Use of algae technology for production of biohydrogen from green microalgae

Possibilities for a practical sustainable process and diversity at both species selection, culturing and gene transcript levels

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Abstract

Algae technology represents an extensive research field which has developed rapidly over the last decades. The research activities extend from algae cultivation including CO_2 capture, production of commercial products such as health food, aquaculture and animal feed, production of valuable metabolites, to conversion of solar energy into energy carriers like biohydrogen or biodiesel. A combination of several aspects of algae technology into a multidisciplinary process is proposed in this work. Valuable metabolites produced by algae include for example carotenoids, unsaturated fatty acids, vitamins, glycerol, components with medical activities and a number of antioxidants. Many of these are secondary metabolites produced as a response to different forms of environmental stress, and they may function as protection mechanisms to avoid damage to the cells. Biohydrogen from green microalgae is an expanding field which has made great progress through the last decade. By exposing some species of algae to environmental stress, e.g. by depriving the algae of sulfur in light, it is possible to produce significant amounts of hydrogen gas. However, this technology is still in its infancy, and there is significant potential for technology development and improvement at every level. In this study, the possibility of producing hydrogen from solar energy by using green microalgae is explored at species selection-, culturing- and gene transcription levels. It is demonstrated that there is a considerable number of species currently known to have potential for hydrogen production, and the same is true for production of valuable metabolites. The effects of different stress reactions on production of the valuable components are described, along with the purpose of their production. This knowledge can be used to evaluate the possibilities for producing hydrogen and high value products efficiently in the same process. Hydrogen production under sulfur deprivation is explored in several species of green algae under controlled conditions, and *Chlamydomonas noctigama* shows the ability to produce hydrogen with efficiency comparable to the model organism *Chlamydomonas reinhardtii*. The ability to produce hydrogen under sulfur deprivation is also explored in relation to the different species' ability to show heterotrophic or mixotrophic growth on acetate. A photobioreactor specifically designed for algae

hydrogen production is described for lab scale research purposes, including considerations for measurement devices and materials choice. Hydrogen production by the algae *C. noctigama* is further explored at molecular level. By using RT-PCR followed by PCR with degenerate primers, mRNA with homology towards green algal hydrogenases was identified. The cDNA sequences were translated to putative amino acid sequences, and analyzed in respect to amino acids characteristic for green algal hydrogenases and amino acids which share characteristics with both hydrogenases and narf-like proteins. These results were used to evaluate the identification of the mRNA sequences found in *C. noctigama*. While other green algae have been shown to contain two different hydrogenases, it is here demonstrated that *C. noctigama* is able to transcribe three distinct genes which share essential characteristics with hydrogenases.

The combination of these results provides valuable insights at several levels of a combined process for production of biohydrogen and other valuable products. Further studies of these topics may result in a sustainable process where solar energy can be converted into hydrogen in an integrated manner, where production efficiencies are sufficient for an economic exploitation of algal technology using algal stress reactions.

Scientific Environment

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List of papers:

Paper I: K. Skjånes, P. Lindblad, J. Muller, (2007). BioCO$_2$ - a multidisciplinary, biological approach using solar energy to capture CO$_2$ while producing H$_2$ and high value products. Biomolecular Engineering 24: 405-413

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Paper III: K. Skjånes, G. Knutsen, T. Källqvist, P. Lindblad, (2008). H$_2$ production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design. International Journal of Hydrogen Energy, 33: 511-521.

Paper IV: K. Skjånes, F.L. Pinto,. P. Lindblad. (2010). Evidence for transcription of three genes with characteristics of hydrogenases in the green alga *Chlamydomonas noctigama*. International Journal of Hydrogen Energy 35: 1074-1088.

Contents

Abbreviations:

AA - arachidonic acid

ATP – adenosine triphosphate

BHT – butylated hydroxytoluene

cDNA – complementary deoxy ribonucleic acid

Fe – iron

gsp – gene specific primer

MAA – mycosporine-like amino acid

PCR – polymerase chain reaction

PSI - photosystem I

PSII - photosystem II

PQ - plastoquinone

PUFA - polyunsaturated fatty acid

Q_A – quinone A

RACE - rapid amplification of cDNA ends

mRNA – messenger ribonucleic acid

RT – reverse transcriptase

RuBisCO - ribulose-1,5-bisphosphate carboxylase

ROS – reactive oxygen species

S - sulfur

TAG – triacyl glycerol

tag - molecular tool for tagging primers

UV light – ultraviolet light

1. Introduction

Today's modern society has made us dependent of having access to continuous supply of energy in order to maintain our lifestyle. Our current energy consumption worldwide is in the proximity of 15 TW, while the energy consumption rate in 2050 has been estimated to be at least 27 TW, and in 2100 >40 TW (Lewis and Nocera 2006). The majority of this energy is at the moment obtained from fossil fuels, and any change requires improved technology for use of alternative energy sources. Efforts are being made all over the world to find alternatives to fossil fuels, including nuclear power, wind power, hydro power, geothermic, biomass conversion and more. There is however no doubt that solar energy is the largest source of renewable energy that we know of today. Several technologies are being explored for use of solar energy as a renewable energy source, including indirect methods like water power, wind power and biomass, and more direct methods for use of the solar radiation. The different fields of technology for use of solar radiation include chemical/ physical methods like photovoltaic, concentrating solar power, thermovoltaic, photochemical and thermochemical, and use of biological approaches such as artificial photosynthesis and biophotolysis (Rajeshwar et al. 2008). Practical use of solar energy requires conversion of the energy into an energy carrier, and one of the promising candidates for alternative energy carriers is hydrogen. Combustion of hydrogen gas is an extremely clean process, emitting only water as a byproduct. Hydrogen is also very light with a high energy to weight-ratio, and it is fairly safe compared to other energy carriers. Technology for use of hydrogen gas for transport has already come quite far, all major car producers now offer hydrogen powered cars with competable properties in terms of speed, space and mileage. However, the majority of hydrogen which is used today, is produced from fossil fuel.

Ever since Gaffron and his co-workers discovered that the green alga *Scenedesmus* was able to produce hydrogen during anaerobic conditions (Gaffron and Rubin 1942), the idea of hydrogen production from solar energy using biophotolysis from algae, has intrigued researchers all over the world. However, it is not until the last decade that possibilities have emerged for the development of hydrogen production technology

from algae with potential for viable efficiencies. Algae produce hydrogen as a response to anaerobiosis in light, as a safety valve to avoid oxidative stress. Hydrogen production is catalyzed by enzymes called hydrogenases, which are easily inactivated by oxygen. This incompatibility between oxygen producing photosynthesis and hydrogen production has until recently made it difficult to sustain a significant production of hydrogen from light by algae. The breakthrough in this field appeared when Melis and co-workers discovered that sulfur deprivation could cause a significant production of hydrogen in light by the green microalgae *Chlamydomonas reinhardtii* (Ghirardi et al. 2000; Melis et al. 2000). Through this mechanism, it became possible to produce high amounts of hydrogen over a period of several days. A number of species of green algae have to date been found to contain at least one hydrogenase (**Paper II**, Table 1), while it currently appears to occur less often in other groups of algae. Due to this fact, this study has focused on green microalgae only.

Algae technology is today used commercially for production of algal biomass for health food, aquaculture, animal feed and industrial purposes. The algae can either be used directly, or metabolites can be extracted and sold individually. The number of species in commercial use today is very low (**Paper II**, Table 2), but there is a significant potential for increasing this number. Some green algae can be found in environments that can cause serious challenges for living organisms, like on Arctic ice and snow, desert crusts, salt marches and in nutrient deficient water (Barsanti et al. 2008). Different adaptation mechanisms allow these algae to survive the harsh conditions, and some of these mechanisms give production of secondary metabolites with potential to be commercially exploited. Hydrogen production can also be considered to be such a mechanism, an emergency reaction induced by stress, which provides a product with high commercial potential.

Important research has been carried out in this field since the mechanism of hydrogen production by sulfur starvation, was discovered (Ghirardi et al. 2009; Melis 2007). The majority of this research has focused on the model organism *C. reinhardtii*, which is where the process was initially detected. Even if substantial progress is continuously made, there are still many unknowns regarding hydrogen production mechanisms and

how the efficiency can be improved. A fundamental understanding of this topic at every level is still needed in order to obtain a sustainable system in the future.

Challenges and possibilities for optimization include:

- Overall analysis of possibilities for increasing practical and economic potential, including combination of several areas of algae technology
- Practical implementation such as design of bioreactors, techniques for harvesting of products, infrastructure, economic feasibility, feasibility regarding technological efficiency
- Hydrogen production efficiency determined by physical/chemical factors such as light intensity, temperature, pH, carbon source and medium composition
- Hydrogen production efficiency determined by selection of species with optimal properties
- Hydrogen production efficiency determined by sulfur deprivation mechanisms
- Hydrogenase enzyme: structure, specific activity, oxygen sensitivity, interaction with electron donor
- Hydrogenase enzyme: presence of different hydrogenases and their respective function in the cell
- Hydrogenase genes: sequences, transcription, regulation, complementing genes and their function, new gene compositions

A number of these topics are addressed in this work.

2. Aim

The aim of this study has been to explore the potential for using algae technology for producing hydrogen from solar energy. This has been studied at several different

levels, including gene characterization, hydrogen production by sulfur deprivation, the possibilities for a complete multidisciplinary process by involving a number of algal technological approaches, and the diverse abundance of algal species with characteristics which potentially can be used for a sustainable practical process. The multidisciplinary process which is proposed, combines hydrogen production from green algae with production of other valuable products which bring added value to the process, thereby increasing the likelihood of commercial success. Hydrogen production during sulfur deprivation has been studied in a selection of green algal species in a purpose-specific designed bioreactor, and hydrogenase enzymes in a selected species have been explored in respect to transcription and sequence characterization.

3. Background –mechanisms for hydrogen production under sulfur deprivation

Some species of green algae are able to produce hydrogen as a response to stress conditions. During anaerobic conditions in light, these algae can release electrons from the system in the form of hydrogen gas, as an emergency reaction to avoid oxidative damage to the cells. The most promising method at the moment for inducing hydrogen production in green algae may be sulfur deprivation (Ghirardi et al. 2009; Melis 2007). By depriving the algal cells of sulfur and exposing them for light, the culture can produce significant amounts of hydrogen for a period of several days, or even for a longer period of time with the proper adjustments of the method (Fedorov et al. 2005; Kim et al. 2010). The mechanisms behind this process are described below, as studied in the model organism *C. reinhardtii*. As discussed below, the majority of research on hydrogen production during sulfur deprivation, has until recently been performed using acetate as a carbon source.

When *C. reinhardtii* is deprived of sulfur, several changes in the cell's metabolism may occur. During lack of an important nutrient like sulfur, production of important cell components such as sulfolipids and proteins may stop, which leads to prevention of cell division and growth. The algae may show increased production of starch for the

first few days, followed by degradation of RuBisCO and the other proteins of the Calvin cycle, which leads to a halt of this CO_2 reducing pathway (Zhang et al. 2002).

When sulfur is removed from the medium and the algae are exposed to light, the photosystem II (PSII) reaction center becomes partly inactivated due to several factors. A sulfolipid in the thylakoid membrane associated with PSII is specifically degraded (Sugimoto et al. 2007), non-reducing Q_A leads to a decreased electron transport between PSII and photosystem I (PSI) (Antal et al. 2007), and the repair mechanisms of the D1 protein are inhibited. These reactions lead to a partial inactivation of PSII, which implies that the production of oxygen is slowed down. The decreased amount of oxygen produced, can be continuously used up by the respiration, hence the culture becomes anaerobic after a couple of days (Wykoff et al. 1998). In this condition, where the Calvin cycle has stopped and no longer can function as a sink for energy, the energy absorbed by the photosystems will exceed the energy that can be consumed. This means that the photosystems are in a reduced state, a condition which may normally lead to production of reactive oxygen species (ROS) and photodamage to the cells. In order to prevent oxidative damage, hydrogen is produced as a way of relieving the reductive pressure. The electrons from the water splitting at PSII follow the electron transport chain in the thylakoid membranes via the plastoquinone (PQ) pool to PSI, they then reduce ferredoxin which transfers the electrons directly to hydrogenase where the hydrogen is produced (Antal et al. 2009; Chochois et al. 2009; Long et al. 2008). (See Figure 3, **Paper II**).

Through this mechanism, *C. reinhardtii* and other green algae species have adapted to stress from nutrient deprivation by releasing excess absorbed energy in the form of hydrogen gas.

While starch is produced during the first phase of sulfur deprivation, these starch reserves are consumed in a fermentative degradation during the hydrogen production phase. There are mainly two possible light dependent routes for electrons to reach the hydrogenase. They can be derived from the water splitting at PSII (PSII dependent pathway), or originate from the fermentative breakdown of starch (PSII independent

pathway) (Chochois et al. 2009). There are some uncertainties regarding the origin of the electrons that are released as hydrogen in light, but it has been suggested that at least some of the hydrogen originates from the PSII independent pathway where electrons enter the electron transport chain in the thylakoid membranes through the PQ pool, passing through PSI before reaching ferredoxin and hydrogenase (Hemschemeier et al. 2008).

Until recently, most of the research in this field used cultures grown under heterotrophic or mixotrophic conditions, but lately efficient hydrogen production using CO_2 as a sole carbon source has also been shown (Tolstygina et al. 2009; Tsygankov et al. 2006). However, in order to obtain an efficient production under autotrophic conditions, at the current stage of development a strict regime of light intensities must be followed. Issues such as energy efficiency of the total process, economy and risk of contamination of the cultures, all indicate that hydrogen production from autotrophic cultures will be a great advantage at many levels.

4. Results and discussion

4.1. A multidisciplinary approach using solar energy to capture CO_2 while producing hydrogen and high value products from algae

There are many areas of algae technology with potential for commercial products, and in many cases there are already established markets for algal production for different purposes. Examples of products with commercial success are algal biomass for health food, aquaculture and animal feed, and extracted metabolites like β-carotene and astaxanthin to be used as colorants and antioxidants (Olaizola 2003; Spolaore et al. 2006). Other uses are still highly experimental, like for example incorporation of algae into construction materials. Although hydrogen production from algae is also still at an experimental stage, there is great interest for this emerging field. CO_2 from exhaust gas from fossil fuel combustion may be used as a carbon source for algal cultivation,

providing an opportunity for CO_2 capture and management. A proposed multidisciplinary system for combining hydrogen production and other areas of algae technology involving CO_2 capture from exhaust gas, is presented in the following.

In the first stage of the proposed multidisciplinary process, CO_2 from exhaust gas is used by algae as a carbon source thereby capturing the CO_2 using solar energy through photosynthesis. The algal biomass is then deprived of sulfur and induced to produce hydrogen under anaerobic conditions in the second stage. After hydrogen production using this technique, the remaining algal biomass can be used for several purposes. The simplest option is to use the biomass directly as health food, aquaculture and animal feed. Biomass can also be used for extraction of valuable biomolecules which often consist of secondary metabolites. Other options for use of the remaining biomass include biofuel production, application as fertilizer, or incorporation into construction materials. The proposed process has been divided into modules representing each separate step (**Paper I**).

4.1.1. CO_2 capture

CO_2 emissions from industry are considered to be an important contributor to increased CO_2 concentrations in the atmosphere, which are believed to cause a man-made global warming effect. Photosynthesis is nature's CO_2 capture mechanism where this inorganic source of carbon is reduced and incorporated into organic carbon and biomass. Microalgae are able to fix CO_2 with efficiencies 10 times higher than that of terrestrial plants measured as biomass production per m^2 (Usui and Ikenouchi 1997). Culturing algae using exhaust gas as a carbon source, entails several potential challenges. These include high temperature, high CO_2 concentration, low pH, presence of toxic components like SO_x, NO_x and CO, and presence of heavy metals. Species of algae vary greatly in terms of optimal growth conditions and in terms of tolerance towards contaminants and toxic compounds. Some strains of green algae are able to grow under both very high CO_2 concentrations (20-100% bubbling of cultures) and

high temperatures (Wang et al. 2008). One example is a strain of *C. sorokiniana* isolated from a hot spring (Sakai et al. 1995), other examples are strains of *Scenedesmus* sp. (de Morais and Costa 2007; Hanagata et al. 1992) and *Chlorococcum littorale* (Satoh et al. 2002). It is hypothesized that tolerance towards high CO_2 is connected to state transition in favor of PSI (Miyachi et al. 2003; Satoh et al. 2002). CO, SO_x and NO_x are contaminants in exhaust gas from fossil fuel combustion that have toxic effects on many organisms. However, some algae are able to tolerate or even thrive under this exposure, the algae may in some cases use these components as nutrients (Brown 1996; Douskova et al. 2009; Lee et al. 2002). Exhaust gas from fossil fuel combustion often contains heavy metals, which represent potential health risks when exposed to the organisms in the environment. Heavy metals cause damage to the cells by binding to enzymes and other important cell components, thereby preventing essential cell functions. Some algae show tolerance towards heavy metals that cause lethal damage in other strains. Mechanisms for heavy metal tolerance involve avoidance by binding to cell wall components or excretion of metal binding organic compounds to the environment. Other mechanisms involve detoxification by binding to metal binding proteins, followed by precipitation in the cytoplasm or vacuoles, and excretion from the cell (Hart and Bertram 1980; Juarez et al. 2008; Kaplan 2004). Degree of toxicity of different heavy metals is sometimes decided by competition to adsorption sites on the plasma membrane (Rachlin and Grosso 1993). Heavy metals resistance can often be found in algae isolated from contaminated environment, while this resistance is gradually lost when the metals are absent from the medium (Vilchez et al. 1997). CO_2 capture from fossil fuel by algae is possible, however it is very important to choose a strain with resistance against high CO_2 concentrations, heavy metals and CO, SO_x and NO_x. The suitable algae can be found by isolating strains from an exhaust gas exposed environment. Important aspects for culturing algae as for example bioreactors and conditions for optimal growth, is thoroughly reviewed elsewhere (Carvalho et al. 2006; Posten 2009; Pulz 2001; Xu et al. 2009) and is not discussed in this work.

4.1.2. Hydrogen production

The second stage in the proposed process, involves hydrogen production after CO_2 capture, by sulfur deprivation. During sulfur deprivation, several changes in the cells' metabolism will occur, as described above and in **Paper II**. In certain species of green algae these changes in the metabolism can lead to production of significant amounts of hydrogen, as an emergency reaction to prevent cellular damages. The mechanism behind hydrogen production during sulfur deprivation is thoroughly discussed in section 3. One great advantage of this method compared to the more traditional conversion of algal biomass to hydrogen, biodiesel or other energy carriers (see below), is that this method does not consume the biomass. Consequently, after hydrogen production by sulfur deprivation, high amounts of algal biomass remain and can be used for other purposes, bringing added value to the proposed multidisciplinary process.

4.1.3. Use of algal biomass

Health food, aquaculture and animal feed: Algae have been used as a food source for centuries, and currently there is an increasing awareness of this food source's potential. Algal biomass can contain a high amount of nutrients such as proteins, minerals, vitamins, unsaturated fatty acids and antioxidants. The production of algae as health food is currently limited to only a few species, and the main products consist of tablets, capsules or powder. Algal biomass is an essential dietary source used in aquaculture, which is a fast growing market with high potential (Pulz and Gross 2004). Applications as feed for animals such as for example poultry, is also significant. The topic of potential and current commercial products is thoroughly discussed in **Paper II**.

Extraction of metabolites: Some algae will induce adaptation mechanisms as a response to stress conditions, and in some cases the algae may produce certain secondary metabolites which have properties of medical or industrial significance.

Some of these metabolites are thoroughly characterized, such as polyunsaturated fatty acids and carotenoids (Del Campo et al. 2007; Guschina and Harwood 2006), others are less studied and in some cases not even identified (Chu et al. 2004; Ördög et al. 2004). Metabolites for industrial use include for example colorants, antioxidants and waxes (Cardozo et al. 2007). Very little is known about the potential for a simultaneous induction of hydrogen production and valuable components. The topic of extractable metabolites is thoroughly discussed in **Paper II**.

Fertilizer: Algal biomass used as a fertilizer has water binding properties and may improve the soil's mineral composition (Riley 2002). This may be a potential use of the algal biomass either directly, or after extraction of metabolites.

Novel industrial materials: Another alternative for use of algal biomass is incorporation into plastic materials. Experimental methods for incorporation of algae into polypropylene (Zhang et al. 2000a), polyethylene (Otsuki et al. 2004) and PVC (Zhang et al. 2000b) have been developed and satisfactory properties have been measured with up to 50 dry weight % algal biomass.

Biofuel: Algal biomass can be converted into biofuel by a variety of different methods. Thermochemical methods such as gasification, pyrolysis or liquefaction or even direct combustion convert the stored energy into gases such as hydrogen or methane, oils, charcoal, electricity, heat and mechanical power (Bridgwater 2003). Biological methods include fermentation of the biomass to produce energy carriers like bioethanol, biomethane, biohydrogen, or extraction of lipids and hydrocarbons to produce biodiesel (Hu et al. 2008; Mata et al. 2010). Advantages of producing biofuels from algae compared to biofuels from energy crops include production possibilities on non-arable land, low water consumption, no competition with food production, and high biomass per area ratio. However, using the algal biomass produced in this multidisciplinary approach for biofuel production, will most likely bring less income to the process than many of the other products mentioned above.

4.1.4. Precautions

Algal biomass which has been cultured using CO_2 from flue gas as a carbon source may contain contaminants, heavy metals creating the most attention due to their very hazardous potential (Satoh et al. 2005). The compositions of flue gas from different sources are likely to vary greatly. However, a study using flue gas from a municipal waste incinerator showed that algal biomass of *Chlorella vulgaris* cultured using flue gas as a CO_2 source, produced algal biomass with only small amounts of mercury, all other heavy metals and other contaminants were below the limit defined for food grade quality by European Union legislation. Treating the flue gas with activated carbon removed this contaminant and resulted in the production of algal biomass with food grade quality (Douskova et al. 2009).

However, use of algal biomass produced by using flue gas as a carbon source should be considered with extreme caution. First of all, great care should be taken in selecting the right algal strain. As described above, many algae have the ability to detoxify heavy metals by intracellular encapsulation, and this can be an adaptive response. This reaction can lead to concentration of heavy metals in the biomass, and due to the algae's ability to adapt to toxic exposure, the ability to concentrate heavy metals may increase over time. Even if all heavy metal contaminants are removed from the flue gas before it is exposed to the algal culture, using this biomass for human consumption may suffer resistance from the market. Furthermore, adding it to the food chain by using it for animal feed can be met with skepticism, even if the contents of heavy metals are below the legal limits.

4.1.5. Efficiencies

The topic of efficiencies in the different areas of algae technology is a vast field and will not be discussed in this work. Although cultivation of microalgae is already used for commercial purposes several places on earth (see **Paper II**, Table 2), careful considerations must be taken into account before a large scale production of algae for

any purpose is initiated. Economic potential for a process depends not only on the market price for the product, which may fluctuate significantly, but also on variables such as labor cost, cost of materials, cost of consumables, transport cost, water supply, electricity and required land area. A recent feasibility study regarding algal biofuel concluded that under the current market conditions, an additional production of high value products from the algal biomass is an important factor for algal biofuel production to succeed economically (Stephens et al. 2010). Not only the economical perspective has to be considered when hydrogen or other forms of biofuel from algae are to be produced, the complete energy efficiency through all steps of the process is also an essential factor for success.

Using the sulfur deprivation method, the amount of algal biomass available after the hydrogen production step depends on the chosen approach. Some approaches include continuous or semi- continuous hydrogen production as described in **Paper II**, while the method most commonly explored at lab scale involves hydrogen production for a limited period of time, which leaves a significant amount of algal biomass. The land area required for producing a significant amount of hydrogen from solar energy is also a topic of discussion and will depend on factors such as bioreactor design and efficiency of the process. However, it is clear that even with a significant increase of the current hydrogen production efficiencies using the sulfur deprivation technique, a hydrogen production plant in commercial operation will result in a large amount of algal biomass which leaves a significant potential for economic exploitation.

4.2. Variation and potential for selection of species of green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process

The multidisciplinary process described in section 4.1 aims to combine many different areas of algae technology in order to capture CO_2 from flue gas, produce hydrogen from solar energy, and suggests many different ways of using the valuable biomass after hydrogen production. As mentioned in sections 4.1.1 and 4.1.4, use of flue gas

for growing algae introduces many uncertainties to an overall process, in particular related to contamination. Also, the different ways of exploiting the algal biomass after hydrogen production are numerous, but some of these have a limited economic potential that may not be sufficient for providing sustainability to the process.

Opposed to combining all the areas of algae technology described above, one alternative can be to cultivate algae using pure CO_2, use the algae for hydrogen production by S-deprivation, and use the remaining algal biomass for high value products. Figure 1 illustrates how a limited number of modules from the overall process described in **Paper I** can be included. The term high value products in this context refers mainly to secondary metabolites with medical activity or industrial interest, but may also include algal biomass for health food, functional food, aquaculture and animal feed. The high value products may consist of secondary metabolites which have been induced during the hydrogen production process, but until now very little is known about how hydrogen production can be combined with induction and production of valuable metabolites. This part of the study aims to analyze the current knowledge on stress reactions in green algae, especially in relation to hydrogen production, and attempts to explore the potential for production of valuable secondary metabolites from different species.

The major environmental factors which affect the algae are light, temperature, nutrient availability, salt and pH as described in **Paper II**. By exposure to sub-optimal conditions, algae will change their metabolism in order to adapt to their new environment. Some green algal species are able to adapt to very harsh environmental conditions like intense sunlight, low temperatures, nutrient deprivation, high salinity and acidic pH (Barsanti et al. 2008). The stress reactions in the cells often lead to production of secondary metabolites which help to protect the cells from damaging effects caused by their environmental conditions. In some cases, these metabolites have a high commercial value, and environmental stress factors can, and already are, used in order to obtain a maximum production.

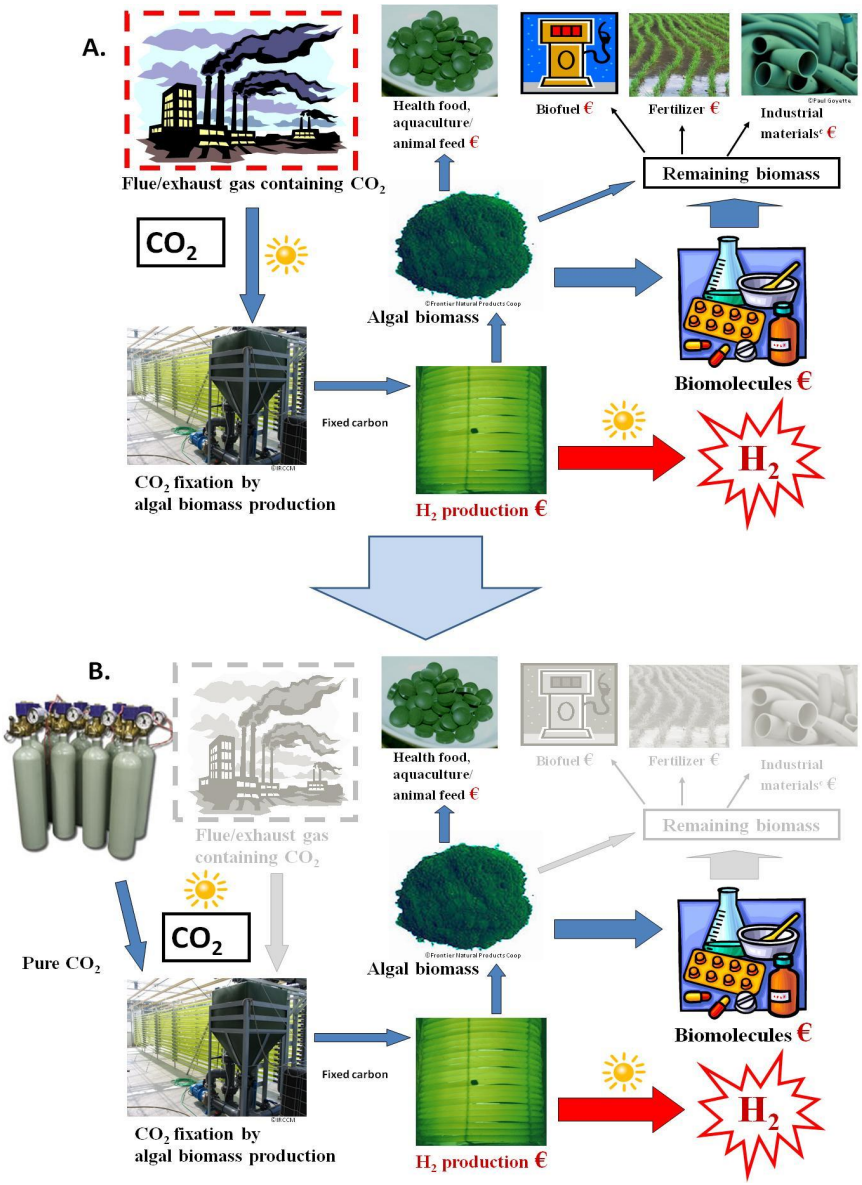

Figure 1. Alternative A): A multidisciplinary process where solar energy is used to capture industrially produced CO_2 and produce algal biomass, hydrogen, secondary metabolites, health food, aquaculture/ animal feed, fertilizer, construction materials and biofuel, is illustrated (from **Paper I**). The limitations of this process are described in the text. Alternative B): In order to increase the likelihood of success, the steps representing the highest uncertainties of the multidisciplinary process are omitted. In this system, clean CO_2 is used as a carbon source to produce algal biomass from solar energy. The algae are deprived of sulfur, and subsequently change their metabolism to convert solar energy into hydrogen. After hydrogen production, the algal biomass may contain valuable secondary metabolites which can be extracted, or used directly as an active ingredient in the biomass.

4.2.1. Stress reactions and adaptation to sub-optimal environmental conditions.

Light intensity: One major environmental factor having a high impact on the metabolism in green algae, is light intensity. When light intensity is above optimal for growth, the cells will undergo a number of changes in order to avoid photoinhibition and damage. The major damaging effects of high irradiation are caused by overexcitation of the photochemical apparatus leading to the formation of reactive oxygen species (ROS). High light intensity damage PSII by inhibiting the synthesis of D1, the important protein of the PSII reaction center, and possibly also causing disruption of manganese ions from the oxygen evolving complex (Nishiyama et al. 2006). The defense mechanisms implemented by algae to increase their tolerance to high light, involve thermal dissipation of the energy absorbed by the antenna systems, state transitions, reduced antenna size, production of antioxidants which quench ROS before they cause damage to the systems, and release of reductive pressure from the photosystems by creating electron sinks, for example in the form of starch, lipid or carotenoid production. The effects of photoinhibition may increase when the algae are exposed to additional stress factors, a fact that algae technology can take advantage of.

Temperature: Although there are no truly thermotolerant green microalgae, several species are able to tolerate very low temperatures. At low temperatures, algae can suffer from increased rigidity of membranes, slow enzymatic reactions causing oxidative stress, and freezing. Cold adapted algae implement several reactions in order to prevent damage. In order to prevent rigid membranes, algae produce increased amounts of polyunsaturated fatty acids which are incorporated into cellular membranes to sustain fluidity. Psychrophilic algae adapted to sub-zero temperatures are also able to produce antifreeze proteins that bind to ice crystals and prevent damage. To compensate for slower enzymatic reactions, the algae may produce more of a given enzyme, or adapt by shifting optimal enzymatic activity towards lower temperature (Morgan-Kiss et al. 2006). Since metabolic rates are generally slower at low

temperatures, and light absorption by the antenna pigments is temperature independent, an imbalance between absorbed and consumed energy will easily occur, causing oxidative stress. The algae can adapt by state transitions, reduced antenna size, or production of energy sinks like starch, lipids or carotenoids. Oxidative damage may also be prevented by production of antioxidants as described below.

pH: All algae are dependent on maintaining a close-to-neutral intracellular pH in order to maintain cellular processes, and most algae have limited abilities to tolerate very high or very low pH. However, some algae have adapted to highly acidic environments by pumping protons out of the cell using efficient ATP-driven H+ pumps, one example being the acidophilic *Chlamydomonas acidophila* (Gerloff-Elias et al. 2006). Under very low pH, as much as 50% of the synthesized ATP has been observed to be consumed by these proton pumps (Bethmann and Schönknecht 2009). Other reactions to low pH include increased fatty acid saturation, production of acid tolerant cell wall proteins, reduction of cell volume, reduction of starch reserves possibly as an energy source, and production of antioxidants.

Salt concentration: Although some algae have adapted to tolerate a very high salt concentration, like for example the halophilic *Dunaliella salina* (Oren et al. 2008), most algae thrive in either freshwater, brackish water or marine environments. When salinities increase above optimum for growth, algae may suffer from hyperosmotic stress, leading to impaired electron transfer between antenna pigments, and in PSII and PSI reaction centers, again leading to photoinhibition and oxidative stress. Among several different adaptive responses to salinity stress, are production of osmolytes or production of secondary carotenoids (Hadi et al. 2008). A common osmolyte in green algae is glycerol, which can be present in high amounts without inhibiting enzymatic activities. Halophilic algae are able to maintain glycerol molecules inside the cell, while less halotolerant species in many cases excrete their osmolytes and therefore are dependent on a continuous production (León and Galván 1994). In some cases algae exposed to high salinities produce high amounts of carotenoids for protection (Orosa et al. 2001).

Nutrient limitation: In nature, nutrient limitation is often the limiting factor for growth. When production of components necessary for growth is inhibited due to lack of nutrients, several adaptation mechanisms occur. Uptake mechanisms for the limiting nutrient are enhanced, and molecules in the cells containing the limiting nutrient, which are non-essential for maintaining cellular processes, are degraded. Under nutrient limitation, photosynthetic activity is decreased, there is photodamage of PSII, formation of non-reducing Q_A inhibiting electron transport from PSII to PSI, and state transition from state 1 to state 2. Nutrient deprived algae may experience photoinhibition at lower light intensity than under optimal nutrient availability. During the oxidative stress caused by nutrient deprivation, many algae will produce energy sinks like starch, lipids or carotenoids. A combination of nutrient deprivation and high light intensity can be used in order to induce an optimal production of valuable carotenoids (Jin et al. 2006), or in some cases polyunsaturated fatty acids (PUFA) (Solovchenko et al. 2008).

The effects of sulfur deprivation has lately been studied more specifically, partly due to the discovery by Melis and co-workers (Ghirardi et al. 2000; Melis et al. 2000), showing that *C. reinhardtii* is able to produce significant amounts of hydrogen in light when deprived of this essential nutrient. The mechanism behind this process is described in section 3.

4.2.2. Secondary metabolites and valuable products from algal biomass

As described above, exposure to sub-optimal levels of most of the major environmental factors can in some cases induce production of secondary metabolites as adaptation mechanisms in green algae, and some of these have a high commercial value. Some of these metabolites from green algae are already in commercial production, while other substances which algae are able to produce in high amounts, are currently on the market either in the form of synthetic molecules, or as products extracted from other organisms. One example of a synthetic product on the market,

where the natural products produced by algae are gaining an increasing share, is β-carotene. In this case it has been shown that the naturally produced pigment has higher health benefits than the synthetic version (Yeum and Russell 2002).

One of the most common responses to oxidative stress in algae is production of antioxidants, which have the ability to quench the ROS and thereby prevent oxidative damage to the cells. ROS can be responsible for many health problems, including age-related diseases, and oral intake of antioxidants have been attributed a long row of health benefits. The most important antioxidants in algae are various carotenoids, although algae are also able to produce several other antioxidants such as vitamins, butylated hydroxytoluene (BHT) and glutathione.

Carotenoids: While primary carotenoids are directly involved in the photosynthesis, secondary carotenoids are produced as a response to different environmental factors such as light intensity, nutrient limitation, temperature, pH and salinity. Secondary carotenoids can be produced in high amounts under environmental stress as a protection mechanism to avoid oxidative damage. The antioxidants extracted from algae with the greatest commercial success at the moment, are the carotenoids astaxanthin and β-carotene produced by *Haematococcus* sp. and *Dunaliella* sp., respectively. Other examples of useful carotenoids produced in high amounts by some species of green algae are lutein, zeaxanthin and canthaxanthin. Oral intake of carotenoids by humans have been attributed many positive health effects, such as anti-cancer, anti-inflammatory and neuroprotective effects, effects on ulcer, cholesterol levels, immune response, eye disease, degenerative disease, arthritis and obesity prevention. Industrial applications of carotenoids from algae include use as antioxidants and coloring agents for food and cosmetics, and as feed additive giving color to for example egg yolks, chicken skin, shellfish and salmon flesh (Bhosale and Bernstein 2005; Del Campo et al. 2007; Guerin et al. 2003; Jin et al. 2003; Lorenz and Cysewski 2000).

Vitamins: Many algae are known to produce high amounts of vitamins which have a number of health beneficiary effects. Vitamin C (ascorbic acid) is a water soluble

vitamin with antioxidant activity, and is essential for collagen, carnitine and neurotransmitter biosynthesis. It can be produced in high amounts by green algae like for example *Chlorella* sp. and *Dunaliella* sp. Vitamin E (α/β-Tocopherol) is a fat soluble vitamin with antioxidant activity, which works synergistically with Vitamin C. Vitamin E is claimed to have medical activity against cancer, heart disease, eye disease and other (Pham-Huy et al. 2008), and is also used as a preservative and as photoprotection in skin cream. It can be produced in high amounts by green algae like for example *Dunaliella tertiolecta* and *Tetraselmis suecica.* Production of vitamins by algae is induced by stress conditions such as nutrient deprivation and high light intensity (Barbosa et al. 2005; Durmaz 2007).

Other antioxidants: Butylated hydroxytoluene (BHT) is an antioxidant produced by for example *Botryococcus braunii* (Babu and Wu 2008), used as a food additive. Glutathione is an antioxidant used as a pharmaceutical compound, with a strong anti-virus activity. It can be produced in high amounts by for example *Dunaliella* sp. (Li et al. 2004). In both cases, these antioxidants have not been thoroughly studied in respect to optimization of their production by application of environmental stress factors. However, their nature as antioxidants indicates that conditions leading to oxidative stress as described above, are likely to promote their production.

Fatty acids: Fatty acids occur in the algal cells for example as glycolipids or phospholipids forming cellular membranes or as storage products such as triacylglycerol (TAG). Polyunsaturated fatty acids (PUFA) are known to have many health beneficiary effects, and some PUFAs are essential for the human diet. Some examples of these effects are anti-inflammatory, anti-thrombotic, anti-arrythmic and hypolipidemic effects, prevention of heart disease, hypertenton, diabetes and many others (Simopoulos 1999). Many green algae are able to produce high amounts of lipids, and in many cases the concentration in the cells may increase significantly during environmental stress like for example nutrient deprivation. Some algae species will mainly produce saturated and monounsaturated fatty acids, but many algae will also produce high amounts of PUFA, one example being *Parietochloris incisa*, which produce high amounts (up to 20% of biomass) of arachidonic acid (AA) during N-

deprivation (Khozin-Goldberg et al. 2002). Other environmental factors influencing the relative amount of unsaturated fatty acids, are low temperature, low light intensity and in certain cases high salinity.

Polysaccharides: Polysaccharides from green algae with medical effects have been extracted, but in many cases the exact structure is unknown. One example of an identified polysaccharide is β-1,3-glucan from *Chlorella,* which has been found to be an active immunostimulator, a free radical scavenger and a reducer of blood lipids in addition to other activities (Iwamoto 2004; Spolaore et al. 2006). Most studies have not explored whether stress conditions are able to induce increased amounts of a given polysaccharide.

Glycerol: Glycerol is produced as an osmolyte during high salinity in some species of green algae, and has applications in cosmetics and pharmaceutical industry among others (Wang et al. 2001). It can be produced in high amounts by for example the halotolerant *D. salina* (Kaçka and Dönmez 2008).

Lectins: Lectins are carbohydrate binding proteins located within protein bodies in the cells, which can be used for applications in medical science. Their presence in other groups besides green algae indicates that the production can be induced by nutrient deprivation and light intensity stress (Liao et al. 2003).

Mycosporine-like amino acids (MAA): These amino acids represent a group of molecules consisting of an amino acid bound to a chromophore that absorbs low wavelength light. They protect the organism against UV radiation and are produced by for example the highly UV-tolerant snow algae *Chlamydomonas nivalis* and other green algal species. Production of mycosporine-like amino acids is induced by exposure to UV-light, but there are indications that N-limitation leads to a decreased production (Karsten et al. 2007; Xiong et al. 1999). This UV-protecting agent is used commercially in skin-care products (Schmid et al. 2006).

Anti-freeze proteins: Cold adapted strains of green algae are often producers of antifreeze proteins which prevent damage occurring as a result of very low temperatures. Antifreeze proteins extracted from algae or other microorganisms can be

used for agricultural, biomedical and industrial applications (Christner 2010; Kang and Raymond 2004; Raymond et al. 2009).

Antibiotics: Some algae are able to produce components with antibiotic activity. In some cases this activity has only been identified in general extracts without determining the identity of the active substrate (Chu et al. 2004; Ördög et al. 2004), in other cases the antibiotic agents have been identified. There are indications that antibiotics are more likely to occur in strains isolated from environments polluted by bacteria, than in strains isolated from cleaner environments (Lustigman 1988).

In addition to the above mentioned metabolites, green algae also produce unidentified components with medical activity or other useful properties. Extracts from *Chlorella* sp., *Dunaliella* sp., *Scenedesmus* sp. and *Chlamydomonas* sp. have all showed activities of pharmaceutical interest (Borowitzka 1995; Ördög et al. 2004), but the potential for increased production by applying stressful environmental conditions has not been explored.

4.2.3. Stress reactions in algae as a tool

It is clear from the above and the results presented in **Paper II**, that all the major environmental factors light intensity, temperature, nutrients, salinity and pH are able to influence the cell content of valuable metabolites. By applying selected stress factors to selected species of algae, it is possible to induce production of large amounts of valuable products. This is a fact that has been exploited commercially for several years, although to a limited extent. Some of the current knowledge about valuable metabolites from green algae and the stress factors found to induce their production, is summarized in Table 1. In many cases, production of specific metabolites and the effect of certain stress factors have either not been explored at all, or have been studied to a very limited extent.

Table 1. Production of some specific high value metabolites from green algae vs. stress reactions. Some species of green algae are able to produce one or more of the listed metabolites during certain forms of environmental stress. Details on which species are able to produce the different metabolites in relatively high amounts, can be found in **Paper II**, Table 1. Unknown: There is scarce information available as to whether the production of these metabolites can be increased in green algae by applying environmental stress factors. In many cases, the studies have only been performed under optimal conditions for growth.

Metabolites from green algae	**Environmental stress factors currently known to induce their production**
Carotenoids	High light, nutrient deprivation, high salinity, low or high temperature
Vitamins	High light, nutrient deprivation
Unsaturated fatty acids	Low temperature, high salinity, nutrient deprivation
Glycerol	High salinity, nutrient deprivation
Lectins	High light, nutrient deprivation
Mycosporine-like amino acids	UV-light
Anti-freeze proteins	Low temperature
Butylated hydroxytoluene (BHT)	Unknown
Specific polysaccharides	Unknown
Glycoproteins	Unknown
Glutathiones	Unknown

4.2.4. Combination with hydrogen production

Many secondary metabolites are produced as response to oxidative stress caused by environmental stress factors. Their function is, as described above, to protect the cells from damage due to oxidative stress and other effects. In many cases, the valuable metabolites that are produced in high amounts under environmental stress function as energy sinks to relieve some of the reductive pressure from the photosystems. When algae produce hydrogen, the excess energy absorbed by the photosystems is released

from the cells, also as a function to relieve the reductive pressure. It is therefore likely that a simultaneous production of hydrogen and large amounts of secondary metabolites used as energy sinks, would result in a less efficient production of both. One solution to this situation, in order to obtain an economically viable process, may be to focus on production of metabolites which have higher commercial value than the typical metabolites that can be produced in large amounts such as astaxanthin, β-carotene and unsaturated fatty acids. Some algae have shown ability to produce metabolites with important medical activity, and the possibility that these may bring sufficient income to the process even if they are produced in low amounts, must be considered. Another possibility could be to create a process where hydrogen is produced for a period of time, followed by a change in the metabolism so that the absorbed energy is used to produce high amounts of a valuable metabolite instead. However, the possibility for production of hydrogen and valuable metabolites in the same process has at the moment not been extensively explored.

4.2.5. Wild type algae species vs. gene modified organisms

Significant research has been carried out in the field of hydrogen production since the mechanism of hydrogen from algae by sulfur deprivation was discovered (Ghirardi et al. 2009; Melis 2007). The majority of research has focused on the model organism *C. reinhardtii*, which is where the process was initially detected. There are several reasons for this, one being the potential for gene modification. *C. reinhardtii* is a thoroughly studied organism, and transformation techniques and other methods for genetic engineering have been developed for this species (Neupert et al. 2009; Walker et al. 2005). The number of possibilities for optimization through genomics is high. There have been significant improvements of hydrogen production efficiencies through modification of *C. reinhardtii* DNA, for example involving starch metabolism and electron transport in the thylakoid membrane (Kruse et al. 2005) or PSII D1 protein modificatio (Torzillo et al. 2009). As discussed in **Paper II**, even if gene modification may be necessary in order to obtain maximal hydrogen production

efficiency, a system based on wild types may in some cases be easier to implement in terms of permits, legislations and consumer acceptance.

In cases where a system based on a wild type species may be considered advantageous, the selection of the right species will be an essential part of the method development. First of all, the algae must have the ability to produce high amounts of hydrogen under sulfur deprivation in light. Secondly, the algae must be able to produce at least one valuable metabolite, and this production cannot compete with the production of hydrogen. The current knowledge about species where hydrogenase or hydrogen production has been detected, hydrogen production during sulfur deprivation, production of specific metabolites and algal species used for other purposes, is listed in **Paper II**, Table 1. This information may be used as a starting point for a screening to find an optimal species to be used in the proposed combined process.

4.3. Hydrogen production from selected species of green algae during sulfur deprivation and considerations for bioreactor design

4.3.1. Bioreactors

In addition to selecting the right species and conditions for production of algal biomass, hydrogen and valuable metabolites, another essential factor for a successful process is the bioreactors involved. Bioreactors for cultivation of algae in large scale are run commercially today (Posten 2009; Pulz 2001). However, bioreactors designed for hydrogen production are currently mainly operated at lab scale. Several factors are important for designing bioreactors with optimal properties, these factors include light penetration, area/volume ratio, agitation, temperature and gas exchange. Optimal properties for a bioreactor to be used for cultivation of algae, are not necessarily equal to optimal properties for a bioreactor to be used for hydrogen production (Dasgupta et al. 2010). For example, in the case of gas exchange, a bioreactor designed for optimal

growth may have addition of CO_2 as an important feature, while a bioreactor for hydrogen production may have some emphasis on trapping and collection of the produced gas.

In this study, a lab scale photobioreactor was designed for studying hydrogen production in green algae. The intention was to provide a system where controlling a number of physical and chemical factors was possible. These factors were light intensity, temperature, pH, stirring, oxygen production and hydrogen production, and the main purpose was to provide a system for research purposes, not intended for future scale-up.

The lab scale photobioreactor for hydrogen production which was designed, constructed and tested in this work is illustrated in **Paper III**, Figure 1. The culture chamber was made of a 1250 ml glass bottle with 4 ports. Port 1 was used for collection of the gas that was produced by the algae. The gas was channeled through a steel tube and into a glass collector filled with distilled H_2O placed in a beaker with distilled H_2O, so that the volume of collected gas could be measured by the volume of water replaced inside the tube. The low solubility of hydrogen in water (~0.0016g H_2/ kg water at 20 ºC, 1 atm) makes it possible to trap hydrogen gas as described, for short periods of time. A pH electrode was inserted through Port 2, measuring the pH in the culture. The pH electrode was connected to a pH control unit which allowed the system to be continuously pH adjusted using HCl addition through Port 3. This port was also used for sampling from the culture and from the headspace above the culture. A polarographic oxygen electrode was inserted through Port 4, and the oxygen level in the culture was continuously logged. The culture chambers were placed in a temperature controlled water bath, with a light panel placed in front. This system allowed for experiments with sulfur deprived green algae, where light, temperature and pH were kept stable, oxygen was continuously monitored, gas produced by the algae was continuously collected, and samples both from the collected gas and from the gas in the headspace of the reactor could be measured manually at any time.

One of the challenges that needs to be approached when designing photobioreactors for hydrogen production, is the choice of materials. Hydrogen is a very small molecule, and can penetrate materials that have low permeability for other gases, and materials that are frequently used for example in tubes for inlets and outlets in standard bioreactors designed for optimal growth. Tubes or connections made of silicon rubber are one example, which have other suitable properties like high flexibility, low cost and transparency. Variations of silicon rubber are frequently used for separation of hydrogen gas from liquid, due to the high permeability for hydrogen (Liang et al. 2002). If the gas in the headspace of the reactor is exposed to a material without sufficient hydrogen trapping properties, the hydrogen gas can escape and the production output may be significantly reduced. In the bioreactor design presented here, all openings were sealed with a silicon based material specifically designed for trapping gases including hydrogen (see **Paper III** for details).

Another challenge regarding choice of materials for bioreactors, is the severe inhibiting effect some materials may have on the organisms. For example are certain rubber and latex materials, and metals commonly found in stainless steel, known to have an inhibitory effect on algae (Jin et al. 1996; Price et al. 1986; Singh and Rai 1991; Williams and Robertson 1989). A preliminary attempt was made in this study to explore the effect of ten different rubber and plastic materials on two species of green algae. The materials tested were: Silicon rubber (two brands), natural rubber (two brands), acryl (Plexiglass, PMMA), polyvinylchloride (PVC), Polycarbonate (PC), polypropylene (PP), polystyrene (PS) and polyethylene high density (PEHD). The materials were tested for effect on growth and stress reactions in *C. reinhardtii* and *C. sorokiniana*. The results, as shown in Figure 2, showed that natural rubber caused a strong inhibition of growth of *C. reinhardtii*, but under the conditions used in this experiment, a similar effect was not observed for *C. sorokiniana*.

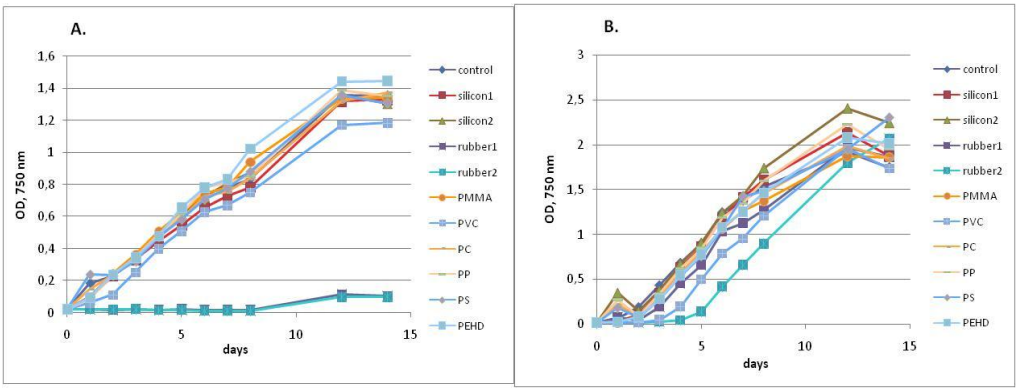

Figure 2. Growth of *Chlamydomonas reinhardtii* (A) and *Chlorella sorokiniana* (B.) under exposure to ten different rubber and plastic materials.

Natural rubber consists mainly of a polymer made of isoprene units. Isoprene is a common component of biological polymers, but is also known to be hazardous as a monomer in high amounts (Fabiani et al. 2007). The toxic effect may have been caused by isoprene monomers, or by undefined impurities of proteins, fatty acids and inorganic compounds which are also known to be present in natural rubber. Due to practical limitations in the lab, these initial experiments were only performed under one single light intensity (~100 µE m^{-2} sec^{-1}) and one fixed temperature (22ºC). As described in **Paper II**, *C. sorokiniana* is known to be highly tolerant to various stress factors like high light intensities, high temperatures, high CO_2, and heavy metals. It is possible that the difference between the strains was caused by an unknown defence mechanism against the toxic components present in *C. sorokiniana* and not in *C. reinhardtii*. Alternatively, *C. sorokiniana* being more tolerant to high light intensities in general, may have had a higher tolerance against the toxic effects from the rubber components under this specific light intensity than if the light intensity had been higher. Similarly, it is possible that the other materials would have had a larger effect on the algae under higher light intensities or other sub-optimal environmental factors. In addition to plastic and rubber, many components of metal alloys are known to have inhibiting or toxic effects on algae (Jin et al. 1996; Singh and Rai 1991). The continuation of this work will include effects of materials on several different species

of algae, at several light- and temperature conditions both above and below each algae's optimum for growth.

When different species of green algae were tested for hydrogen production during sulfur deprivation, an interesting phenomenon in the bioreactor was observed. Due to the very high oxygen sensitivity of hydrogenase, hydrogen production from wild type algae is dependent on anaerobic conditions in the culture as described above. However, in experiments with 4 of the 7 species that showed hydrogen production during sulfur deprivation, traces of hydrogen were observed during the aerobic phase (**Paper III**, Table 2). This effect was particularly evident for *Chlamydomonas noctigama*, where hydrogen was detected during the aerobic phase in 32 out of 33 experiments. It was not observed during any of the experiments with *C. reinhardtii*. When parallel experiments were performed with and without electrodes inserted into the culture, the hydrogen production during the aerobic phase was only observed in the cultures where electrodes were inserted. The cultures without inserted electrodes produced no detectable hydrogen during this phase, suggesting that the hydrogen production during aerobic conditions were related to the electrodes. A suggested hypothesis for explaining this phenomenon is the formation of a biofilm on the surface of the membrane in front of the oxygen electrode. The polarographic type oxygen electrode functions by reducing oxygen, which may cause the development of an anaerobic microhabitat on the electrode membrane. Algal cells trapped inside the biofilm would thereby be in an anaerobic environment allowing hydrogen to be produced, even if oxygen was present in the rest of the culture. Although this artefact does not represent a problem for a practical implementation of this method for hydrogen production from algae, it may be important to be aware of the danger of false positive results during research where this type of electrode is used. Exploring the reasons for differences between the species may be an interesting topic for future studies.

4.3.2. Hydrogen production during anaerobic incubation and sulfur deprivation

A total of 21 strains of green algae isolated form marine, brackish water and fresh water sources, were tested for ability to produce hydrogen during anaerobic conditions. 7 of the strains were found to produce hydrogen under these conditions, in addition to the model organism *C. reinhardtii* (**Paper III**, Table 1). The intention of this initial screening was to identify strains with the ability to produce hydrogen, for further studies. No efforts were made to identify strains that were unable to produce hydrogen.

The 7 strains were tested for hydrogen production during sulfur deprivation by harvesting algal cells in exponential phase of growth, and re-suspending in medium without sulfur. The experiments were performed using the closed bioreactors described above, with continuous oxygen logging and pH adjustment. After 1-3 days of sulfur deprivation most of the strains in most of the experiments had decreased oxygen production to a level where the produced oxygen was used up by respiration, and the cultures entered into an anaerobic stage, as shown in Figure 3. In other cases it took 5-6 days before anaerobic conditions occurred. This difference between experiments could be caused by factors like for example culture density, pigment content of cells, starch accumulation and acetate consumption. Although the algae were harvested in exponential growth phase, the cultures did not always have the exact same density between experiments at the point of harvesting. The importance of small differences in cultures after pre-growth can be a topic for further studies. Oxygen was typically present in the headspace above the cultures 1-2 days after the cultures became anaerobic, showing that the oxygen diffused slowly into the culture and was consumed immediately by the cells close to the surface.

Although some cultures were able to produce traces of hydrogen during the aerobic stage of sulfur deprivation as described above, hydrogen production in amounts measured as ml did not start until ~2-3 days after anaerobiosis in the cultures. While the model organism *C. reinhardtii* produced 80-140 ml hydrogen from a 1,15 l culture, *C. noctigama* produced typically 30-80 ml hydrogen, *Chlamydomonas euryale*

produced a maximum of 22 ml, *Chlorella pyrenoidosa* produced maximum of 10 ml and the other strains produced only smaller amounts of hydrogen.

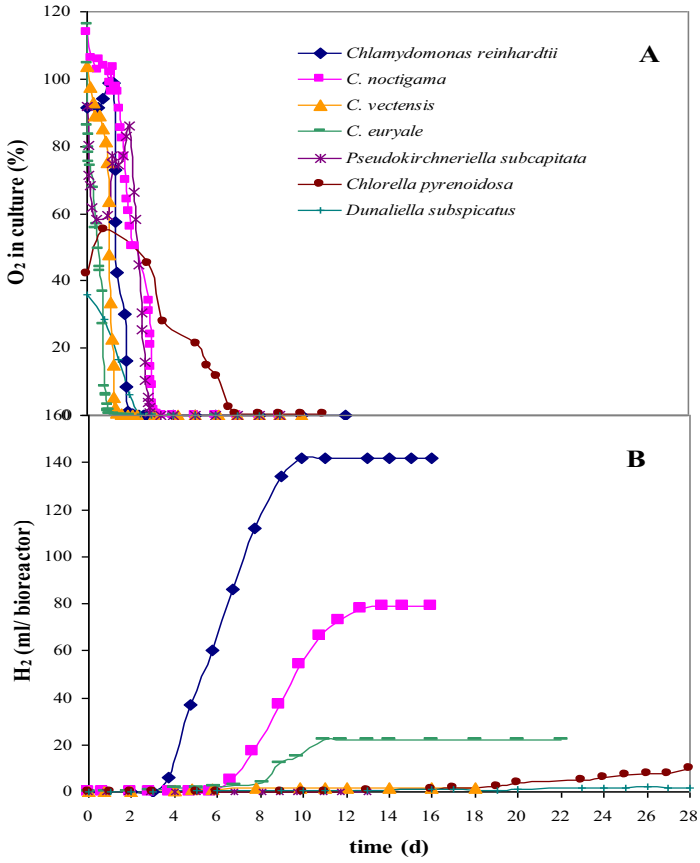

Figure 3. O_2 concentration (A) and hydrogen production (B) in cultures of green algae during S-deprivation. (From **Paper III**).

All of the 7 strains produced hydrogen during sulfur deprivation, although only *C. noctigama* produced hydrogen in amounts comparable to *C. reinhardtii*. Since no attempts were made to optimize conditions such as light-, temperature-, pH, nutrient composition for each strain, the amounts of hydrogen produced from the tested strains in these experiments can only be interpreted as an indication of the potential for

hydrogen production. More thorough investigations are necessary in order to determine which strain have the optimal performance in a practical process.

Hydrogen production is for example highly dependent on light intensity. Decreased PSII activity and thereby oxygen production is caused by a photoinhibition effect, explained in detail in **Paper II**. If each cell receives light intensity below optimum for this reaction, for example because of shading by a high cell density or by a high pigment content in surrounding cells, the reversible inactivation of PSII reaction center will be slow and, consequently, it will take longer before the culture becomes anaerobic. On the other hand, if each cell receives light above optimum for this reaction, the inactivation of the PSII reaction centers will become irreversible, and hydrogen production will not be sustained. Investigations have shown that careful selection of light intensities through pre-growth of the cultures before sulfur deprivation, and then through the aerobic and anaerobic phases after sulfur deprivation, can be used to control this method for obtaining an optimal hydrogen production. Application of low light intensities during pre-growth, followed by higher light intensities during the oxygen consumption stage allows for accumulation of a high amount of starch, and a lower intensity during the hydrogen production stage allows for a maintained production of hydrogen (Tolstygina et al. 2009; Tsygankov et al. 2006).

The majority of previous work in the field of hydrogen production during sulfur deprivation has been performed using the model organism *C. reinhardtii*, with acetate as a carbon source in the medium, allowing for a heterotrophic or mixotrophic metabolism (Melis 2007). Ability to use acetate or glucose for increased growth rate was included in the initial screening of species where hydrogen production had been detected (**Paper III**, Table 1). The intention of this was to look for a possible connection between algae with ability to produce hydrogen during sulfur deprivation and ability to utilize acetate for enhanced growth. The only two strains that did not show enhanced growth on acetate in these experiments were *Pseudokirchneriella subcapitata* and cf. *Oocystis*. The latter was not tested for hydrogen production during

sulfur deprivation, but *P. subcapitata* did indeed show very low production of hydrogen under these conditions in the single experiment where this strain was tested.

Tsygankov and co-workers have shown that it is possible to omit acetate from the medium and use CO_2 as a single carbon source, and still obtain significant amounts of hydrogen. However, this is not straightforward, and requires a sophisticated light regime (Tolstygina et al. 2009; Tsygankov et al. 2006). Although a method for producing hydrogen from *C. reinhardtii* during autotrophic conditions now has been developed, the maximal hydrogen production reported is still significantly lower than what has been reported using the original method with acetate as a substrate (Kosourov et al. 2007; Laurinavichene et al. 2004). The advantages of having a completely photoautotrophic process for producing hydrogen, are many. First of all, the organic carbon- and energy source would otherwise have to be obtained from a separate cost- and energy demanding process before it could be used for hydrogen production. Since the purpose is to produce hydrogen from solar energy, this step should be made redundant. Secondly, growing algae in large scale systems without contamination can sometimes be a challenge. With acetate present in the medium, contaminating organisms like bacteria or fungus may thrive and sometimes outcompete the algae. Finding an efficient and stable method for producing hydrogen from autotrophic grown algal cultures, is a very important topic that should be thoroughly investigated in the future. The importance of this relates to not only the practical implementation of the method, but also to the economical and energy efficiency aspects of a sustainable system.

4.4. Transcription of genes with characteristics of hydrogenase in the green algae *Chlamydomonas noctigama*

4.4.1. Hydrogenases in algae

Hydrogen production in green algae is catalyzed by FeFe hydrogenases, which are small, bidirectional enzymes with high activities and very high sensitivity against oxygen (Vignais 2008). The corresponding structural genes, annotated *HYDA*, are encoded in the nucleus, translated in the cytoplasm and transported into the chloroplast by a transit peptide. There they are assembled by the HydEFG maturation system into active enzymes located in the stroma of the chloroplasts (Böck et al. 2006; Posewitz et al. 2004). FeFe hydrogenases can be found in both bacteria and algae, and parts of the enzyme are very similar between the two groups. While the so called H-cluster part of the enzymes where the active site is located are very similar, the major difference between the two groups is the presence of an F-cluster part of the enzyme in bacterial FeFe hydrogenases. This F-cluster is the electron donor to the active site, while in hydrogenases which do not contain the F-cluster, the active site receives electrons directly from ferredoxin. Most algal hydrogenases do not have an F-cluster, although certain strains of *Chlorella* have very recently been found to contain a hydrogenase which includes an F-cluster (Posewitz personal communication).

Exposure to oxygen leads to a complete and irreversible inactivation of algal FeFe hydrogenase by destruction of the (4Fe-4S) domain of the active site H-duster (Erbes et al. 1979; Stripp and Happe 2009). This sensitivity against oxygen represents a challenge when the goal is to produce hydrogen from solar energy using the photosynthetic apparatus. Oxygen sensitivity of algal hydrogenases is an important topic which is being explored from many angles. Approaches include attenuated P/R ratio (Ruhle et al. 2008), selection of mutants with enhanced resistance towards oxygen by random mutagenesis (Flynn et al. 2002) or designing hydrogenase with decreased sensitivity to oxygen. The latter can for example be done by narrowing the oxygen channels of the enzyme, which has been shown in prokaryotic FeFe

hydrogenases (Ghirardi et al. 2006). CO is a competitive inhibitor and leads to reversible inactivation of algal FeFe hydrogenase by a non-destructive binding to the H-cluster 2Fe domain. This reversible inactivation is more efficient than the destructive inactivation by oxygen, and may therefore have a protective effect on the hydrogenase (Stripp and Happe 2009). The sulfur deprivation approach, which is studied in this work and described above, leads to anaerobic conditions in the culture by a partial inactivation of the oxygen producing PSII, thereby providing an environment for efficient hydrogen production.

Green algae characterised so far have been found to encode two distinct hydrogenases, named HYDA1 and HYDA2. To our knowledge, more than two hydrogenases have so far not been detected in species of green algae. The two hydrogenases are coded by *HYDA1* and *HYDA2* genes, which have different promoter regions and are transcribed and regulated differently in response to environmental conditions (Forestier et al. 2003). However, their specific functions in the cell during hydrogen production is still not unravelled. Experience from prokaryotes show that a number of very different hydrogenases can be produced by the same organism, and may represent very different functions. As an example, *Desulfovibrio vulgaris* Hildenborough has a total of six different hydrogenases (Heidelberg et al. 2004). The presence of a number of different hydrogenases within the same prokaryotic organism are hypothesized to help the organism to cope with changes in metal availability, exposure to hydrogenase inhibitors or varying environmental concentrations of molecular hydrogen. Alternatively, hydrogenases may function in different metabolic pathways (Caffrey et al. 2007). Eukaryotes have the presence of a gene with some homology towards hydrogenase, however the proteins translated from these genes show no hydrogenase activity. They are frequently referred to as Narf-like or hydrogenase-like proteins, and share both similarities and distinct differences with algal FeFe hydrogenases (Stejskal et al. 2003).

In this work, transcription of three genes from *C. noctigama* with strong similarities to hydrogenase were detected and analyzed, results are presented in **Paper IV**. It was

shown that the three transcripts all have higher similarities to other green algal hydrogenase sequences than to transcripts from known narf-like genes.

4.4.2. Approach for identifying presence of putative hydrogenase transcripts in *C. noctigama*

The approach that was used to identify transcripts from *C. noctigama* with homology to hydrogenase included RT-PCR, 3'-RACE, PCR and 5'-RACE methodology, followed by cDNA sequencing. First strand cDNA was produced using RT-PCR with a poly-T antisense primer with a tag, and a 3'-RACE product was amplified using a degenerate sense primer designed from a conserved sequence of hydrogenase RNA from other species, and an antisense tag primer. The product from the 3'-RACE reaction was cloned and sequenced, and aligned with the known hydrogenase sequences. Two different sequences with homology to hydrogenases were identified in separate clones from the same band, and tentatively named *HYDA1* and *HYDA2*. Nested PCR was performed with gsp sense primers and the same tag primer as previous. The nested PCR from *HYDA1* resulted in amplification of two products, one identical to the original *HYDA1*, and a second product that had homology to hydrogenases, but were identical to neither *HYDA1 n*or *HYDA2*. The third sequence was tentatively named *HYDA3*.

Longer parts of each of the three sequences were found by using the first strand cDNA produced with the poly-T primer described above. Antisense gsp primers designed from the non-conserved regions towards the 3'-ends, and degenerate sense primers designed from conserved regions upstream were used to amplify parts of all three transcripts. New gsp antisense primers were used for 5'-RACE reactions and 5'-ends were amplified. The cDNA sequences from all three transcripts were assembled and aligned with cDNA sequences from *C. reinhardtii, Chlamydomonas moewusii, Chlorella fusca* and *Scenedesmus obliquus*. The results showed that the putative hydrogenase sequences from *C. noctigama HYDA1, HYDA2* and *HYDA3* all had homology to the known hydrogenases in the conserved regions of the transcripts, and no homology in the non-conserved regions.

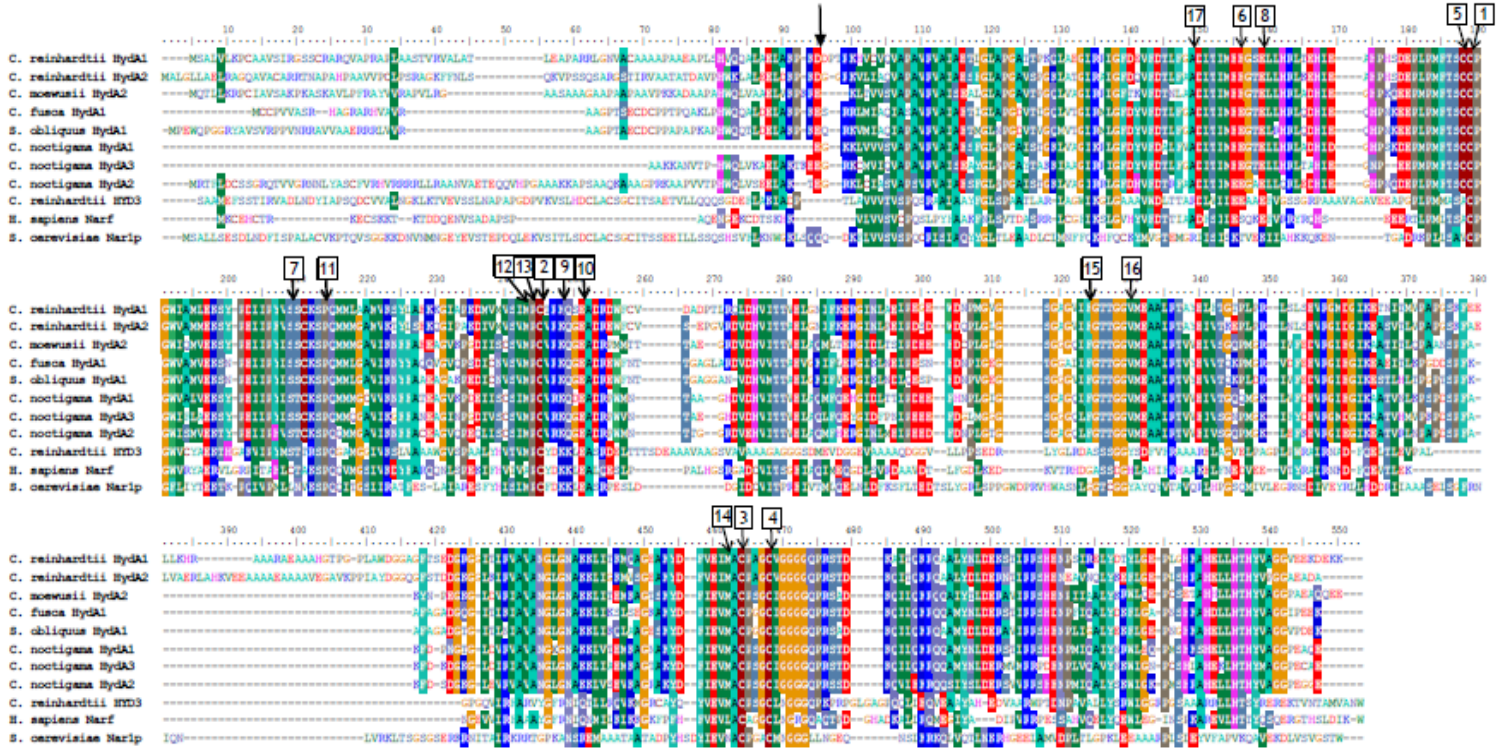

Figure 4. Alignment of amino acid sequences from *Chlamydomonas reinhardtii* HYDA1 and HYDA2, *Chlorella fusca* HYDA, *Chlamydomonas moewusii* HYDA2 and *Scenedesmus obliquus* HYDA, compared to putative hydrogenases from *Chlamydomonas noctigama*. Also included are non-hydrogen producing members of the Fe-only-hydrogenase family represented by *C. reinhardtii* HYD3/ prelamin A binding protein (HYD3), *Homo sapiens* nuclear prelamin A recognition factor (Narf) and *Saccharomyces cerevisiae* Nuclear architecture related protein (Nar1p). Numbered arrows refer to important amino acids. See **Paper IV** for details.

The three cDNA sequences from *C. noctigama* were translated into putative amino acid sequences, and aligned with amino acid sequences from the species listed above. In addition, the sequences were aligned with narf-like sequences from *C. reinhardtii, Saccharomyces cerevisiae* and *Homo sapiens*. The alignment is showed in Figure 4. The homology between all the sequences was calculated as % identities, and the homology was found to be significantly higher between the sequences from all the known hydrogenases and the putative hydrogenases from *C. noctigama*, than between these and the narf-like sequences (**Paper IV**, Table 2).

4.4.3. Characteristics of putative hydrogenases from *C.noctigama*

The numbered arrows in Figure 4 point to some of the amino acids that are believed to have important roles in the proton transport to and from the active site, or the structural integration of the active site (Fontecilla-Camps et al. 2007; Nicolet et al. 2000; Peters et al. 1998). See **Paper IV**, Table 3 for the identity of the selected amino acids. It is clear that several of the most important amino acids are present both in the known hydrogenases, and the putative hydrogenases from *C. noctigama*, but are not present in the narf-like sequences. The four cysteine units believed to form bonds between the peptide and the Fe-S centres are present in all the sequences. A fifth cysteine unit, which is believed to be involved in the proton transport pathways (Meyer 2007), is present in all the known hydrogenases and the three putative hydrogenases from *C. noctigama*, but are absent from all the three narf-like sequences included in this comparison. Three other amino acids believed to be involved in the proton transport pathways, are present in all the hydrogenases and putative hydrogenases, but are absent from some of the narf-like sequences. Among seven amino acids that are hypothesised to form the hydrophobic integration of the active site, all are conserved in the hydrogenases and putative hydrogenases, while only two are present in all the narf-like sequences. These results show that the three amino acid sequences of the putative hydrogenases detected in *C. noctigama* have a higher similarity to known green algal hydrogenases than to hydrogenase-like or narf-like sequences.

The three transcripts identified from *C. noctigama* were induced under the conditions that were used in this study. It should be noted that if other hydrogenases were present, but not expressed under these conditions, they would not be detected.

There are currently no indications suggesting a relationship between the number of hydrogenases in an alga and their ability to produce high amounts of hydrogen during S-deprivation or other stress conditions. However, our current understanding of the roles and functions of the different hydrogenases in algae is very limited. The discovery of expression of multiple possible hydrogenases in a single species, represents an opportunity for gaining further understanding, which in turn can be used for optimization of the hydrogen production process.

5. Main findings

- A high number of algal species may have potential for a combined production of hydrogen and other valuable products. The species presented here can function as a starting point for a screening where the goal would be to identify an alga which can perform well in a hydrogen production process using the sulfur deprivation technique, combined with production of a component with medical activity or other industrial interest which would result in a high economic potential for the process.

- A number of valuable secondary metabolites produced in high amounts as responses to environmental stress, function as electron sinks. This implies that combining production of large amounts of stress-induced metabolites might be unfavorable in combination with simultaneous hydrogen production. One solution to this problem would be to induce production of metabolites which have a higher economic potential than the current commercial products from algae. These could be produced in small amounts which does not compete with the hydrogen production, and still provide the commercial value this process would need. One other solution would be to have a two-stage process where

hydrogen production is followed by a second step where large amounts of valuable metabolites are produced.

- Careful considerations must be taken when designing bioreactors when it comes to both materials choice and measurement devices. Certain materials may have inhibiting effects on algal growth, a factor which should be thoroughly tested at lab scale before an algal bioreactor up-scaling. The negative effect of natural rubber on the growth and survival of algal cultures was particularly clear in this study. It was also shown that some species of algae were able to produce trace amounts of hydrogen under 100% oxygen saturation in the culture, a characteristic that is here hypothesized to be caused by an anaerobic microhabitat in front of the oxygen electrode. This artifact should be taken into account when laboratory findings are interpreted, in cases where hydrogen production is studied with oxygen electrodes inserted into the culture.

- Several species of green algae have the possibility to produce hydrogen during sulfur deprivation in light. The majority of research on hydrogen production from green algae during sulfur deprivation, has until recently mainly been performed on the model organism *C. reinhardtii*. In this study, it has been demonstrated that all the 7 species tested were able to produce hydrogen under the conditions used here, although some in only small amounts. Both fresh water and brackish water species were used. All the species tested for hydrogen production under sulfur deprivation were able to use acetate for heterotrophic or mixotrophic growth.

- *C. noctigama* has the potential to produce significant amounts of hydrogen during sulfur deprivation in light. This part of the study was performed under physical and chemical conditions which had previously been found to be optimal for production of hydrogen from *C. reinhardtii*. These conditions included light intensity, temperature, pH, and the presence of organic substrate. Testing the potential of optimization of these conditions for a more efficient

hydrogen production was not a part of this study. Neither was the possibility for hydrogen production from autotrophic grown algae.

- *C. noctigama* has the ability to express three distinct genes with characteristics of hydrogenases under anaerobic conditions. To our knowledge, only two genes are known to be expressed in other species of green algae. By identifying and sequencing mRNA from *C. noctigama*, the putative amino acid sequences were deduced. By comparing characteristic amino acids in the sequences found in these experiments, with hydrogenases from other green algae and with sequences from narf-like or hydrogenase-like proteins, it was found that the sequences from *C. noctigama* shared significant characteristics with green algal hydrogenases, and showed less similarity towards the other amino acid sequences in this comparison.

6. Topics for further studies

- Examine the potential for species of green algae to produce valuable metabolites during hydrogen production by sulfur deprivation.
- Examine the potential for species of green algae to produce valuable metabolites after hydrogen production in a two stage process.
- Use of CO_2 vs. acetate as a carbon source for algae producing hydrogen during sulfur deprivation.
- Find optimal composition of algal biomass for hydrogen production, for example in respect to starch content. Explore methods for cultivation of algae in order to obtain optimal biomass composition.
- Design of bioreactors for hydrogen production from algae, in respect to parameters such as light transfer, agitation, temperature control, gas capture and collection, and testing of materials for hydrogen leakage and toxic effects.

- Ability of algal strains to create biofilms on solid surfaces and the possibility for forming anaerobic microhabitats in aerobic cultures.
- Expression of hydrogenase genes under different conditions in selected species.
- Production of hydrogenase proteins under different conditions in selected species.
- Identify roles of different hydrogenases in green algae.

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