

# **Bacterial symbionts enhance photo-fermentative hydrogen evolution of *Chlamydomonas* algae**

Ph.D. Thesis

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## Introduction

Since the beginning of the industrial revolution, the carbon-dioxide content of the atmosphere has been continuously growing because of the rising utilization of fossil fuels. At the beginning coal was used as the dominant energy carrier, later crude oil and natural gas received more and more attention. Beside the problem of the growing amount of carbon-dioxide which is significantly responsible for the greenhouse effect, the utilization of these fossil fuels is connected to numerous other unsolved problems. The available sources of fossil fuels are limited, but the intensity of the consumption is continuously growing. The source and the site of utilization are mostly localized distantly, thereby causing further environmental pollution and geopolitical crisis. However, the extracted energy from their burning constitutes the essential part of the economies. Already many alternatives exist for the replacement of the fossil fuels. Nowadays, the technologies, which can put the renewable energy sources (sun, wind and water energy) to use, are getting more widespread. The cardinal drawback of these renewable technologies is the difficulty of energy storage. The utilization of the molecular hydrogen could provide an environmental friendly solution for the renewable energy storage. Its huge advantage is that during the burning of the hydrogen the only side-product is pure water. The hydrogen's energy storage capacity is very high, one gram hydrogen can store 122 kJ energy. It is 2.75 times higher than the yield stored by the fossil fuels. The large-scale transportation of the hydrogen gas is solvable by the utilization of the already existing systems. Moreover, it can be stored in metal gas bottles under high pressure. Beside the utilization as a fuel, it can be used for oil refinery, food and fertilizer production. Hydrogen can be generated in very high purity from water by electrolysis, but it consumes a lot of energy. Thus, nowadays it is produced from natural gas or methane by the technology of gas reforming. For the relay of this technology, many biohydrogen producing methods are currently under research and development. Among these investigations, the research of the biohydrogen production by green algae already shows promising results.

The FeFe hydrogenases can be activated under proper environmental conditions in certain *Chlamydomonas*, *Scenedesmus* and *Chlorella* species. The FeFe hydrogenases can convert the light energy into biohydrogen with 10 % efficiency. However, they are highly sensitive for the presence of oxygen, which inhibits the continuous photolytic hydrogen production. The indirect photolysis can solve this problem by dissecting the hydrogen evolution and oxygen production phases. First, the energy of the illumination is stored in starch, then the starch is degraded and utilized. Thus, hydrogen evolution is separated from

the photosynthetic oxygen production temporally. The most prevalent method of algal indirect photolytic hydrogen productions based on sulfur deprivation of the culture medium. During sulfur deprivation, the algae cells first accumulate carbohydrates and nutrients, then they start degrading their protein stores to survive the nutrient stress. It causes major reduction in the activity of the II. photochemical system (PSII), which finally inhibits further oxygen production of the algae cells. The reduced oxygen evolution and the active cell respiration allow the closed system to become anaerobic, thus enhancing the activation of hydrogenases. The major disadvantage of this method is that the algae cultures must be grown up first, then the original medium must be changed to a sulfur-free medium. This process definitely requires extra time, material and energy, which makes the starting of the industrial application more difficult. The hydrogen production process is an approximately five day-long period. The term of hydrogen evolution can be prolonged by the re-addition of sulfur for a few weeks, but it causes the reduction of the hydrogen production efficiency. The oxygen consumption period takes at least one day, which also reduces the industrial feasibility of the hydrogen production. Besides, the living cell number is continuously decreasing because of the nutritional stress effect.

## Aims

Hydrogen is a remarkable candidate between the numerous energy sources and carriers. Currently, several biohydrogen producing methods exist, which are under investigation. This thesis focuses on the properties of the hydrogen producing algal-bacterial cultures and on the relationship between its microbial partners.

Specific aims:

1. Selection of hydrogen producing algae strains, assessment of algal biomass production of strains from the Mosonmagyaróvár Algal Culture Collection.
2. Identification of the bacterial partners of *Chlamydomonas* sp. 549 algae and selection of bacterial partners enhancing the algal hydrogen and biomass production most efficiently.
3. Investigation of the effects of the natural and artificial bacterial partners on the properties of *Chlamydomonas* sp. 549 (hydrogen production, culture respiration ratio, algae propagation).
4. Analysis of hydrogen evolution in pure and mixed algal cultures under dark and light circumstances, with or without the combination of sulfur-deprivation.
5. Examination of the photosynthetic system in *Chlamydomonas* sp. 549 using fluorescence measurements and analysis of the hydrogen evolution pathways in the algae.

## Methods

Molecular- and microbiological methods and analytical tools were used for the investigations of the algal-bacterial mixture. Gas chromatography was used for the measurements of the hydrogen and oxygen concentrations in the headspace of the bottles. The dissolved oxygen content in the liquid phase of the cultures was measured by oxygen electrode. The living cell numbers of the bacterial partners were monitored by counting colony forming unit (CFU). The changes in the algae cell numbers were detected by the utilization of microscope and Bürker-chamber. The biomass productions of the certain pure and mixed cultures were followed by spectrophotometer. pH meter and HPLC were used for the investigation of the media. The alterations and functionality of the photosynthetic system was followed by chlorophyll fluorescent measurements. The *Chlamydomonas* sp. 549 strain was maintained on TP (Tris-Phosphate) plates supplemented with rifampicin, while the bacterial partners were separated and maintained on LB plates. Capillary Sanger sequencing of the bacterial 16S rDNA PCR products was used to identify the bacterial partners.

## Results

1. The effects of three natural bacterial partners (*Rhodococcus* sp., *Brevundimonas* sp., *Leifsonia* sp.) were tested on the biomass and hydrogen production of *Chlamydomonas* sp. 549. The highest biomass yields were obtained by the mixture of *Rhodococcus* sp. - *Chlamydomonas* sp. 549. The algal-bacterial investigations were broadened by adding wild-type and hydrogenase deficient *Escherichia coli* and *Ralstonia eutropha* bacterial strains to the algae culture as consortial partners. We clearly showed, that *Chlamydomonas* sp. 549 is the only hydrogen producer in the algal-bacterial consortium. The highest total accumulated hydrogen yield was achieved by the utilization of the hydrogenase deficient *E. coli*  $\Delta hypF$  ( $1196.06 \pm 4.42 \mu\text{L L}^{-1}$ ).
2. The hydrogen evolution characteristics of *Chlamydomonas* sp. 549 – *E. coli*  $\Delta hypF$  and *Ch. reinhardtii* cc124 - *E. coli*  $\Delta hypF$  mixed cultures were analysed. Significant differences were observed between the total accumulated hydrogen amounts (*Ch. reinhardtii* cc124 - *E. coli*  $\Delta hypF$ :  $5800.54 \pm 65.73 \mu\text{L L}^{-1}$  vs. *Chlamydomonas* sp. 549 – *E. coli*  $\Delta hypF$ :  $1196.06 \pm 4.42 \mu\text{L L}^{-1}$ ) and in the dynamics of hydrogen evolution as well. The hydrogen production lasted approximately 24 h in both *Chlamydomonas* sp. 549 – *E. coli*  $\Delta hypF$  and *Ch. reinhardtii* cc124 - *E. coli*  $\Delta hypF$  mixed cultures, then the hydrogen yields in the headspace started decreasing. In the case of the *Ch. reinhardtii* cc124 - *E. coli*  $\Delta hypF$  mixed culture the hydrogen production re-started after the fourth day. The oxygen level in the cultures containing bacterial partner decreased in 4 hours to that level, where it could not inhibit the operation of the hydrogenases (3 % in the headspace, 1 – 2  $\mu\text{mol L}^{-1}$  in the liquid phase). In conjunction with the acetic acid consumption, which was consumed in 2 days by the mixed cultures, the oxygen level started increasing after the second day both in the liquid phase (from 1 – 2  $\mu\text{mol L}^{-1}$  to 1300 - 1600  $\mu\text{mol L}^{-1}$ ) and in the headspace (from 3 % to 60 %). Parallel to the acetic acid consumption, the pH level increased from pH 7.3 to pH 8.7.
3. Under illumination, the increase of the *Chlamydomonas* sp. 549 cell number lasted 4 days, although a faster algal cell number increase was observed in the mixed *Chlamydomonas* sp. 549 – *E. coli*  $\Delta hypF$  compared to the pure *Chlamydomonas* sp. 549 culture. The *Rhodococcus* sp. was chosen for the colony forming unit (CFU)

investigations. The main conclusion of the CFU result was that the presence of the algal partner under illumination had a positive effect on bacterial living cell number until the headspace of the sealed bottle is saturated with hydrogen. In light circumstances, connection was observed between the high bacterial cell number and the algal hydrogen production in the mixed algal-bacterial cultures on the first day of the experiment. Along with the stop of algal hydrogen production, the living cell number of the *Rhodococcus* sp. was dramatically reduced.

4. The algal-bacterial consortial hydrogen producing method was investigated in combined experiments with the hydrogen producing method of sulfur-deprivation and dark fermentation. No significant differences were observed in dark fermentative hydrogen production between the axenic *Chlamydomonas* sp. 549 and the mixed *Chlamydomonas* sp. 549 – *E. coli*  $\Delta$ *hypF* cultures ( $6114 \pm 369 \mu\text{L L}^{-1}$  vs.  $5621 \pm 645 \mu\text{L L}^{-1}$ ). The total accumulated hydrogen yield and the dynamics of the hydrogen production were compared in the sulfur-deprived *Chlamydomonas* sp. 549 and *Ch. reinhardtii* cc124 axenic cultures, and *Chlamydomonas* sp. 549 – *E. coli*  $\Delta$ *hypF* and *Ch. reinhardtii* cc124 - *E. coli*  $\Delta$ *hypF* mixed cultures. Significant differences were detected between the total accumulated hydrogen yields of the pure algae and mixed algal-bacterial cultures. The mixed cultures containing *E. coli*  $\Delta$ *hypF* bacterial partner produced significantly more hydrogen than the pure algae cultures (*Chlamydomonas* sp. 549:  $193.5 \pm 66.81 \mu\text{L L}^{-1}$  vs. *Chlamydomonas* sp. 549 - *E. coli*  $\Delta$ *hypF*:  $2637.49 \pm 555.42 \mu\text{L L}^{-1}$  and *Ch. reinhardtii* cc124:  $25028.1 \pm 3943.47 \mu\text{L L}^{-1}$  vs. *Ch. reinhardtii* cc124 - *E. coli*  $\Delta$ *hypF*:  $47241.3 \pm 4660.69 \mu\text{L L}^{-1}$ ). Based on these results, it is hypothesized that the presence of the bacterial partner might have a positive effect on sulfur-deprived algal hydrogen production. Notable differences were observed in the total accumulated hydrogen yields, the oxygen level and in the dynamics of the hydrogen evolution between the sulfur-deprived pure and mixed cultures of both *Chlamydomonas* sp. 549 and *Ch. reinhardtii* cc124. The chlorophyll fluorescent measurements and acetic acid analyses suggested, that the different acetic acid consumption rates of the two algae strains and the different decrease of the PSII activities are responsible for the observed variations. The activity of the PSII started to decrease after the fourth day in *Chlamydomonas* sp. 549, while in the case of the *Ch. reinhardtii* cc124 the PSII activity already decreased on the first day of the experiment. The acetic acid was consumed in approximately two days by

*Chlamydomonas* sp. 549, while total consumption of the acetic acid took four to five days for *Ch. reinhardtii* cc124.



## **Discussion**

The results clearly indicated the importance of the proper selection of the algae and bacterial partners for the most efficient hydrogen production. The investigated methods allow us to combine different bacterial and algal partners and various hydrogen-producing approaches in order to achieve the possible highest biohydrogen output. A remarkable advantage of our algal-bacterial co-culturing approach is that the system achieves rapid oxygen level reduction without the need of any interventions. The algal biomass can continuously grow, which also accelerates the rate of hydrogen production. In low light circumstances, the algal partner can consume the carbon sources with high efficiency and maintains the bacterial partner for an extended period of time..

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### Publications related to the topic of the thesis

Lakatos, G., Deák, Zs., Vass, I., Rétfalvi, T., Rozgonyi, Sz., Rákhely, G., & Maróti, G. (2014). Bacterial symbionts enhance photo-fermentative hydrogen evolution of *Chlamydomonas* algae. *Green Chemistry*, 16(11), 4716-4727.

impact factor: 6.83

Wirth, R., Lakatos, G., Maróti, G., Bagi, Z., Minárovics, J., Nagy, K., Kondorosi, É., Rákhely, G., Kovács, K. L. (2015) Exploitation of algal-bacterial associations in a two-stage biohydrogen and biogas generation process. *Biotechnology for Biofuels* (under publication)

impact factor: 6.22

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### **Hungarian publications**

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