating its effects on cellular metabolites such as carotenoids and glycogen. My research findings show that combining the immobilization of cyanobacteria with recovery air-treatments not only improved the $H_2$ production yield but also extended the potential production period. It was also revealed that the entrapment and placement of cyanobacteria under $H_2$ photoproducing conditions contributes to oxidative stress. Alginate-entrapped cyanobacteria, when maintained under $H_2$ photoproducing conditions, employ various acclimation strategies to counteract C/N imbalance and the inevitable oxidative stress. Most interestingly, these acclimation strategies were observed to be strain specific. The native strain *Calothrix* 336/3 was resilient against C/N imbalance, whereas the Δ*hupL* mutant of *Anabaena* was adversely affected by C/N imbalance. Additionally, *Calothrix* has a diverse range of oxygenated carotenoids (ketocarotenoids and hydroxycarotenoids) than *Anabaena* 7120. The accumulation of hydroxycarotenoids in *Calothrix* may be the reason for this strain resilience against the photoinhibition and oxidative stress encountered under $H_2$ photoproducing conditions. Glycogen levels were also measured due to the conserved nature of this compound as a preferred storage material in several of the model strains of cyanobacteria when a carbon source is available. The results were again strain specific: while the *Anabaena* strains demonstrated accumulation of internally stored carbohydrates (glycogen associated), *Calothrix* tended towards non-glycogen stored carbohydrates, which are likely to be carbohydrate-enriched EPS.

The current drawback of this model system is the compromised porosity of the alginate films, which results in the accumulation of oxygen in the films during photosynthesis, gradually leading to oxidative damage. Therefore, the engineering of a superior artificial film with improved porosity and stability would lower the current challenges related to combating oxidative stress, and as a result enhance and prolong $H_2$ photoproduction in cyanobacteria using our model system. To overcome this bottle-neck of $O_2$ accumulation inside the alginate films, our group is currently investigating novel, renewable and biodegradable materials that can be optimized with controllable porosity and

mechanical stability (Jämsä *et al*., 2018). Such an alternative may be more suitable for $H_2$ photoproduction over the long term from an industrial perspective. In addition, the development of such a material would open other applications such as the retrieval of targeted compounds and limited resources like metals and other industrially important compounds that are washed away with the effluents.

**Paper IV** of my thesis was dedicated to exploring (a) the effect of Martian-like atmospheric composition on the growth of different types of cyanobacteria (filamentous and unicellular) and (b) the $H_2$ photoproduction capability of the $N_2$-fixing filamentous cyanobacteria, *Anabaena cylindrica*, when exposed to low pressure conditions. All tested strains were able to tolerate an atmosphere with 100% $CO_2$, and growth was enhanced in some cases in a species-specific manner. Most interestingly, 100% $CO_2$ atmosphere under a low pressure of 100 mbars affected $H_2$ production in *Anabaena cylindrica* positively. However, there were some limitations to this study, making the results obtained here simply indicative of the effects caused by the Martian-like atmospheric conditions. Further investigation at several time points over an extended period of experimentation will be required to estimate the cellular adaptations that will occur over time. Overall, I was able to show that cyanobacteria could be a potential candidate for the production of biomass, oxygen and even $H_2$ under Martian like conditions, and this research encourages further screening and investigation to find the most suitable candidates.

In my doctoral research project, I have touched on very ambitious topics, such as the possibility of using cyanobacteria as a sustainable production unit for biohydrogen, carotenoids, and sugars for industrial applications, and as a support component for manned expeditions to far away destinations, such as Mars. These research areas have a lot of potential, especially when considering the current energy demands and the ongoing search for alternative fuel sources that are sustainable and have a small carbon footprint.

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