Establishment of genuinely photoautotrophic hydrogen production in the green alga, *Chlamydomonas reinhardtii*

Introduction

Biohydrogen produced by algae may become a genuinely carbon-free energy carrier because upon the combustion of H_2, only pure water is produced. [Fe-Fe]-type hydrogenases found in green algae are one of the most active molecular catalysts known for H_2 production. In the natural environment, the anoxic conditions established for instance during the night favor the expression of hydrogenases in green algae. Upon illumination, H_2 production is started, serving as a safety valve for the photosynthetic electron transport. It is a temporary process because the activation of the Calvin-Benson-Bassham (CBB) cycle indirectly results in increased O_2 evolution which promptly inhibits H_2 production.

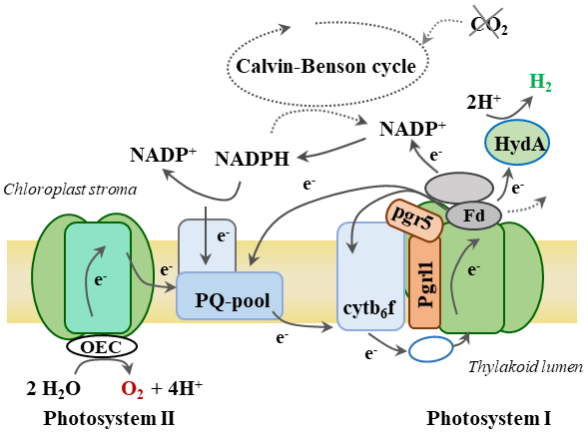

Fig. 1. The photosynthetic H_2 production in green algae

Results

The method of choice for inducing H_2 production in green algae is sulphur deprivation, which, however, results in the degradation of the photosynthetic apparatus and is largely dependent on organic substrates. The sulphur deprivation induced H_2 production can be maintained only for a few days (Nagy et al., Plant Cell Environ, 2016; Nagy et al., Plant J, 2018). Therefore, there was a need to develop an alternative approach, which is at least as efficient as the sulphur deprivation procedure but solves most of the issues of biotechnological applicability.

We suggested that for achieving an efficient photoautotrophic H_2 production, the Calvin-Benson cycle has to be kept inactive. At the same time, O_2 should be eliminated without inactivating linear electron transport (Nagy et al., Biotechnol Biofuels, 2018).

Dark anaerobic incubation treatments to induce hydrogenase expression were carried out with the CC124 strain of *Chlamydomonas reinhardtii*, because it is a relatively efficient H_2 producer and has been successfully used under various conditions. After growing the cultures for 3 days in TAP medium, the cells were transferred to culture media with (TAP, HSA) or without acetate (TP, HS) with a chlorophyll (chl) (a+b) content set at 50 μg/ml. Hydrogenase expression was induced by a 4-h dark anaerobic incubation during which N_2 flushing was applied to remove both O_2 and CO_2. We subjected the cultures to relatively high light intensities (320 μmole photons m^{-2} s^{-1}) after the dark anaerobic induction. Upon light exposure, H_2 production was observed during the first hour in all growth media. In acetate-containing media (TAP and HSA), prolonged illumination did not result in further H_2 production, whereas in acetate-free media (HS, TP) a sustained H_2 production was observed. The amount of O_2 in the headspace of the vials rapidly accumulated in the presence of acetate, whereas in its absence, the O_2 concentration remained at a low level. As cultures kept in HSA or TAP had low H_2 yields and both TP and HS cultures produced large amounts of H_2, the increase in H_2 production efficiency is attributed to the absence of acetate. Therefore, we continued using for the HS media, which is commonly used as a minimal media for studying algal physiology.

Next, we compared the efficiency of H_2 production following a dark anaerobic induction in HS medium with the classical sulphur deprivation method that is largely dependent on acetate. Our results demonstrate that anaerobic induction of hydrogenases in minimal (i.e. acetate-free) media can result in sustained H_2 production, with yields that are higher than in TAP-S media at equal chl(a+b) contents and under the same illumination conditions.

Our system has fundamental advantages compared to earlier methods: i) following a few h of anaerobic dark incubation, H_2 production starts promptly upon illumination; ii) as opposed to sulphur deprivation, it does not require media exchange; iii) it does not require acetate, thus it is fully photoautotrophic; iv) because no organic carbon source is required, the risk of bacterial contamination is low; v) the cultures remain photosynthetically active during the H_2 production phase and they can be easily recovered afterwards; vi) it is based on linear electron transport and the electrons originate from the water-splitting activity of PSII, as demonstrated by a DCMU-treatment, and has relatively high light energy conversion efficiency; vii) during the growth phase, CO_2, as an industrial by-product, can be utilized; and, viii) it can make use of relatively high light intensities (320 μmole photons $m^{-2}s^{-1}$).

Another key factor to reach a sustained H_2 production is to protect the hydrogenases from O_2, which may also shift the balance between O_2 and H_2 production. We applied and iron-salt-based O_2 absorbent, which decreased the O_2 concentration in the headspace below 0.1%. The decrease in O_2 concentration was accompanied by two-fold increase in H_2 production, reaching c. 200 µl H_2 ml^{-1} culture in 96 h (Nagy et al, Biotechnol Biofuels, 2018; Nagy and Tóth, WO2018146167).

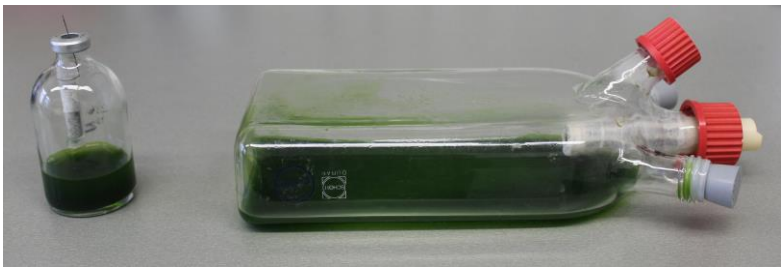

Fig. 2. H_2 production in serum bottles (bulk) and thin cell layer photobioreactors (TCL-PBR).

As a follow-up of this study, we aimed at improving our anaerobiosis-induction based H_2 production method for upscaling at high light. We compared the amount of H_2 produced in hypovial bottles (bulk cultures) and in horizontally placed 1-L Roux bottles in which a thin layer of alga culture (approx. 5 mm) was placed (TCL-PBR, this is our photobioreactor steup). The experiments were conducted at medium (50 µg Chl(a+b)) and high (150 µg Chl(a+b)) cell densities and at medium (350 µmol photons m^{-2}s-1) and high (1000 µmol photons m-2s-1) light intensities. In absolute terms, the highest amount of H_2 produced was achieved by very high density thin layer cultures (about 500 µl H_2/ml), whereas there were not major differences whether the cultures were placed in medium or very high light. The essence of our findings is that thin TCL-PBRs are much better suited for long-term H_2 production than batch reactors, enabling approx. three-fold increase in H_2 production yield. In a TCL-PBR, the application of very high cell density and light intensity also enables maximizing the amount of produced H_2 per surface, highly relevant for outdoor applications. In the following experiments, we tested some photosynthetic mutants in a TCL-PBR.

We compared the H_2 production of the L159I-N230Y photosystem II *Chlamydomonas reinhardtii* mutant, its CC-409 background strain, and the CC-124 control strain in TCL-PBR at sunlight intensity (1000 µmol photons $m^{-2}s^{-1}$) and high Chl content (150 µg Chl(a+b)) in a 6-day experiment. The H_2 production of all three strains was comparable in the first 24 h, approx. 200 µl/ml. After the first day, there was a steady decrease of H_2 production by the CC-124 and

the CC-409 strains, whereas it remained fairly stable in the L159I-N230Y mutant, which declined more consistently only by day 6 (144 h). In total, the L159I-N230Y strain produced 50% more H_2 compared to CC-409 and CC-124. The Chl content remained approx. at the initial level in the L159I-N230Y strain, whereas it decreased by approx. 30% and 50% in the CC-409 and CC-124 strains, respectively. The cell density increased slightly in the first 24 h in all strains, then remained relatively stable throughout the experiment. These data show no substantial cell damage or lysis occurred, but Chl became partially degraded in the CC-409 and CC-124 strains.

Regarding the composition of the photosynthetic apparatus, an important observation is that various PSII subunits, including PsbA, CP47, PSBO, and PSBP rapidly degraded in the CC-124 strain, while they were much more stable in the CC-409 and L159I-N230Y strains. A similar trend occurred for the PsaA subunit of PSI. The amount of HydA strongly decreased (by about 70%) in the CC-124 strain already after 24 h of H_2 production, whereas it was more stable in the other two strains. In agreement with these data, the F_V/F_M parameter, an indicator of photosynthetic efficiency, also remained relatively high in the CC-409 and L159I-N230Y strains, further suggesting that they are equally more resistant to high-light exposure during anaerobiosis-induced H_2 production than the CC-124 strain.

In our photoautotrophic anaerobiosis-induced H_2 production system, we have also tested the *pgrl1* and *prg5* photosystem I cyclic electron transport mutants. The *pgr5* mutant performed very well at 1000 μmol photons m^{-2} s^{-1} and 150 μg Chl(a+b)/ml: on the first day, it produced already remarkably more H_2 than the CC-124 strain (by about 65%), and the difference became much larger towards the end of the experiment. On days 4 and 5, the *pgr5* mutant produced approx. 5-fold more H_2 than CC-124; the total amount of H_2 was 2.5 times as much as that of the CC-124 strain. The *pgrl1* mutant showed a similar trend, but the improvement was only about 50% more as compared to the CC-124 strain.

Remarkably, the Chl(a+b) content of the *pgr5* mutant remained unchanged during six days of H_2 production at a very intense light, whereas that of the *pgrl1* mutant decreased slightly, and in the case of the CC-124 strain, a substantial decrease was observed; the cell densities remained relatively stable for all three strains apart from a moderate increase in the first 24 h.

The photosynthetic apparatus was preserved in the PSI-CET mutants, especially in the *pgr5* mutant: in this strain, about 50% of PsbA was detected by the end of the experiment, and practically all CP47, PSBO, PSBP subunits remained unchanged. Regarding HydA, a large portion (about 70%) of HydA was degraded within 24 h in CC-124 and in the *pgrl1* mutant. In

stark contrast, HydA was retained at almost 100% in the *pgr5* mutant throughout the 6-day H_2 production experiment.

After six days of H_2 production, the *in vitro* hydrogenase activity remained at a considerably high level in all strains. In the CC-124 strain, about 34% of the initial *in vitro* activity was retained, whereas in the *pgrl1* mutant 11% remained. Remarkably, 44% of the initial *in vitro* HydA activity of the *pgr5* mutant remained by the end of the experiment.

Anaerobiosis-induced photoautotrophic H_2 production in TCL-PBR is increased approx. three-fold as compared to traditional bulk cultures. TCL-PBR also enables continuous H_2 production at sunlight intensity. The sustained H_2 production at sunlight intensity is in stark contrast with the literature, making the present protocol a significant step toward upscaling algal H_2 production. H_2 productivity was enhanced in a PsbA mutant and in mutants deficient in PSI-CET. The *pgr5* mutant performed surprisingly well: Its photosynthetic apparatus and HydA were retained for six days and it produced as high as 1.2 ml H_2/ml culture, which is the highest H_2 photoproduction achieved so far (Nagy et al, Bioresource Technol, 2021).

Ongoing research

We are further improving our system to establish computer-controlled online sampling for gas chromatography measurements with automatic headspace flushing.

In order to gain more insights into the mechanism underlying the anaerobiosis-induced H_2 production, we intend to apply system biology methods. We have started the proteomic and metabolomic analysis of the wild-type (CC-124) and the excellent hydrogen producer *pgr5* mutant strains.

Outcomes:

Our results were presented in the form of poster or oral presentations at several national and international conferences. The most important conferences we attended were AlgaEurope conference held in Paris (France) in 2019 and Chlamy2020+1, the 19th International Conference on the Cell and Molecular Biology of Chlamydomonas held in Six-Fours-les-Plages (France) in 2021.

Based on the main results of our project two papers were published in highly ranked international journals (Nagy et al., Biotechnol Biofuels, 2018 (IF:5.4); Nagy et al., Bioresource Technol, 2021 (IF:9.64)) and our anaerobiosis-induced H_2 production method was patented (WO2018146167 - Photoautotrophic and sustainable production of hydrogen in algae) at the European Patent Office.

We are negotiating with industrial companies (Air Liquide, Tungsram) about the potential utilization of our hydrogen production protocol.

Miscellaneous

Other three papers related to our project were published (Vidal-Meireles et al., New Phytol, 2017 (8.5); Nagy et al., Plant J, 2018 (5.7); Podmaniczki et al., Physiol Plantarum, 2021 (IF:4.5).

I was granted a Nessling Foundation research fellowship and I was working as a postdoctoral fellow at the University of Turku from May 2018 to October 2019, in the group of Dr. Yagut Allahverdiyeva-Rinne. In Turku, I learned new techniques, such as MIMS, to study the hydrogen production in a flavodiiron protein mutant of *Chlamydomonas reinhardtii*. Based on our results, we published two papers (Jokel, Nagy et al., Biotechnol Biofuels, 2019 (IF:5.4; Kosourov et al., Proceeding Natl Acad Sci USA, 2021(IF:11.205). I also investigated the H_2 metabolism in cyanobacteria and a manuscript is under preparation.