Thesis of the PhD dissertation

Implementation of efficient photoautotrophic H_2 production in the green alga *Chlamydomonas reinhardtii*

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Introduction

Since the industrial revolution that began in the 17th century, fossil fuels, including coal, oil and natural gas have been operating the technological and transport networks that drive our society. However, supplying the world with fossil fuels with finite geological reserves threatens our long-term energy supply and puts a huge strain on the environment. Additionally, the energy demand has been increasing worldwide due to population growth. In addition to the problem of providing energy supply, the use of fossil fuels also compromises our own health through chemical and particle pollution. They are not only dangerous to our health, but emissions of carbon dioxide and other greenhouse gases are closely linked to global warming, thus they threaten the long-term sustainability of the Earth's climate as well. Therefore, more attention has to be paid to renewable energy sources.

Natural resources from which energy can be gained and are re-produced within a few years, are called renewable energy sources. Their importance comes from the fact that they do not cause significant pollution and are in line with the principles of sustainable development. Such alternative energy sources include wind energy, geothermal energy, hydro-, biomass- and solar energy. The utilization of renewable energy technologies offers an excellent opportunity to reduce greenhouse gas emissions and slow down the process of global warming by replacing conventional energy sources.

Significant research focuses on the widest possible usage of H_2. H_2 gas is a clean energy carrier, the combustion of which does not produce carbon dioxide, only water. However, about 99% of H_2 is currently produced from fossil fuels, which cause enormous air pollution; therefore, there is a need to develop H_2 production methods based on renewable energy sources. One of the promising ways of doing this is the utilization of green algae, which can produce H_2 with high efficiency linked to their photosynthesis.

Aims of the study

In nature, it has been observed that algae that have been darkened for a few hours produce H_2 for a few minutes upon illumination. The length and efficacy of this short period can be increased by sulfur removal under laboratory conditions, which, however, is accompanied by a number of negative side effects that prevent the industrial application of algal H_2 production.

In the course of my work, I aimed to develop and optimize a technique based on anaerobic induction that is more efficient and less harmful to algal cells. Its elements are as follows:

1. Exploring key steps needed to maintain continuous H_2 production
2. Development and optimization of a novel H_2 production method
3. Testing the sustainability of the system
4. Increasing the efficiency of H_2 production by designing a new type of photobioreactor
5. Identification and characterization of mutant strains involved in photosynthesis with increased H_2 production

Material and methods

1. Maintenance and growth of *Chlamydomonas reinhardtii* strains

In our experiments, we examined and compared the H_2 production of five different strains of *Chlamydomonas reinhardtii*, namely: CC-124, CC-409, L159I-N230Y, *pgrl1*, and *pgr5*. Algae were grown in solid or liquid Tris-acetate phosphate (TAP) medium in an algae growth chamber. After three days of cultivation, the cultures were centrifuged and placed in Sueoka's high salt medium (HS). In experiments carried out in 100 ml serum vials, the chlorophyll content was adjusted to 50 μg/ml chl (a+b) and 30 ml of culture were placed in them. In the Roux-type photobioreactor (TCL-PBR) glasses, the chlorophyll content was adjusted to 50 or 150 μg/ml chl (a+b) and 100 ml of culture were placed in the vessels. Before sealing, an O_2 absorbent was placed in special containers in the airspace of the vials. The cultures were placed in the dark for four hours and O_2 and CO_2 were removed from their gas spaces by 20 minutes of N_2 blowing. After four hours of dark anaerobic induction, cultures were placed under 350 or 1000 μmol m^{-2} s^{-1} photon flux density, 23-25 °C and were shaken at a speed of 130 rpm.

2. Chemical treatments

In some experiments, three hours after the cultures were exposed to light, various chemical treatments were applied.

3. O_2 absorbents

The absorbents used in our experiments to remove O_2 from the air were a mixture of sodium chloride and iron. The 504B 100CC Loose (Oxyfree, Tianhua Tech. CO., Ltd. Nanjing, China) mixture was employed in the experiments using serum bottles. The 50CC Loose (O2Zero, Global Reach Ltd, London, UK) absorbent was employed in the experiments using TCL-PBR vials. 1.3 g of O_2 absorbent was added to the serum bottles, while 20 g of O_2 absorbent was used in the TCL-PBR vials.

4. Applied methods:

- Spectroscopic determination of chlorophyll content
- Determination of H_2 and O_2 production by gas chromatography
- Fast chlorophyll *a* fluorescence transient (OJIP) measurements
- Western blot analysis
- Determination of cell number
- *In vitro* hydrogenase activity measurements

Results

1. Exploring key steps needed to maintain continuous H_2 production

To induce hydrogenase expression, a so-called dark anaerobic induction treatment was applied. The *C. reinhardtii* strain CC-124 was chosen as model organism. This strain was already known as an efficient H_2 producer that is capable to produce H_2 under a wide variety of experimental conditions. H_2 production in green algae requires reducing equivalents and anoxic conditions. Reducing equivalents may derived from the photosynthetic electron transport, from starch breakdown and from the subsequent fermentation. The H_2 production has to share the reducing agents pool with competitive reactions. The main competitor for electrons is CO_2 assimilation. The N_2 atmosphere used to ensure anaerobiosis also removes CO_2, the substrate of the Calvin-Benson-Bassham (CBB) cycle, thereby the competitor electron sink is inhibited, allowing increased electron flow towards HydA.

The presence of acetate is necessary for the accumulation of starch, but its metabolism leads to the formation of CO_2, which is able to partially restore the activity of the CBB cycle. To avoid this, we omitted acetate from the medium. In the absence of acetate and so an ample amount of starch, the photosystem II (PSII) -dependent electron transport becomes the main electron source. The water-splitting-based linear electron transport is a more efficient electron source than starch breakdown; however, it is accompanied by O_2 release which prevents the establishment of anoxic conditions. Therefore, to exploit the linear electron transport as a source of sustainable electron source, we opted to remove the released O_2 rapidly and efficiently rather than deactivating PSII by sulfur deprivation. For this purpose, an iron salt-based O_2 absorbent was applied, which reduced O_2 concentration below 0.1% in the headspace of the cultures.

2. Development and optimization of a novel H_2 production method

The method developed by us has fundamental advantages compared to previous methods, namely, under illumination following a few hours of anaerobic dark incubation, H_2 production starts immediately. In contrast to sulfur deprivation, it does not require the replacement of the nutrient solution. It does not depend on the degradation of starch and does not require acetate as it is a photoautotrophic process. Since there is no need for organic carbon sources, the risk of bacterial contamination is much lower. Cultures remain photosynthetically active during H_2 production and can be easily regenerated. The process is based on linear electron transport, the electrons are derived primarily from the water-splitting activity of PSII and has a relatively high light-to-H_2 energy conversion efficiency. In the growth phase, CO_2, which is an industrial by-product, can be used. Relatively high light intensity (320 μmol photon/m^2/s) can be used for the production of H_2.

3. Testing the sustainability of the system

The sustainability of our H_2 production system was characterized by the status of the photosynthetic apparatus during a 96-hour experiment. We found that the chl (a+b) content was reduced by only 10% during the experiment. The F_v/F_m value, which is a

parameter of PSII efficacy, showed a slow decrease during the 96-hour period but remained relatively high, above 0.4, both for control samples and in the presence of O_2 absorbent. The amount of PsbA (a major protein of PSII), PetP (a subunit of the $cytb_6/f$ complex), and PsaA (a PSI reaction center protein) remained largely unchanged throughout the experiment in both samples. Since the cells remained photosynthetically active in our experiment, we tried to regenerate them after the H_2 production period and used them again in a new experiment. When these regenerated cells were subjected to a second, dark anaerobic induction period, the yield of H_2 production was similar to that measured in the first cycle.

4. Increasing the efficiency of H_2 production by designing a new type of photobioreactor

We thought that the yield of H_2 production, achieved by anaerobic induction in HS nutrient solution, could be further improved. To achieve this, we used a photobioreactor in which we optimized the gas-liquid as well as the culture volume-surface ratio, the conditions for illumination and algae mixing, and the efficient removal of the produced gases. Thin-Cell-Layer Photobioreactors (TCL-PBRs) are culturing systems with a light path of less than 10 mm and a high surface area-volume ratio. These properties allow TCL-PBR to operate at very high cell densities (1000 μg chl (a+b)/ml culture) and improve photosynthetic efficacy per unit surface of the irradiated area. In our work, to obtain a thin-cell-layer, we kept the alga cultures in a horizontally placed 1-liter Roux bottles (TCL-PBR). The thickness of the cell suspension was approximately 5 mm in the vials. The surface area - volume ratio was increased fourfold (from 0,059 cm^{-1} to 0,24 cm^{-1}), while the gas-liquid volume ratio was increased threefold (from 3 to 9) relative to serum bottles.

5. Identification and characterization of mutant strains involved in photosynthesis with increased H_2 production

There is great potential in the application of the protocol we have developed for various photosynthetic mutants. After optimization of the photobioreactor, three photosynthetic mutants were tested, which were affected in PSII (L159I-N230Y) or in PSI cyclic electron transport (*pgrl1* and *pgr5* mutants). In our experiments sunlight intensity (1000 μmol photon/m^2/s) and high chlorophyll content (150 μg/ml) were used.

The H_2 production of all three strains was very similar in the first 24 hours, but the H_2 production of wild-type CC-124 and CC-409 strains showed a continuous decrease, while the L159I-N230Y mutant remained rather stable and it only decreased by day 6. Strain L159I-N230Y produced 30% more H_2 than CC-409, although the condition and performance of their photosynthetic apparatus were similar.

Next, we examined the *pgr5* and *pgrl1* mutant strains with the protocol we developed. For the *pgr5* mutant, H_2 production was multiple compared to the wild-type CC-124 strain. The photosynthetic apparatus of the *pgr5* mutant was not significantly damaged even by the intensity of sunlight, and the amount of hydrogenase enzyme was preserved.

Summarizing our results, we can conclude that a new method based on anaerobic induction has been developed, which has several advantages over the previously used sulfur removal protocol. Due to this method and the photobioreactor used in our later experiments, we successfully increased the amount of H_2 production by optimizing the gas-liquid ratio, the cell layer thickness, and the removal of the produced gases. Testing various photosynthetic mutant strains of *Chlamydomonas reinhardtii*, we found that the *pgr5* mutant proved to be the most efficient H_2 producer under the conditions we set up.

Replacing fossil fuels in a way that ensures sustainability and has no environmental or health impact is an increasingly urging problem today. With the obtained knowledge, it may become possible to make use of the H_2-producing capacity of green algae on an industrial scale.

Publications

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Articles which serve the basis for the thesis

Nagy V., Vidal-Meireles A., Podmaniczki A., Szentmihályi K., Rákhely G., Zsigmond L., Kovács L., Tóth S.Z. (2018): The mechanism of photosystem-II inactivation during sulphur deprivation-induced H_2 production in *Chlamydomonas reinhardtii.*
Plant J 94: 548-561.
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Nagy V., Podmaniczki A., Vidal-Meireles A., Tengölics R., Kovács L., Rákhely G., Scoma A., Tóth SZ. (2018): Water-splitting-based, sustainable and efficient H_2 production in green alga as achieved by susbtrate limitation of the Calvin-Benson-Bassham cycle. Biotechnol Biofuels 11: 69
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Nagy V.* Podmaniczki A.*, Vidal-Meireles A., Kuntam S., Herman É., Kovács L., Tóth D., Scoma A., Tóth SZ. (2021): Thin cell layer cultures of *Chlamydomonas reinhardtii L159I-N230Y, pgrl1 and pgr5* mutants perform enhanced hydrogen production at sunlight intensity. Bioresour Technol 333: 125217
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