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ENERGY INNOVATIONS SMALL GRANT PROGRAM
Renewable Energy Technologies

RENEWABLE HYDROGEN FUEL PRODUCTION BY MICROALGAS PHOTOSENTHESIS

FEASIBILITY ANALYSIS

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PREFACE

The Public Interest Energy Research (PIER) Program supports public interest energy research and development that will help improve the quality of life in California by bringing environmentally safe, affordable and reliable energy services and products to the marketplace.

The PIER Program, managed by the California Energy Commission (Commission), annually awards up to $62 million of which $2 million/year is allocated to the Energy Innovation Small Grant (EISG) Program for grants. The EISG Program is administered by the San Diego State University Foundation under contract to the California State University, which is under contract to the Commission.

The EISG Program conducts four solicitations a year and awards grants up to $75,000 for promising proof-of-concept energy research.

PIER funding efforts are focused on the following six RD&D program areas:

- Residential and Commercial Building End-Use Energy Efficiency
- Industrial/Agricultural/Water End-Use Energy Efficiency
- Renewable Energy Technologies
- Environmentally-Preferred Advanced Generation
- Energy-Related Environmental Research
- Strategic Energy Research

The EISG Program Administrator is required by contract to generate and deliver to the Commission a Feasibility Analysis Report (FAR) on all completed grant projects. The purpose of the FAR is to provide a concise summary and independent assessment of the grant project using the Stages and Gates methodology in order to provide the Commission and the general public with information that would assist in making follow-on funding decisions (as presented in the Independent Assessment section).

The FAR is organized into the following sections:

- Executive Summary
- Stages and Gates Methodology
- Independent Assessment
- Appendices
    - Appendix A: Final Report (under separate cover)
    - Appendix B: Awardee Rebuttal to Independent Assessment (Awardee option)

For more information on the EISG Program or to download a copy of the FAR, please visit the EISG program page on the Commission's Web site at:
http://www.energy.ca.gov/research/innovations

or contact the EISG Program Administrator at (619) 594-1049 or email eisgp@energy.state.ca.us.

For more information on the overall PIER Program, please visit the Commission's Web site at http://www.energy.ca.gov/research/index.html.

Executive Summary

Introduction

The ability of unicellular green algae to produce hydrogen (H_2) gas in the presence of sunlight has been recognized by the scientific community as being a possible way to generate hydrogen gas for energy production. Below is a list of important known factors specific to green algal H_2-production.

1. Photosynthesis can operate with a photon conversion efficiency of >80%,
2. Microalgae can produce H_2 photosynthetically with a photon conversion efficiency of >80%,
3. Molecular Oxygen (O_2) acts as a switch by which H_2 production is turned off,
4. Sixty years of research has failed to overcome the incompatibility of simultaneous O_2 and H_2 production by photosynthesis.

Recent work has shown that the absence of Sulfur (S) from the growth medium of the green algae Chlamydomonas reinhardtii (*C reinhardtii*) causes a specific but reversible decline in the rate of oxygenic photosynthesis but does not affect the rate of mitochondrial respiration.
The absence of sulfur from the growth medium triggers a metabolic switch, one that selectively and reversibly turns-off photosynthetic O_2 production. In the presence of S, green algae photosynthesize normally (H_2O oxidation, O_2 evolution and biomass accumulation), however, in the absence of S and absence of O_2, photosynthesis in *C. reinhardtii* produces H_2 rather than O_2. Further refinement of this method may lead to full understanding of the green algae hydrogen-related metabolism and ultimately the generation of H_2 gas for the fuel and chemical industries.

Objectives

The goal of this project was to determine the feasibility doubling the hydrogen production efficiency of *C. reinhardtii* from the level of ~10% of the theoretical maximum to the level of ~20% of the theoretical maximum. The following project objectives were established:

1. Improve the H_2 production by shifting forward the equilibrium of the reversible hydrogenase catalyzed reaction.
2. Design and test cell growth media that accentuate the metabolism of H_2 production.
3. Test the effect of diurnal cycles on starch mobilization and H_2 production.
4. Identify the rate-limiting step in the H_2 production process.

Outcomes

1. Two approaches were investigated attempting to shift forward the equilibrium of the reversible hydrogenase catalyzed reaction:
   a. The atmospheric air within the photobioreactor was purged with argon to remove dissolved O_2 prior to initiation of H_2 production. Independent measurements have shown that such degassing is sufficient to reduce the O_2 content of the culture to less than 2% of saturation. This purge resulted in almost immediate initiation of H_2 production, while H_2 production by the control culture was delayed for another 10 to 15 hours, hence this approach shifted the reaction forward, reducing the wait time by roughly 10-15% of the hydrogen production cycle length.
   b. The pH balance of the water in the gas collection device was modified with the thesis that the water was saturated with H_2 gas and perhaps contained a significant amount. No detectable increase in the collected amount of H_2 gas was observed.
2. Multiple designs of cell growth media were tested to accentuate the metabolism of H_2 production.
   a. Determined the effects of salinity:
      i. On chlorophyll content and cell viability. Cell density increased then remained constant with moderate levels of salinity.
      ii. On cellular photosynthesis and respiration. Neither photosynthesis nor respiration appeared to be effected by reasonable amounts of salinity.
      iii. On H_2 production. A moderate level of salinity, 10 mM NaCl enhanced the rate and yield of H_2 gas by 30% and 40% respectively over the control.
   b. Determined the effects of ATP biosynthesis uncouplers as follows:
      i. Methylamine-hydrochloride. Over a very narrow range centered on 5 mM Methylamine, H2 production increased as much as 20-25% over the control.
      ii. Gramicidin. This chemical was not sufficiently soluble to allow investigation of concentrations greater than about 5 micromoles. However, at that level the effect was similar to that of Methylamine, above.
      iii. FCCP. This chemical significantly reduced the production of H_2.
   c. Determined the effect of trace amounts of S; Used 10, 50 and 100 micromoles S samples. The addition of S was observed to delay the onset of H_2 production. However the total production increased with increase of S until the delay of onset became the dominant factor. Culture with 50 micromoles S added produced about 35% greater H_2 yield than produced by the cultures with no S added.
3. The *C reinhardtii* were cyclically deprived of S and supplied with S, for three complete cycles, and the rate of production of H_2 was recorded. The result was that the single culture of *C reinhardtii* produced about three times as much H_2 in three cycles as the control cultures did in a single cycle. This illustrated the capacity of the *C. reinhardtii* to recover from the catabolic effects of H_2 production when S is provided between H_2 production cycles.
4. The rate limiting step in the H_2 production process was identified by Western Blot analysis of the total cell protein extracts from *C reinhardtii*. This showed that the hydrogenase gene is expressed sparingly under S deprivation conditions.

5. In all cases the gas produced by the *C reinhardtii* algae was collected in a tube (upside down burette) filled with water. The gas collected in this manner was reported as hydrogen. Gas chromatographic analysis of the gas collected showed ~90% H_2, ~10% nitrogen with traces of CO_2 and O_2.

Conclusions

This research indicates that H_2 production rates approaching 20% of theoretical maximum could be achieved by proper combination of the techniques explored in this project.

1. The equilibrium of the reversible hydrogenase catalyzed reaction can be shifted forward.
   a. There is significant benefit, 8 - 12% production improvement to be gained by rapid transition from aerobic to anaerobic environment in the photobioreactor medium, compared to a "natural" transition.
   b. The question of H_2 solubility in water was not definitively answered.
2. H_2 production can be increased by improving the design of the cell growth media.
   a. Moderate levels of salinity of the culture do not adversely affect cell viability and do increase H_2 production by ~40%. However, there may still be room for improvement with further study.
   b. Due to the modest effect and the high cost of ATP biosynthesis inhibitors, their use is not the method of choice for manipulation of H_2 production.
   c. Sulfur titration holds the promise of improving the yield of H_2 production by as much as 35% in this two-stage photosynthesis and H_2 production method. Its utility in a production environment is a concern.
3. It may be possible to extend the production of H_2 by a single culture ad infinitum by alternately supplementing and depriving the culture of its organic sulfur. Three cycles were demonstrated.
4. The sparse expression of the hydrogenase gene within the *C reinhardtii* is responsible for the limited H_2 production. Genetic engineering should be applied to increase the expression of this gene.
5. The technique for removal of nitrogen from the product gas must be identified.
6. It is evident that incremental improvements in the yield of hydrogen production can be accrued upon R&D of the method. However, further improvements need to be achieved to make "hydrogen production by sulfur-deprivation of green algae" a commercial reality.

Benefits to California

Both small-scale (industrial and commercial) and larger (utility) solar energy conversion plants (photobioreactors) can be envisioned utilizing the Two-Stage Photosynthesis and H_2-Production process. Remote photobioreactors could be installed as modules in arid areas where sunlight is plentiful and alternative uses of land are minimal. Such a process of H_2 gas production would be sustainable, environmentally friendly and economically attractive compared to most other hydrogen production alternatives.

In addition to H_2, a valuable and clean fuel, the Two-Stage Photosynthesis and H_2-Production process will generate green algal biomass as a significant "Value-Added Bioproduct" that will enhance the economics and competitiveness of the process.

Recommendations

Further Research and development should address the specific biological and engineering challenges facing commercialization of this technology.

- Further basic research should be done to improve understanding of the cellular metabolism and basic biochemistry that support this process.
- This project demonstrated that hydrogen production of 20% of theoretical is achievable, however, further research should be done to improve the hydrogen production further.
- Preliminary designs for the scale-up of the process should be created, and a preliminary life cycle cost analysis performed. This analysis should compare the cost of producing electricity by this method to the cost of PV. Further, this analysis should compare the cost of producing hydrogen by this method compared to the electrolysis method.

The technology developed in this project will take 8 to 12 years to advance to the state of market readiness.

Stages and Gates Methodology

The California Energy Commission utilizes a stages and gates methodology for assessing a project's level of development and for making project management decisions. For research and development projects to be successful they need to address several key activities in a coordinated fashion as they progress through the various stages of development. The activities of the stages and gates process are typically tailored to fit a specific industry and in the case of PIER the activities were tailored to be appropriate for a publicly funded energy research and development program. In total there are seven types of activities that are tracked across eight stages of development as represented in the matrix below.

Development Stage/Activity Matrix

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	Stage 8
Activity 1								
Activity 2								
Activity 3								
Activity 4								
Activity 5								
Activity 6								
Activity 7								

A description the PIER Stages and Gates approach may be found under "Active Award Document Resources" at: http://www.energy.ca.gov/research/innovations and are summarized here.

As the matrix implies, as a project progresses through the stages of development, the work activities associated with each stage needs to be advanced in a coordinated fashion. The EISG program primarily targets projects that seek to complete Stage 3 activities with the highest priority given to establishing technical feasibility. Shaded cells in the matrix above require no activity, assuming prior stage activity has been completed. The development stages and development activities are identified below.

Development Stages:		**Development Activities:**	
Stage 1:	Idea Generation & Work Statement Development	Activity 1:	Marketing / Connection to Market
Stage 2:	Technical and Market Analysis	Activity 2:	Engineering / Technical
Stage 3:	Research & Bench Scale Testing	Activity 3:	Legal / Contractual
Stage 4:	Technology Development and Field Experiments	Activity 4:	Environmental, Safety, and Other Risk Assessments / Quality Plans
Stage 5:	Product Development and Field Testing	Activity 5:	Strategic Planning / PIER Fit - Critical Path Analysis
Stage 6:	Demonstration and Full-Scale Testing	Activity 6:	Production Readiness / Commercialization
Stage 7:	Market Transformation	Activity 7:	Public Benefits / Cost
Stage 8:	Commercialization		

Independent Assessment

For the research under evaluation, the Program Administrator assessed the level of development for each activity tracked by the Stages and Gates methodology. This assessment is summarized in the Development Assessment Matrix below. Shaded bars are used to represent the assessed level of development for each activity as related to the development stages. Our assessment is based entirely on the information provided in the course of this project, and the final report. Hence it is only accurate to the extent that all current and past work related to the development activities are reported.

Development Assessment Matrix

Stages / Activity	1 Idea Generation	2 Technical & Market Analysis	3 Research	4 Technology Development	5 Product Development	6 Demonstration	7 Market Transformation	8 Commercialization
Marketing								
Engineering / Technical								
Legal/ Contractual								
Risk Assess/ Quality Plans								
Strategic								
Production. Readiness/								
Public Benefits/ Cost								

The Program Administrator's assessment was based on the following supporting details:

Marketing/Connection to the Market

Melis Energy, a California Corporation was formed in January, 2001 in order to advance the commercialization of the process of hydrogen production by microalgal photosynthesis. A detailed and fully documented business plan does not yet exist.

Engineering/Technical

This project successfully demonstrated that Two-Stage Photosynthesis and H_2-Production process can be operated at efficiencies approaching 20% of theoretical with current techniques. That is, through the simultaneous use of sulfur titration, moderate levels of salinity in the culture and through rapid transition from aerobic to anaerobic environment in the photobioreactor medium, though not demonstrated explicitly, the outcomes indicated that the H_2 production rate should approach 20% of theoretical.

Legal/Contractual

There is no indication of patent or invention disclosure filing, nor is there any certainty that such filings are appropriate in this case.

Environmental, Safety, Risk Assessments/ Quality Plans

This aspect of hydrogen gas production was not addressed by this project although it may be reasonably assumed that this technology will have unique and potentially serious risks associated with it. Initial drafts of the following Quality Plans are needed prior to initiation of Stage 4 development activity; Reliability Analysis, Failure Mode Analysis, Manufacturability, Cost and Maintainability Analyses, Hazard Analysis, Coordinated Test Plan, and Product Safety**.**

Strategic

This product has no known critical dependencies on other projects under development by PIER or elsewhere.

Production Readiness/Commercialization

There was no activity reported in this area beyond that to which technical feasibility relates. However, Melis Energy was formed in January, 2001 in order to advance the commercialization of this process.

Public Benefits

Public benefits derived from PIER research and development are assessed within the following context:

- Reduced environmental impacts of the California electricity supply or transmission or distribution system.
- Increased public safety of the California electricity system
- Increased reliability of the California electricity system
- Increased affordability of electricity in California

The primary benefit to the rate payer from this research will be reduced environmental impacts of the California electricity supply or transmission or distribution system. In order to supply this benefit, hydrogen for electrical generation must directly compete with photovoltaic technology. Projected costs of PV range below $50 per square meter at 10% to 15% efficiency. This project did not provide sufficient information to formulate comparative cost estimates.

Appendix A: Final Report (under separate cover)

Appendix B: Awardee Rebuttal to Independent Assessment (none submitted)

Appendix A to FAR 99-06

ENERGY INNOVATIONS SMALL GRANT (EISG) PROGRAM

EISG FINAL REPORT

RENEWABLE HYDROGEN FUEL PRODUCTION BY MICROALGAL PHOTOSYNTHESIS

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Inquires related to this final report should be directed to the Awardee (see contact information on cover page) or the EISG Program Administrator at (619) 594-1049 or email eisgp@energy.state.ca.us.

Acknowledgement Page

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Executive Summary

1. Introduction

Recent work has shown that lack of sulfur from the growth medium of green algae caused a hitherto unknown switch in cellular photosynthesis. In sealed cultures, instead of oxygen gas, green algae produced hydrogen gas (Melis et al., 2000). It was shown that, under such sulfur-deprivation conditions, it is possible to photoproduce and to accumulate significant volumes of H_2 gas, using the green alga *Chlamydomonas reinhardtii*, in a sustainable process that can be employed continuously for several days. Thus, progress was achieved by a temporal separation of the reactions of (I) normal photosynthesis, cell growth, carbohydrate accumulation and O_2 production from (II) anaerobic photosynthesis and H_2 production. This process became known as the "Two-Stage Photosynthesis and H_2-Production" process (Melis et al., 2000). The novel application of this two-stage protocol revealed the occurrence of hitherto unknown metabolic, regulatory and electron-transport pathways in the green alga *C. reinhardtii* (Melis et al., 2000; Zhang et al. 2001), leading to the significant and sustainable light-dependent release of H_2-gas by the cells.

The temporal sequence of events in this two-stage photosynthesis and H_2-production process is as follows.

- Green algae are grown photosynthetically in the light (normal photosynthesis) until they reach a density of 3-6 million cells per ml in the culture.
- Sulfur deprivation is imposed upon the cells in the growth medium, either by carefully limiting sulfur supply in the medium so that it is consumed entirely, or by permitting cells to concentrate in the growth chamber prior to replacement of the growth medium with one that lacks sulfur nutrients. Cells respond to this S-deprivation by fundamentally altering photosynthesis and cellular metabolism in order to survive (Davies et al., 1996; Hell, 1997; Zhang et al., 2001).
- Inorganic S-deprivation in *C. reinhardtii* exerts a distinctly different effect on the cellular activities of photosynthesis and respiration. The activity of photosynthesis, measured from the light-saturated rate of O_2 evolution, declines quasi-exponentially with a half-time of 15-20 h to a value less than 10% of its original rate (Wykoff et al., 1998). However, cellular respiration, measured from the rate of O_2 consumption in the dark, remains fairly constant over the S-deprivation period (Melis et al., 2000). In consequence, the absolute activity of photosynthesis slips to a level below that of respiration after about 24 h of S-deprivation. Following this cross-point, sealed cultures of S-deprived *C. reinhardtii* quickly consume all dissolved oxygen and become anaerobic (Ghirardi et al., 2000), even though they are maintained under continuous illumination.
- Under S-deprivation conditions, sealed (anaerobic) cultures of *C. reinhardtii* produce H_2 gas in the light but not in the dark. The volume and rate of photosynthetic H_2 production was monitored from the accumulating H_2 gas in an inverted graduated burette, measured from the

volume of water displacement. A rate of 2.0-2.5 mL H_2 production per liter culture per hour was sustained in the 24-70 h period. The rate declined gradually thereafter.

2. Project Objectives

A project objective was to improve in the laboratory the novel Two-Stage Photosynthesis and H_2-Production process and to develop/employ conditions for the continuous production of hydrogen gas. A further objective was to obtain better insight into the biochemistry of the process and the physiology of the cells as they produce H_2 gas under sulfur deprivation. This knowledge is pivotal in efforts to increase the yield and commercialize the process.

3. Project Outcomes

A summary of what we have learned from the conduct of this work is given below:

- In the presence of S, green algae do normal photosynthesis (H_2O oxidation, O_2 evolution and biomass accumulation). The absence of sulfur from the growth medium of green algae acts as a metabolic switch, one that <u>selectively</u> and <u>reversibly</u> turns-off photosynthetic O_2 production.
- In the absence of S and absence of O_2, photosynthesis in *C. reinhardtii* slips into the H_2 production mode.
- Reversible application of the switch (presence/absence of S) permits the algae to alternate between O_2 production/biomass accumulation and H_2 production (temporal separation of the two processes), thus bypassing the incompatibility and mutually exclusive nature of the O_2 and H_2 producing reactions.
- Interplay between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway is essential for this light-mediated H_2-production process.
- The release of H_2 gas serves is an alternative (and novel) light-mediated "respiration" by the cells, needed for the survival of the organism under sulfur-deprivation conditions.

4. Conclusions

Upon further refinement, the "Two-Stage Photosynthesis and H_2-Production" process may serve in the generation of H_2 gas for the fuel and chemical industries. There are no absolute technical barrier for the application of this technology in the commercial production of H_2 gas.

5. Benefits to California

Both small-scale (industrial and commercial) and larger (utility) solar energy conversion plants (photobioreactors) can be envisioned utilizing the Two-Stage Photosynthesis and H_2-

Production process. Remote photobioreactors could be installed as modules in arid areas where sunlight is plentiful and alternative uses of land are minimal. Such a process of H_2 gas production would be sustainable, environmentally friendly and economically attractive compared to most other alternatives.

In addition to H_2, a valuable and clean fuel, the Two-Stage Photosynthesis and H_2-Production process will generate green algal biomass as a significant "Value-Added Bioproduct" that will enhance the economics and competitiveness of the process. In sum, the process will find application in the:

- Generation of a valuable, clean and renewable fuel.
- Global warming mitigation and reduction in the level of atmospheric polution.
- Production of biomass from which "High-value Bioproducts and Biochemicals" could be extracted.

6. Recommendations

The "Two-Stage Photosynthesis and H_2-Production" process brings together two normally separate fields: Energy and Biotechnology. The successful development and employment of this method will boost California's competitiveness in the fields of Energy, Agriculture and Chemistry.

It is thus recommended that every effort be made to advance this technology to its fullest potential

Abstract

Sulfur-deprivation in green algae causes reversible inhibition in the activity of photosynthesis. In the absence of sulfur, rates of photosynthetic O_2 evolution drop below those of O_2 consumption by respiration. In consequence, sealed cultures of the green alga *Chlamydomonas reinhardtii* become anaerobic in the light, induce the "Fe-hydrogenase" pathway of electron transport and photosynthetically produce H_2 gas. In the course of such H_2 gas production, cells consume significant amounts of internal starch and protein. Such catabolic reactions may sustain, directly or indirectly, the H_2-production process. Reversible application of the switch (presence/absence of S) permits the algae to alternate between O_2 production/biomass accumulation and H_2 production (temporal separation of the two processes), thus bypassing the incompatibility and mutually exclusive nature of the O_2 and H_2 producing reactions. Repetitive application of the switch could be the basis of a commercial H_2-production system. Profile analysis of selected photosynthetic proteins during H_2-production showed a precipitous decline in the amount of Rubisco as a function of time in S-deprivation. It is suggested that, under S-deprivation conditions, electrons derived from a residual photosynthetic H_2O-oxidation activity and from endogenous substrate catabolism feed into the hydrogenase pathway, thereby contributing to the H_2-production process in *Chlamydomonas reinhardtii*. Interplay between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway is essential for this light-mediated H_2-production process. The promise and application of this technology in the fields of Energy, Agriculture and Chemistry are discussed.

Keywords *Chlamydomonas reinhardtii* – Green algae – Hydrogen production – Hydrogenase – Photosynthesis – Sulfur deprivation

Main Report

Introduction

Background and Overview

Hydrogen gas is considered as the ideal fuel for a California future in which air pollution has been eliminated, global warming has been arrested and our environment has been protected in an economically sustainable manner. Hydrogen fuel would team with electricity to provide attractive options to Californians in transportation and power generation. The easiness of interconversion between these two forms of energy suggests a future in which the electric power grid would become a carrier, user and producer of hydrogen, depending on local conditions and many interdependent and only partially predictable technological developments. Achieving this vision as a practical reality within the next century will require a significant effort in order to attain the necessary infrastructure in H_2 production and utilization technologies. The most challenging problem in establishing hydrogen as a fuel for the future is the renewable generation of large quantities of hydrogen gas. Thus, processes that are presently conceptual in nature, or at an early development stage in the laboratory, need to be encouraged, tested for feasibility and assessed to identify the most promising alternatives.

Since the pioneering discovery by Gaffron and co-workers over 60 years ago (Gaffron 1939; Gaffron and Rubin, 1942), the ability of unicellular green algae to produce H_2 gas in the light has been mostly a biological curiosity. Historically, hydrogen evolution activity in green algae was elicited upon a period of anaerobic incubation of the cells in the dark (Greenbaum, 1982; Roessler and Lien, 1984; Happe and Naber, 1993). A high specific activity hydrogenase enzyme was expressed under such incubation of the cells and catalyzed a light-mediated H_2-evolution. The monomeric form of the enzyme, which belongs to a novel class of Fe-hydrogenases (Voordouw et al., 1989; Adams, 1990; Meyer and Gagnon, 1991; Peters et al., 1998) is encoded in the nucleus of the unicellular green algae but the mature protein is localized and functions in the chloroplast stroma (Happe et al., 1994). Light absorption by the photosynthetic apparatus is essential for the generation of hydrogen. Ferredoxin PetF, the natural electron donor, links the Fe-hydrogenase to the photosynthetic electron transport chain of the green algae (Florin et al., 2001). However, light absorption also results in the oxidation of H_2O and the release of molecular O_2, which is a powerful inhibitor of the Fe-hydrogenase.

In addition to this PSII-dependent H_2 photoevolution, which implicates water as the source of electrons and produces 2:1 stoichiometric amounts of H_2 and O_2, a second mechanism has been described in the literature (Gfeller and Gibbs, 1984). The alternative pathway entails catabolism of endogenous substrate and an oxidative carbon pathway for the generation of reducing power in the chloroplast of the green algae. Reductant (electrons) from such endogenous substrate catabolism feeds into the photosynthetic electron-transport chain between the two photosystems, and probably at the level of the plastoquinone pool. Light absorption by PSI and the ensuing electron transport elevates the redox potential of these electrons to the redox equivalent of ferredoxin (Gibbs et al., 1986). Thus, following a sufficiently dark anaerobic incubation of the culture, high amounts of H_2 can be transiently detected upon a subsequent illumination of the algae (Happe and Naber, 1993; Ghirardi et al., 1997).

The transient only nature of H_2-production is attributed to the light-dependent simultaneous generation of O_2 by the photosynthetic apparatus. Oxygen is a powerful inhibitor of the Fe-hydrogenase (Ghirardi et al., 2000). Current technological developments in this field

have not yet succeeded in overcoming this mutually exclusive nature of the O_2 and H_2 photoproduction reactions. Thus, the physiological significance and role of the Fe-hydrogenase in green algae, which normally grow under aerobic photosynthetic conditions, has long been a mystery. Given the O_2 sensitivity of the Fe-hydrogenase and the prevailing oxidative environmental conditions on earth, questions have been asked as to whether the hydrogenase is anything more than a relic of the evolutionary past of the chloroplast in green algae, and whether this enzyme and the process of photosynthesis can ever be utilized to generate H_2 gas for commercial purposes (Zhang et al., 2001). Nevertheless, the ability of green algae to photosynthetically generate H_2 gas has captivated the fascination and interest of the scientific community because of the fundamental and practical importance of the process. Below is an itemized list of the properties and promise of photosynthesis in green algal H_2-production, and the problems that are encountered with current technology:

- Photosynthesis can operate with a photon conversion efficiency of ≥80% (Ley and Mauzerall 1982).
- Microalgae can produce H_2 photosynthetically with a photon conversion efficiency of ≥80% (Greenbaum 1988).
- Molecular O_2 acts as a <u>powerful</u> and <u>effective</u> switch by which the H_2-production activity is turned off.
- This incompatibility in the simultaneous O_2 and H_2 photoproduction could not be overcome in 60 years of related research.

Recent work has shown that lack of sulfur from the growth medium of the green alga *Chlamydomonas reinhardtii* causes a specific but reversible decline in the rate of oxygenic photosynthesis (Wykoff et al. 1998) but does not affect the rate of mitochondrial respiration (Melis et al., 2000). In sealed cultures, such imbalance in photosynthesis-respiration resulted in a net consumption of oxygen, quickly causing anaerobiosis in the growth medium, a condition that automatically elicited H_2-production by the cells (Melis et al., 2000). It was shown that, under such conditions, it is possible to photoproduce and to accumulate significant volumes of H_2 gas, using the green alga *Chlamydomonas reinhardtii,* in a sustainable process that can be employed continuously for several days. Thus, progress was achieved by circumventing the sensitivity of the Fe-hydrogenase to O_2 through a temporal separation of the reactions of O_2 and H_2 production, via a so-called "Two-Stage Photosynthesis and H_2-Production" process (Melis et al., 2000). The novel application of this two-stage protocol revealed the occurrence of hitherto unknown metabolic, regulatory and electron-transport pathways in the green alga *C. reinhardtii* (Melis et al., 2000; Zhang et al. 2001), leading to the significant and sustainable light-dependent release of H_2-gas by the cells.

Upon further refinement, this method may serve in the elucidation of the green alga hydrogen-related metabolism and in the generation of H_2 gas for the fuel and chemical industries. A summary of the advancement in the state-of-the-art in this field is given below:

- The absence of sulfur from the growth medium triggers a metabolic switch, one that <u>selectively</u> and <u>reversibly</u> turns-off photosynthetic O_2 production.
- In the presence of S, green algae do normal photosynthesis (H_2O oxidation, O_2 evolution and biomass accumulation). In the absence of S and absence of O_2, photosynthesis in *C. reinhardtii* slips into the H_2 production mode.

<u>Project Objectives</u>

The discovery of sustainable H_2 production that bypasses the sensitivity of the reversible hydrogenase to O_2 is a significant development in the field. It may lead to exploitation of green algae for the production of H_2 gas as a clean and renewable fuel. However, the actual rate of H_2 gas accumulation was at best 15-20% of the photosynthetic capacity of the cells, when the latter is based on the capacity for O_2 evolution (Melis et al., 2000). The relatively slow rate of H_2-production suggests that there is room for significant improvement in the yield of the process, by as much as one order of magnitude. Similarly, other improvements must be made to optimize the process under conditions of mass culture of the algae. For example, optical problems associated with the size of the chlorophyll antenna and the light-saturation curve of photosynthesis must also be addressed (Melis et al., 1999) before green algae can achieve high photosynthetic solar conversion efficiencies in mass culture. Moreover, H_2-production by S-deprived algae cannot be sustained forever. The yield begins to level off after about 70 h in S-deprivation. The cells need to go back to normal photosynthesis after about 100 h of S-deprivation in order to be rejuvenated by replenishing lost endogenous substrate (Ghirardi et al. 2000). Thus, it was the objective of this research to perform experimentation by which (a) to improve the yield of the process and (b) to address the continuity of H_2-production. The work was divided into four specific tasks and was implemented in its entirety. A listing of the tasks is given below:

Task 1. Improve the H_2 production by shifting forward the equilibrium of the *reversible hydrogenase* catalyzed reaction.
Task 2. Design and test cell growth media that accentuate the metabolism of H_2 production.
Task 3. Test the effect of diurnal cycles on starch mobilization and H_2 production.
Task 4. Identify the rate-limiting step in the H_2 production process.

Project Approach and Outcomes

Task 1: To improve the yield of H_2 production by shifting forward the chemical equilibrium of the *reversible hydrogenase* catalyzed reaction

Subtask 1: Degassing of the photobioreactor was tested for its effect on the Stage 1→ Stage 2 H_2–production process.

Experimentation addressed the effect of oxygen partial pressure on the onset of the hydrogen production process. **Figure 1** shows the results of an experiment in which the green algal samples were grown in the photobioreactor until they reached a density of $3x10^6$ cells per milliliter of culture. Sulfur was removed from the growth medium at time 0 h and the culture was incubated for 24 hours to permit cells to consume internally stored sulfur. The photobioreactor was either degassed for 10 min by a steady stream of argon to purge dissolved atmospheric air from the medium (**Fig. 1, Degassed**), or sealed without any prior degassing (**Fig. 1, Control**). Subsequently, culture bottles (Roux type) were sealed with silicone stoppers tightly fitted with a syringe. Capillary Teflon tubing was attached to the syringe for the collection of gasses. The Teflon tubing was used to conduct H_2 gas (evolved by the algae in the photobioreactor) to an upside-down burette filled with water. The upside-down water-filled

burette was immersed in a beaker also filled with water. Hydrogen gas produced by the culture eventually accumulated in the inverted burette by displacing a equal volume of water. It was measured directly from the graduated divisions of the burette.

It is evident from the results of **Figure 1** that degassing of the culture accelerates the onset of H_2 production by the cells. This can be attributed to the removal of dissolved oxygen from the growth medium (lowering of the oxygen partial pressure in the photobioreactor). Independent measurements with an oxygen electrode have shown that such degassing is sufficient to lower the oxygen partial pressure from near saturation levels (270 mmoles O_2 per liter medium) to less than 5 mmoles O_2 per liter medium (results not shown). Absence of O_2 is an absolute prerequisite for the H_2-production process. The rate of H_2 production in the two samples (Fig. 1, Degassed and Control), measured from the slope of the two lines, was approximately the same, suggesting that the rate of H_2 production is not influenced by the early removal of oxygen from the photobioreactor. However, for the duration of this single batch experiment, initiation of the process occurred sooner and the yield of H_2 production was systematically greater in cultures that were degassed than in control ones. This was a direct consequence of the early removal of atmospheric oxygen from the cultures. In conclusion, this experimentation clearly shows that, in a Stage 1→Stage 2 process in which photosynthetic O_2 evolution and carbon accumulation (Stage 1) alternate with consumption of cellular metabolites and concomitant H_2 production (Stage 2), the effectiveness of the switch from Stage 1 to Stage 2 largely depends on the removal of oxygen from the photobioreactor medium. **Thus, future experimental designs seeking to improve the yield of H_2 production in this Two-Stage process need focus on how best to make the transition from aerobic to anaerobic environment in the photobioreactor medium.**

Subtask 2: The research will measure the effect of the pH of the water medium in the burette, to assess the optimal pH conditions for the release of H_2 gas in the collection space.

In the Stage 1→Stage 2 process described above, hydrogen gas is generated by the green algae in a 1 L sealed culture. It passes through a volume of water contained in the collection apparatus (beaker and inverted burette) before it can be collected in the gaseous form. Water in the collection apparatus is in equilibrium with atmospheric air. Inevitably, the volume of H_2 gas collected is equal to that produced *minus* the amount of H_2 dissolved in the growth medium of the photobioreactor and in the water of the collection apparatus (beaker and inverted burette). The solubility of molecular hydrogen to degassed water is 0.77 mmoles per liter, and presumably lower in the presence of other gasses (e.g., nitrogen in the photobioreactor; nitrogen and oxygen in the collection apparatus). Moreover, H_2 gas produced photobiologically in Stage 2 is expected to purge other gases from the medium, both in the photobioreactor and in the collection apparatus. These considerations suggest that there is a complicated and dynamic interaction between the various gases (and the atmosphere) in the collection apparatus, one that might influence the yield of H_2 gas collected. Therefore, experimental conditions that limit the solubility of molecular H_2 in water might help increase the yield of H_2 production in a Stage 1→Stage 2 approach.

Figure 2 shows the result of a first experimental attempt to influence the H_2 solubility in water. The pH of the water in the collection apparatus was adjusted from the normally neutral (pH=7.0 in Fig. 1) to acidic (pH<2.0 in Fig. 2, solid circles) or basic (pH>12 in Fig. 2, open

circles). The yield and rate of H_2 gas collected were measured under these two different pH conditions. The results shown in Fig. 2 failed to show any significant difference in either rate or yield of H_2 gas collection in the inverted burette as a function of the pH of the medium. **These results suggest one of two possibilities: (1) H_2 solubility in water is not an important variable for the determination of yields and rates in H_2 production, or (2) changes of the pH in the H_2 collection apparatus is not the method of choice by which to influence H_2 solubility in water.** New insights and experimental designs are needed to conclusively address the question of H_2 solubility in water.

Task 2. <u>To design and test cell growth media that accentuate the metabolism of H_2 production. *Effect of salinity on H_2-production*</u>

The *reversible hydrogenase* pathway of electron transport, which leads to the generation of H_2 gas, is thought to function in order to generate ATP energy for the metabolic needs of the cell [Schlegel and Schneider 1978]. If this hypothesis is correct, then, physiological and environmental conditions that require a greater cellular expenditure of ATP may cause a stimulation in the rate of electron transport via the *reversible hydrogenase* pathway, thereby resulting in greater yields of H_2 gas production. Salinity is an environmental condition that requires expenditure of cellular ATP to prevent accumulation of unwanted Na^+ cations in the cytosol of green algae. Accordingly, **the research investigated the yield of H_2 gas production as a function of NaCl concentration in the bioreactor medium**. A complete analysis of the Two-Stage process in different salinity concentrations is presented and discussed below.

<u>Subtask 1: Effect of salinity on chlorophyll content and cell viability.</u>

The Chl content of the cells and the viability of *C. reinhardtii* were measured as a function of time upon sulfur deprivation in cultures that contained different amounts of NaCl. **Fig. 3** shows that, independent of salinity and as a function of time in the absence of sulfur, the cell density in the culture increased from about $4x10^6$ cells/mL at 0 h to about $6x10^6$ cells/mL at 60 h. Subsequently, cell density stayed constant or declined slightly during the 60-100 h period. These result show that no significant loss in cell viability occurs throughout the long sulfur-deprivation stress.

Fig. 3 also shows that the Chl content of the culture increased transiently during the 0-10 h sulfur deprivation period but declined steadily thereafter. After 100 h of sulfur deprivation, only about 50% of Chl content had remained in the cells, suggesting regulated catabolism of some of the Chl-protein complexes in the cell [Melis et al. 2000]. Thus, the Chl content per cell declined from about $2x10^{-15}$ mol Chl/cell to about $1x10^{-15}$ mol Chl/cell after 100 h of sulfur deprivation. These results show that some cell division does occur during the first 60 h of sulfur deprivation but that a gradual loss of Chl also occurs during the sulfur deprivation period.

<u>Subtask 2: Effect of salinity on cellular photosynthesis and respiration.</u>

When *Chlamydomonas reinhardtii* cultures are deprived of inorganic sulfur, the capacity of the cells for O_2 evolution and CO_2 fixation decline significantly with a half-time of about 15-20 h in the light [Wykoff et al. 1998], without a concomitant loss in the capacity of respiration [Melis et al. 2000]. The absolute activity of photosynthesis, measured from the light-saturated rate of O_2 evolution in *C. reinhardtii* (**Fig. 4**, Pmax), declined bi-exponentially from ~120 mmol O_2 (mol Chl)$^{-1}$ s^{-1} at t=0 h to about 5 mmol O_2 (mol Chl)$^{-1}$ s^{-1} at t=100 h. Cellular respiration,

measured from the rate of O_2 consumption in the dark (Fig. 3, R), remained fairly constant over the 0-100 h period. It is important to note that the absolute activity of photosynthesis decreased below that of respiration in *C. reinhardtii* after about 25 h of sulfur deprivation. Interestingly, this pattern did not change as a function of salinity, at least in the 0-15 mM NaCl concentration. At higher concentrations, the activity of photosynthesis and the ability of cells to grow were adversely affected [Neale and Melis 1989].

Subtask 3: Effect of salinity on hydrogen production.

It is known that, sometime after about 25 h of sulfur deprivation, a sealed *Chlamydomonas reinhardtii* culture would quickly become anaerobic in the light, due to the significantly greater rate of respiration than photosynthesis of the cells. This was indeed confirmed by measurements with a Clark-type O_2 electrode (results not shown). Under these conditions, *C. reinhardtii* resorts to the reversible hydrogenase pathway for electron transport, leading to H_2-production. **Fig. 5** shows results from such measurements with sulfur-deprived cultures of *C. reinhardtii* suspended under different salinity conditions. It is evident that cultures under mild salinity conditions (5 and 10 mM) perform better than the control (0 mM NaCl) and the 15 mM NaCl sample. The optimal concentration of NaCl for maximum H_2-production was 10 mM. The results show that, at this salinity, the H_2-production activity of the 10 mM NaCl sample was enhanced, on the order of about 30% in the initial rate and 40% in the yield, compared to that of the control.

These results are consistent with the hypothesis that the reversible hydrogenase pathway serves to drive the formation of ATP energy for the metabolic needs of the cells. **They also suggest that further refinement in the design of cell growth media, including the application of mild stress conditions, may lead to further enhancement in the rate constant and yield of H_2-production by green algae.**

Task 2. To design and test cell growth media that accentuate the metabolism of H_2 production. *Effect of ATP biosynthesis uncouplers*

The *reversible hydrogenase* pathway of electron transport, which leads to the generation of H_2 gas, is thought to function in order to generate ATP energy for the metabolic needs of the cell. If this hypothesis is correct, then, physiological and environmental conditions that require a greater cellular expenditure of ATP may cause a stimulation in the rate of electron transport via the *reversible hydrogenase* pathway, thereby resulting in greater yields of H_2 gas production. In the previous report, salinity was used as an environmental condition that requires expenditure of cellular ATP to prevent accumulation of unwanted Na^+ cations in the cytosol of green algae (please see January-March 2000 report). In this reporting period, a complementary approach was taken: the ATP generation process was uncoupled from the electron-transport pathway leading to H_2-production. The goal of this investigation was to see whether such uncoupling might release the "load" and therefore permit greater rates of H_2-production. Three different classes of such uncouplers were tested and the results are described below for each category.

Subtask 1: Effect of methylamine-hydrochloride.

Methylamine-hydrochloride acts upon sequestering itself in the interior (lumen) of the photosynthetic membranes. There, it acts by binding protons as the latter are deposited during electron transport. This binding prevents the overaccumulation of protons in the lumen and

alleviates a potentially inhibitory effect it may have in the process of electron transport. In this experimentation, variable amounts (0-50 mM) of methylamine-hydrochloride (CH_3NH_2-HCl, pH 7.4) were added to the green algal culture *(Chlamydomonas reinhardtii)* 24 h after sulfur deprivation and immediately prior to sealing of the cultures. Hydrogen accumulation was monitored by the inverted burette method. **Fig. 6** presents a family of curves showing initiation of H_2-production at about 27 h. In several such experiments, control cultures (Fig. 6, 0 mM) and cultures in the presence of low amounts of methylamine (less than 2 mM) produced H_2 with similar rates and yields. Methylamine concentrations in the 2-7 mM range enhanced the rate and yield, albeit by modest amounts. The initial rate of H_2-production in the presence of 5 mM methylamine was greater than that of the control by 20-25%. Yields at 96 h were 10-20% better in the presence of 5 mM methylamine than in the control. Thus, the results with methylamine support the notion of enhanced initial rates (by 20-25%) due to the uncoupling of the H_2-production from the ATP biosynthesis process.

Subtask 2: Effect of gramicidin.

Gramicidin acts by inserting itself across the lipid bilayer of the photosynthetic membranes, thereby forming channels that permit the efflux of protons from the lumen, as the latter are deposited during electron transport. The unregulated leakage of protons from the lumen has essentially the same effect as that of methylamine, albeit by a different mechanism. **Fig. 7** shows that, qualitatively, gramicidin brings about the same result in terms of hydrogen production as methylamine (Fig. 6). A serious limitation encountered by gramicidin, however, was due to the low solubility of this chemical in the water phase of the growth medium. This constraint limited the use of gramicidin to only low concentrations (up to 5 μM). When higher concentrations of gramicidin were injected into the water phase of the growth medium, gramicidin precipitated from the solution in the form of white crystals. Nevertheless, the qualitatively similar results with methylamine and gramicidin support the notion that dissipation of the proton gradient from the interior of the photosynthetic membranes (lumen) to the outside space could enhance the process of electron transport and hydrogen production in green algae.

Subtask 3: Effect of FCCP.

Carbonyl Cyanide p-Trifluoromethoxyphenyl-hydrazone (FCCP) represents yet another class of ATP biosynthesis inhibitors acting in a mode that does not necessarily involve dissipation of sequestered protons from the chloroplast lumen. Whatever the mode of action of this ATP biosynthesis inhibitor, results in **Fig. 8** show an effect dissimilar to that of the other uncouplers. FCCP appears to only inhibit the rate and yield of H_2-production. Even low μM quantities had a dramatically inhibitory effect, suggesting that the mode of action of this ATP biosynthesis inhibitor is detrimental to the H_2-production process. At a concentration of 5 μM FCCP, the rate and yield of H_2-production were lowered by about 80%.

These results strengthen the hypothesis that the reversible hydrogenase pathway serves to drive the formation of ATP energy for the metabolic needs of the cells. Thus, greater expenditure of cellular ATP or uncoupling of the ATP biosynthesis from the electron transport process helps to enhance the rate of H_2-production. **However, these measurements also suggest that use of ATP biosynthesis inhibitors is not the method of choice for the experimental manipulation of H_2-production, especially under conditions of green algal mass culture.** This is due to both the modest effect and the high cost of these chemicals.

Task 3. ***Testing the effect of cycling the Stage 1 → Stage 2 process to extend the production of H_2 gas by green algal cultures.***

Anaerobiosis in C. reinhardtii cultures upon sulfur deprivation. As explained previously, anaerobic incubation of green algae is required to induce the expression of the gene that encodes the reversible hydrogenase and of other genes that are essential for H_2 production. It is also known that O_2 evolved by photosynthesis is sufficient to lower or to fully inhibit the activity of the reversible hydrogenase. This incompatibility between the simultaneous O_2 and H_2 production reactions can be overcome upon temporal separation of the two reactions by physiological and fully reversible means. This was achieved upon incubation of the green algae in the absence of sulfur (S)-containing nutrients.

S nutrient deprivation exerts a distinctly differential effect on oxygenic photosynthesis and mitochondrial respiration in green algae. When cultures of the green alga *C. reinhardtii* are deprived of inorganic S, the light-saturated rates of O_2 evolution and CO_2 fixation decline significantly within 24 h in the light. The absolute activity of photosynthesis, measured from the light-saturated rate of O_2 evolution in *C. reinhardtii* (**Fig. 9**), declined bi-exponentially from ~45 mmol O_2 (mol chlorophyll)$^{-1}$ s^{-1} at t = 0 h to ~2 mmol O_2 (mol chlorophyll)$^{-1}$ s^{-1} at t = 100 h. The reason for such loss of activity is traced to the requirement of sulfur, needed for the frequent repair of the H_2O-oxidizing PSII complex. In the absence of sulfur, which is an essential component of cysteine and methionine, protein biosynthesis is impeded and the PSII repair process is blocked.

Cellular respiration, measured from the rate of O_2 consumption in the dark (**Fig. 9**), remained fairly constant at ~13 mmol O_2 (mol chlorophyll)$^{-1}$ s^{-1} over the 0-50 h period and declined slightly thereafter. It is important to note that the absolute activity of photosynthesis decreased below that of respiration in *C. reinhardtii* after ~22 h of sulfur deprivation.

Sometime after ~22 h of S-deprivation, a sealed *C. reinhardtii* culture is expected to quickly become anaerobic in the light, because of the significantly greater respiratory, than photosynthetic, activity of the cells. In sealed cultures, this leads to **anaerobiosis**, even under saturating illumination. Anaerobiosis is necessary and sufficient to induce the H_2-production process in green algae.

Photoproduction of H_2 upon S-deprivation in C. reinhardtii. Under conditions of S-deprivation in sealed cultures, i.e., low levels of oxygenic photosynthesis and oxidative phosphorylation, the green alga *C. reinhardtii* resorts to the production and release of H_2 in order to sustain the electron transport process. Electrons from the residual activity of the H_2O-oxidizing enzyme and from endogenous substrate pass through the plastoquinone pool, the cytochrome *b-f* complex and PSI, with electron transport coupled to the reversible hydrogenase pathway. This process results in the release of molecular H_2 and generates ATP energy, which is needed for the maintenance and repair functions of the cell. **Figure 10** shows such measurements with S-deprived cultures of *C. reinhardtii*. Cells in a Roux bottle were incubated in S-deprived media under continuous illumination. Cultures were sealed 24 h after S

deprivation at a time when the rate of photosynthetic O_2 evolution was determined to be equal to, or less than, that of respiration. H_2 gas accumulation was observed in the light (**Fig. 10, A**) but not in the dark. The rate of H_2 accumulation was constant at ~2.5 ml h^{-1} for the first 25-35 h before starting to level off. Gas chromatographic analyses of the gas collected showed a ~90% H_2 with the remainder being mostly nitrogen (N_2) with traces of CO_2 and O_2.

The initial rate of H_2 gas accumulation (~2.5 ml H_2 h^{-1}), was equivalent to 7 mmol H_2 (mol chlorophyll)$^{-1}$ s^{-1}. This rate is significantly slower than the capacity of electron transport in the photosynthetic apparatus, which can be estimated from the rate of light-saturated O_2 evolution at the onset of S-deprivation [45 mmol O_2 (mol chlorophyll)$^{-1}$ s^{-1}; **Fig. 9**]. The estimated H_2 to O_2 ratio (H_2:O_2, mol:mol) of 0.16:1 suggests a lower than optimal yield of H_2 production vis-a-vis the capacity of the thylakoid membrane for electron transport.

Reversibility and reproducibility of the S-deprivation and/or H_2-production sequence of events was demonstrated by cycling a single *C. reinhardtii* culture between the two stages (oxygenic photosynthesis in the presence of S and H_2 production in its absence) for up to three full cycles. **Figure 10** shows the result of such a 'cycling of the stages' in which, at the end of H_2 production in cycle A (**Fig. 10, A**), the culture was supplemented with inorganic S (t = 100 h). Addition of inorganic S caused prompt inhibition in H_2 production (**Fig. 10**, beginning of cycle B), due of the ensuing S-induced activation of oxygenic photosynthesis (100 h < t < 130 h). Subsequently, the culture was driven to anaerobiosis upon S deprivation (130 h < t < 160 h) and H_2 production (160 h < t < 220 h). **Figure 10 (C)** shows a third temporal cycling of the Stage 1 → Stage 2 process.

CONCLUSION

The successful Cycling of the Stages, as outlined in this report (Fig. 10), shows that it is possible to extend the production of H_2 gas by a single culture of green algae *ad infinitum* upon alternatively supplementing and depriving the culture of inorganic sulfur. The principle described in this report can be applied to mass cultures of green algae for the sustained production of H_2 gas. This Cycling of the Stages approach would significantly lower the cost of producing H_2 gas commercially as it would alleviate the need of replacing the culture of algae at the end of individual Stage 1 → Stage 2 cycles.

Task 3. *Testing the effect of supplemental sulfur on the Stage 1 → Stage 2 hydrogen production process in green algal cultures.*

Introduction

Hydrogen photo-production upon S-deprivation of green algae cannot be sustained indefinitely. This is because, concomitant with the H_2-production process, significant catabolism of endogenous substrate occurs (Melis et al. 2000). Endogenous substrate catabolism appears to be essential as it sustains, directly or indirectly, the H_2-production process. Significant loss of endogenous substrate gradually leads to a slow-down of the rate of H_2-production. Hydrogen production upon S-deprivation could be sustained, on the average, for only about 5-days before the process was gradually inhibited. At this point, cells must be permitted to go back to normal

photosynthesis in order to replenish needed metabolites before they can be subjected to S-deprivation and H_2-production for another cycle (please see Progress Report, 30 September 2000).

The present report focuses on the application of limited amounts of sulfur to the growth medium in *C. reinhardtii* as a way by which to maintain a low-level metabolic activity in the cells and, thus, to possibly prolong the H_2-production process. This report examines the merit of utilizing micro-amounts of sulfur as a way by which to continuously sustain the H_2-production process beyond the 5-day limit imposed upon the process by current technology.

Results

Lack of sulfur from the growth medium brings about a prompt but reversible inhibition of oxygenic photosynthesis. The reason for such inhibition is the apparent chloroplast inability to do high rates of *de novo* protein biosynthesis, needed for the frequent replacement of the D1/32 kD reaction center protein in the H_2O-oxidizing PSII complex (Mattoo et al. 1987). In the absence of sulfur, which is an essential component of cysteine and methionine, protein biosynthesis is impeded and the PSII repair cycle is blocked (Wykoff et al. 1998). It was of interest to investigate the above phenomenology under conditions of limited sulfur supply. Thus, S-titration of photosynthesis and H_2-production was undertaken.

Figure 11 plots the efficiency of PSII primary photochemistry in *C. reinhardtii*, measured from the variable to maximal (Fv/Fmax) fluorescence yield ratio (Kitajima and Butler 1975), as a function of time after transferring the culture from a replete medium to a TAP medium containing variable amounts of sulfur (regulated S-deprivation conditions). In the absence of S (0 μM), Fv/Fmax declined exponentially in the light with a half-time of about 17 h, from about Fv/Fmax=0.58 at t = 0 h to about Fv/Fmax=0.08 at t = 60 h. At longer periods of incubation under S-deprivation (t > 60 h), Fv/Fmax remained constant at about the 0.08 level.

In the presence of limited amounts of S (10, 50 and 100 μM), there was a lag in the decline of the Fv/Fmax ratio, roughly proportional to the S-concentration used (Fig. 11). The half-times measured for the media containing 10, 50 and 100 μM sulfur were 18, 28 and 68 h, respectively. Since care was exercised to eliminate contribution of non-photochemical quenching in the Fv/Fmax ratio measurement (see Materials and methods), the results in Fig. 11 support the notion that S-deprivation interferes with the repair of PSII from the frequently occurring photo-oxidative damage in *C. reinhardtii* (Wykoff et al. 1998, Melis 1999).

The activity of photosynthetic H_2O oxidation and O_2 evolution also declined with time in S-deprivation. Kinetic patterns of such inhibition showed that, in the absence of S (0 μM), H_2O oxidation activity declined with a half-time of about 13 h (Fig. 12A, P), from about 40 mmol O_2 (mol Chl)$^{-1}$ s^{-1} at t = 0 h, to about 3-4 mmol O_2 (mol Chl)$^{-1}$ s^{-1} after about 60 h of S-deprivation. Interestingly, photosynthetic O_2 evolution activity appeared to become stabilized at this low-level for times t > 60 h (see also Melis et al. 2000, Ghirardi et al. 2000). This is consistent with the Fv/Fmax ratio measurements in Fig. 11, suggesting that a 5-10% of the water oxidizing activity is retained, even after prolonged S-deprivation. When cells were suspended in media with limited amounts of sulfur (10, 50 or 100 μM), H_2O oxidation activity declined with longer half times (15, 22 and 50 h, respectively). The results suggest that, in the presence of even limited amounts of inorganic sulfur, onset of inhibition in PSII activity is delayed. Thus, PSII inhibition appears to depend strictly on sulfur availability. Kinetic patterns of such inhibition were consistent with the S-titration results shown in Fig. 11.

The activity of mitochondrial respiration was not significantly affected by the regimen of S-deprivation (Fig. 12A, R). It declined only gradually as a function of time in S-deprivation from about 12 mmol O_2 (mol Chl)$^{-1}$ s^{-1} at t = 0 h to about 6 mmol O_2 (mol Chl)$^{-1}$ s^{-1} at t = 120 h. The absolute activity of photosynthesis dropped below that of respiration at times that strongly depended on sulfur availability. The incubation time required for this "crossover" point to occur was 23, 27, 36 and 85 h for the samples suspended in the presence of 0, 10, 50 or 100 µM S, respectively. Sealed *C. reinhardtii* cultures became anaerobic soon after the crossover point between photosynthesis and respiration (not shown).

Hydrogen gas production and accumulation was detected soon after the establishment of anaerobiosis in the culture, commencing at about 27 h in S-deprivation (Fig. 12B). The rate of H_2-production was fairly constant at 2.8 ml h^{1} during the 27-60 h period. It gradually declined thereafter. The yield and rate of H_2-production were similar in the 0 and 10 µM S samples. In the 50 µM S sample, H_2-production commenced later (at about 40 h) and lasted longer, leading to greater yields. In the 100 µM S sample, H_2-production commenced much later (at times longer than 100 h) and, therefore, did not appear to be promising for the purposes of this application.

CONCLUSION

Pre-calibrated supplemental but limiting amounts of sulfur may boost the yield of hydrogen production in a green algal culture to amounts beyond what has been collected under similar conditions in the total absence of sulfur. Depending of bioreactor geometry and size, such sulfur titration may hold the promise of improving the yield of hydrogen production in this two-stage photosynthesis and hydrogen production method.

Task 4. ***Identifying the rate-limiting step in the H_2 production process***

Given the significant changes in total cellular protein content during H_2-production in *Chlamydomonas reinhardtii* (Melis et al. 2000), and in order to gain insight as to the rate limiting step in this process, we focused our attention on the activity and concentration of key photosynthetic proteins. Total cellular protein content was examined upon cell disruption by sonication, followed by protein solubilization and SDS-PAGE analysis. In such experiments, a TAP-grown culture of *C. reinhardtii* was transferred to TAP-S conditions, and cells were harvested after different periods of sulfur deprivation (0-120 h). Proteins were resolved on the basis of equal Chl loading (4 nmol per lane), as previously described (Zhang et al. 1997). Fig. 13 shows the Coomassie-stained profile of cellular proteins. Notable changes occurred as a function of time in S-deprivation (a) in the composition and amount of the LHC II proteins and (b) in the amount of Rubisco. Both the electrophoretic mobility and the amount of the LHC II apoproteins changed especially at times longer that 60 h in S-deprivation. The amount of Rubisco appeared to decline precipitously as a function of time in S-deprivation (Fig. 14). Two apoproteins, migrating to about 66 kD and 40 kD, respectively, appeared to increase in quantity as a function of S-deprivation. The latter were apparently soluble proteins since examination of an isolated thylakoid membrane fraction from sonicated *C. reinhardtii* (not shown) did not contain them.

To gain further insight into the various protein changes, specific polyclonal antibodies against the LHC II apoproteins, RbcL subunit, D1 protein of PSII, *psaA/psaB* reaction center

proteins of PSI, and the hydrogenase of *C. reinhardtii* were employed in Western blot analyses of total cell protein extracts. Fig. 14 (LHC II) confirms changes in the composition and amount of the LHC II upon S-deprivation. It is of interest to note that, under the conditions of this experiment, alterations in LHC II were noted only at times t > 60 h, suggesting a threshold for the triggering of these changes. It is also of interest to note that such threshold coincides with the leveling-off of the Fv/Fmax ratio (please see Fig. 11) and of the H_2O-oxidation activity in the photosynthetic apparatus (Fig. 12) under conditions of S-deprivation. A more detailed analysis of the effect of S-deprivation on the composition and amount of the LHC II was not undertaken in this work.

The Western blot of Fig. 14 (Rubisco) shows a precipitous decline in the amount of this protein as a function of time in S-deprivation. Only traces of this important cellular protein were present at about 48 h of S-deprivation. Within the detection limit of this method, no Rubisco could be detected at times t > 60 h. This result is consistent with the findings of Ferreira and Teixeira (1992) who reported a nearly complete degradation of Rubisco in sulfur-starved *Lemna minor* L. The finding suggests that reductant generated from the residual photosynthetic H_2O oxidation activity at times t > 48 h is probably not utilized in the Calvin cycle for CO_2 fixation and reduction. Rather, electrons derived upon the residual photosynthetic H_2O oxidation activity must be channeled exclusively through the hydrogenase pathway and thus quantitatively contribute to the generation of molecular H_2.

The level of PSII (D1) and PSI *(psaA/psaB)* reaction center proteins (Fig. 14) declined with time in S-deprivation, consistent with the Q_A and P700 measurements that were reported earlier (Melis et al. 2000). Interestingly, cross-reaction between an antibody against the hydrogenase (Happe et al. 1994) and a protein band migrating to about 43 kD (Fig. 14, hydrogenase) consistently increased in the 0-60 h S-deprivation period. Rather irreproducible results were obtained at S-deprivation times longer than 60 hours. In some cases, levels of the hydrogenase appeared to decline at times t > 60 h (Fig. 14, hydrogenase, upper panel), whereas, in other cases, the intensity of the cross-reaction gradually increased up to 80 h and declined thereafter (Fig. 14, hydrogenase, lower panel). Moreover, although titer of the immune serum against the hydrogenase was strong (Happe et al. 1994), nevertheless, cross-reaction between the 43 kD band and the antibody was always faint. To overcome this difficulty, gels for the Western blots of the hydrogenase (Fig. 14, Fe-hyd) were loaded with 12 nmol Chl per lane, as opposed to the usual 4 nmol Chl, and the nitrocellulose membranes were exposed to the coloring agents for a longer-than-usual period of time. These observations show that the hydrogenase gene is expressed sparingly under our S-deprivation conditions, thereby limiting the yield of H_2-production under these conditions (Progress Report of 31 December 2000). Fig. 15 shows a quantitative summary of the Western blot results, with the cross-reactions of the various proteins normalized to the maximum (=1.0) detected during the S-deprivation kinetic course.

CONCLUSION

Experimental findings in this work show that the hydrogenase gene (hydA) is expressed sparingly under S-deprivation conditions. This was evidenced by the faint cross-reaction between polyclonal antibodies and hydrogenase in extracts from H_2-producing cells. The rather low concentration of the hydrogenase is therefore what impedes the rate and yield of H_2-production under our conditions. To remedy this limitation, genetic

engineering approaches need be applied by which to over-express the *hydA* gene in order to permit a greater concentration of the hydrogenase enzyme and, therefore, to achieve greater rates of H_2-production in green algae.

- Reversible application of the switch (presence/absence of S) permits the algae to alternate between O_2 production and H_2 production (temporal separation of the two processes) (Ghirardi et al., 2000), thus bypassing the incompatibility and mutually exclusive nature of the O_2 and H_2 producing reactions.
- Interplay between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway is essential for this light-mediated H_2-production process.
- The release of H_2 gas serves to sustain baseline levels of chloroplast and mitochondrial electron transport for the generation of ATP, which is needed for the survival of the organism under sulfur-deprivation stress conditions.

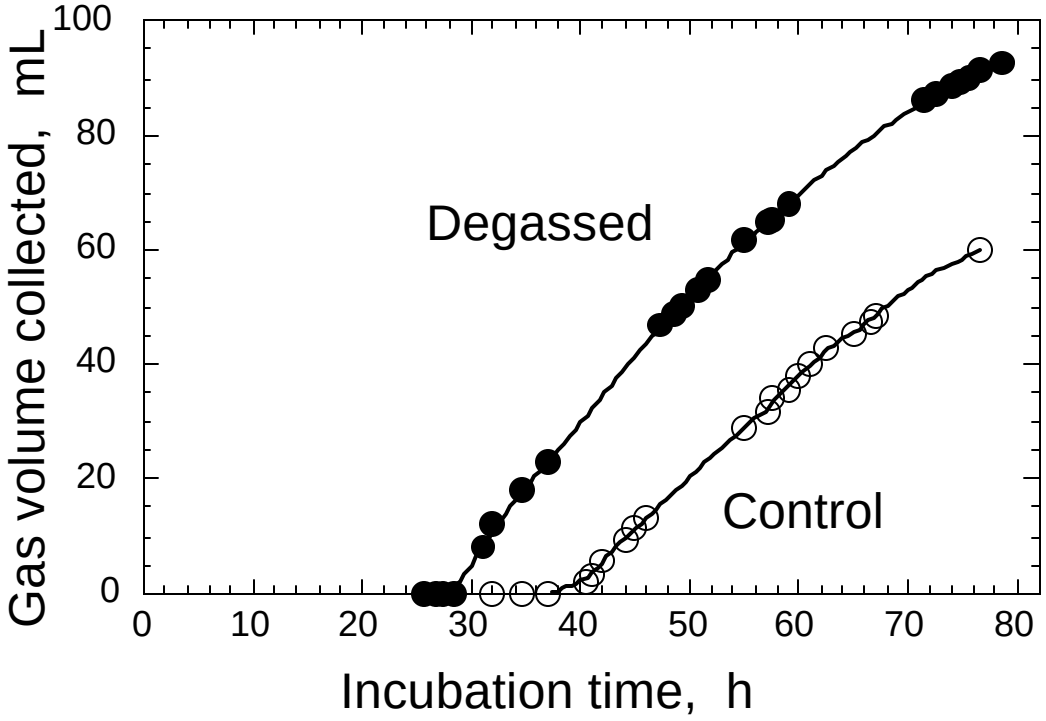

Figure 1. Hydrogen gas volume accumulated by displacement of water in an inverted burette as a function of cell incubation time in the absence of sulfur in the green alga *Chlamydomonas reinhardtii*. The culture was sealed at about 25 h after suspension of the cells in a sulfur-free medium with (solid circles) or without (open circles) prior degassing with argon. Values correspond to 1 L culture.

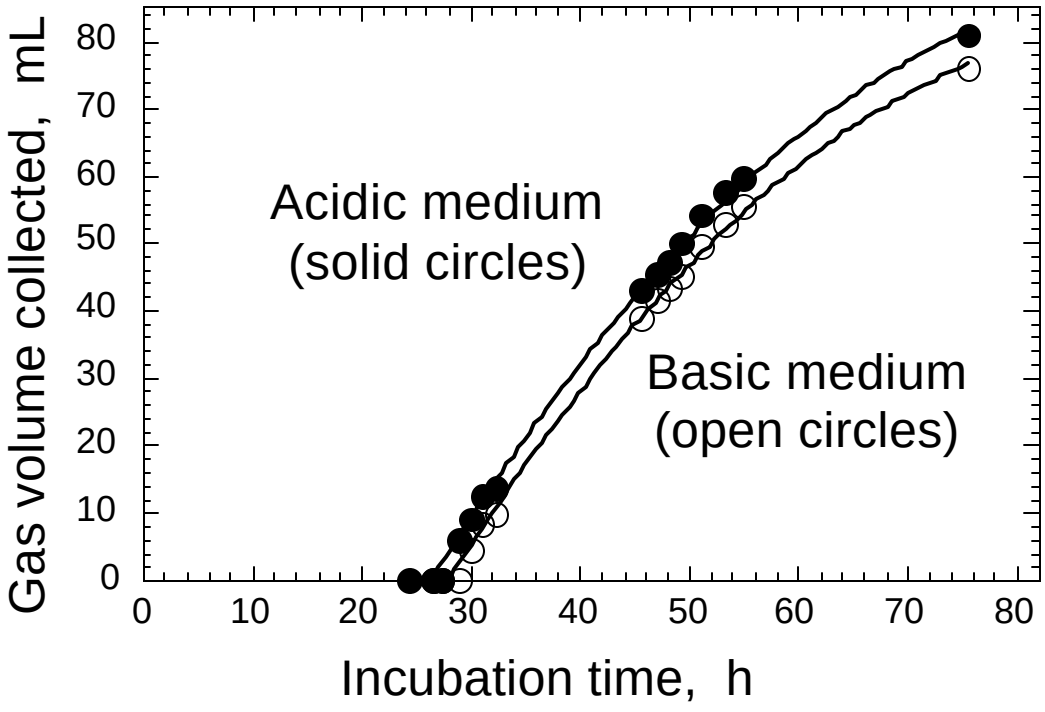

Figure 2. Hydrogen gas volume accumulated by displacement of water in an inverted burette as a function of cell incubation time in the absence of sulfur in the green alga *Chlamydomonas reinhardtii*. Cultures were sealed at about 25 h after suspension of the cells in a sulfur-free medium after briefly degassing with argon. pH of the water in the H_2 collection medium (water contained by the beaker and inverted in the inverted burette) was adjusted to an acidic or basic value upon addition of HCl or NaCl (0.1 M final concentration), respectively. Values correspond to 1 L culture.

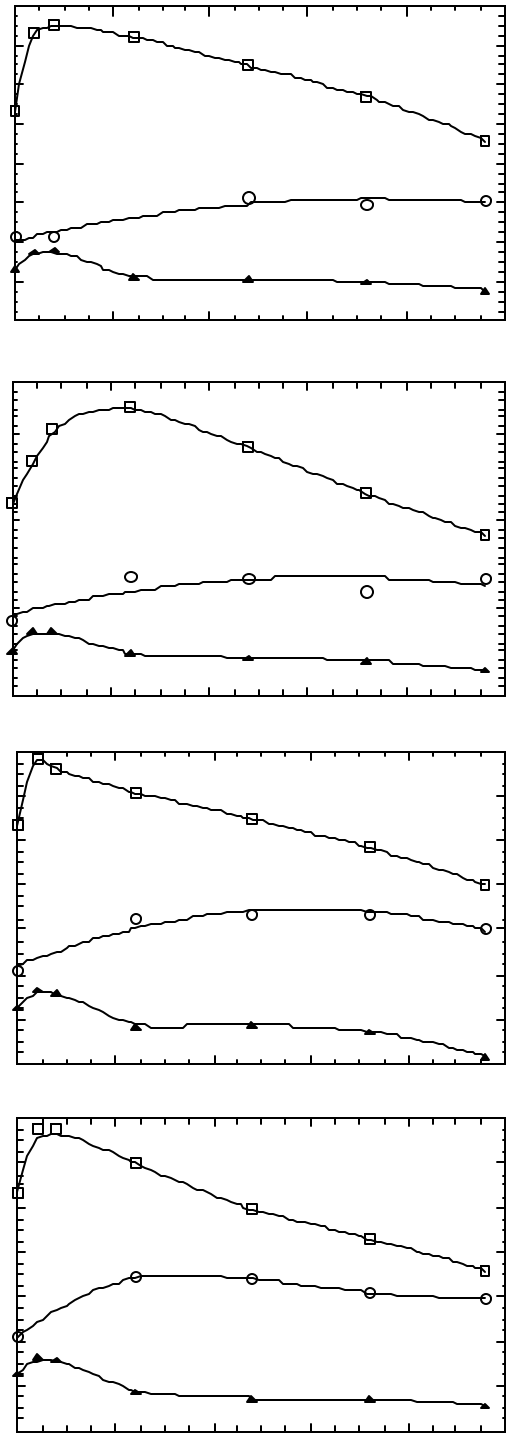

Figure 3.

Chlorophyll concentration, cell density and chlorophyll content per cell in a sulfur-deprived *C. reinhardtii* culture.

Initial values, at t=0 h, were Chl=7.7 μM, Cell/mL=2.8×10^{6}, Chl/cell=2.8×10^{-15} mol/cell.

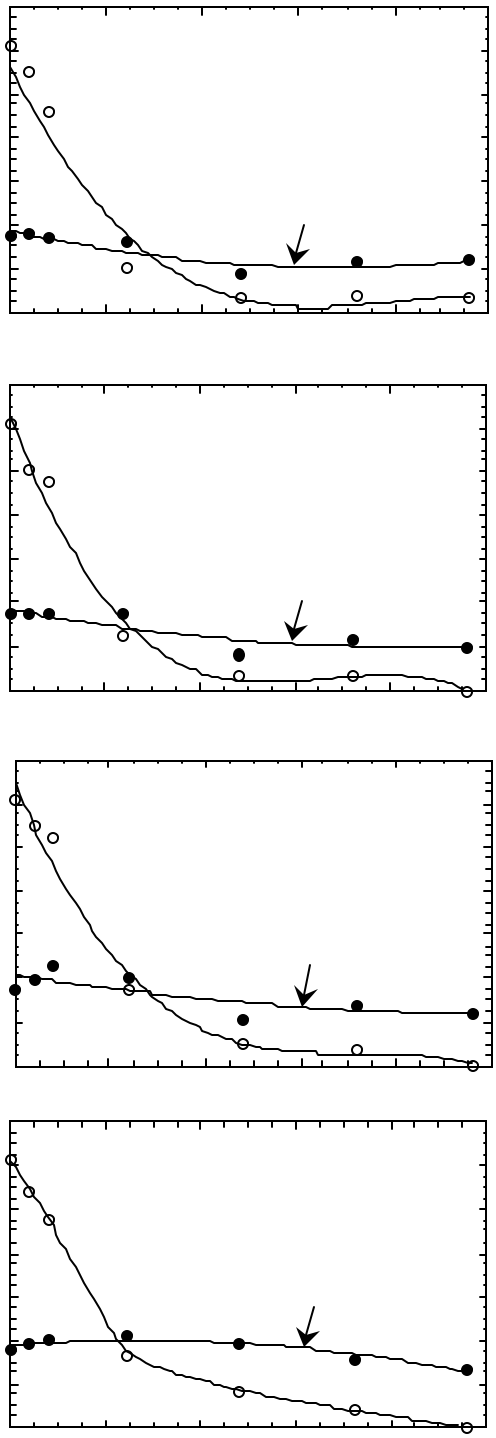

Figure 4.

Absolute activity of oxygenic photosynthesis (P) and oxidative respiration (R) in *C. reinhardtii* cells suspended in a medium devoid of sulfur. Incubation under sulfur-deprived conditions started at 0 h. Cells were suspended in the presence of 10 mM $NaHCO_3$, pH 7.6. The rate of cellular respiration (R) was recorded in the dark, followed by a measurement of the rate of light-saturated photosynthesis (P). Rates of photosynthesis were corrected for the rate of dark respiration.

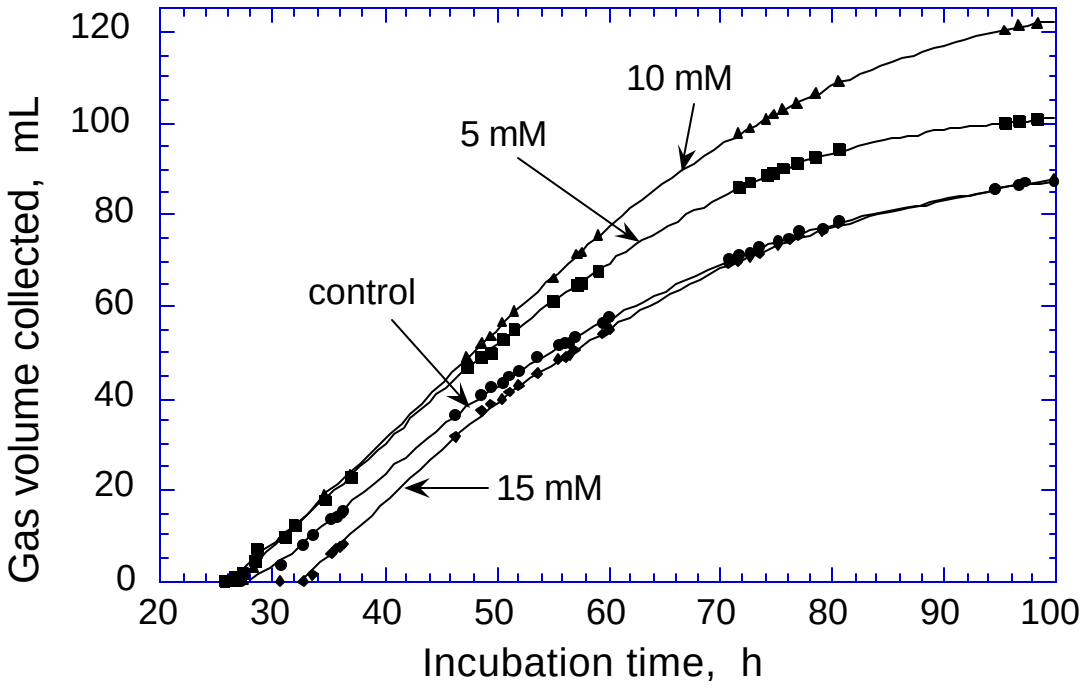

Figure 5. Hydrogen gas volume accumulated by displacement of water in an inverted burette as a function of cell incubation time in the absence of sulfur in the green alga *Chlamydomonas reinhardtii*. The culture was sealed at about 25 h after suspension of the cells in a sulfur-free medium. Values correspond to 1 L culture. Gases were collected in inverted burettes by displacement of water. The salinity of the cultures is indicated in mM. Control=0 mM NaCl.

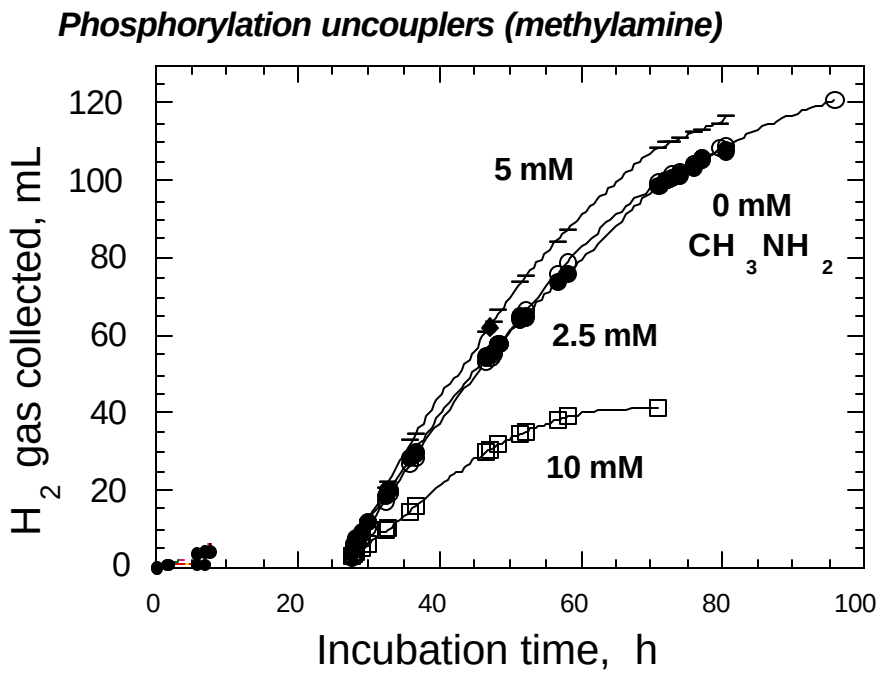

Figure 6. Hydrogen gas volume accumulated by displacement of water in an inverted burette as a function of cell incubation time in the absence of sulfur in the green alga *Chlamydomonas reinhardtii.* Values correspond to 1 L culture. Cultures were sealed at about 24 h after suspension of the cells in a sulfur-free medium. Immediately prior to sealing of the cultures, methylamine-hydrochloride was added to the medium (concentration range from 0-10 mM). C=0 mM CH_3NH_2-HCl. Culture characteristics, at t=0 h, were Chl=11 μM, Cell/mL=$4x10^6$.

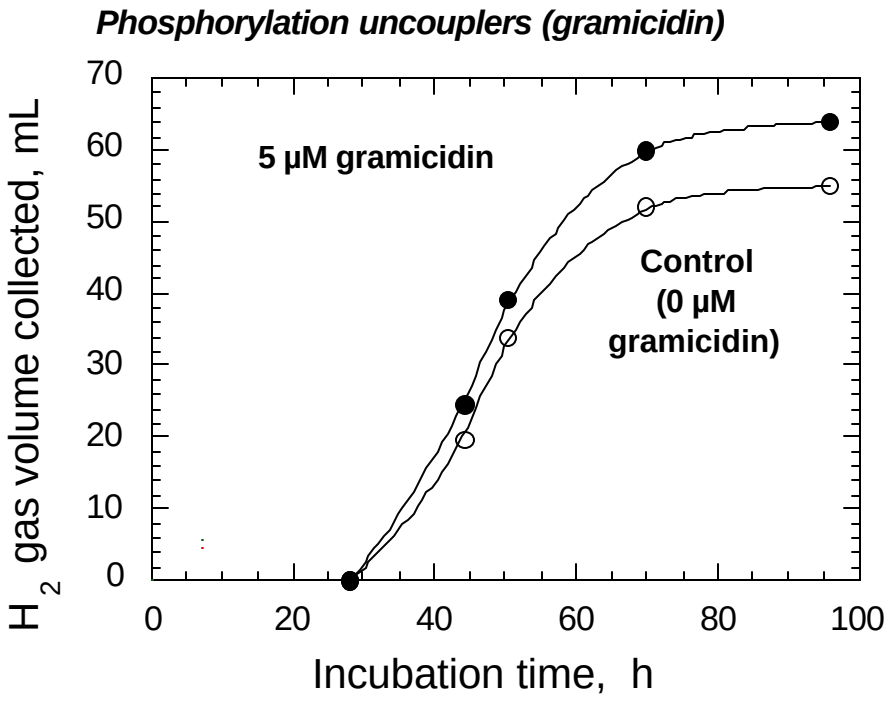

Figure 7. Hydrogen gas volume accumulated by displacement of water in an inverted burette as a function of cell incubation time in the absence of sulfur in the green alga *Chlamydomonas reinhardtii*. Values correspond to 1 L culture. Cultures were sealed at about 24 h after suspension of the cells in a sulfur-free medium. Immediately prior to sealing of the cultures, gramicidin was added to the medium (concentration range from 0-5 µM). Culture characteristics, at t=0 h, were Chl=7 µM, Cell/mL=2.5×10^6.

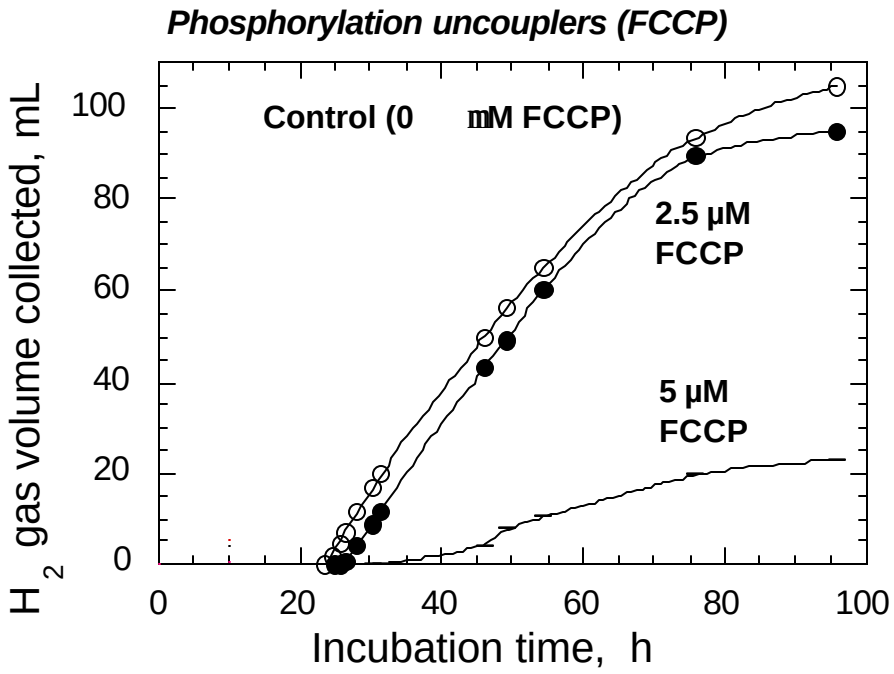

Figure 8. Hydrogen gas volume accumulated by displacement of water in an inverted burette as a function of cell incubation time in the absence of sulfur in the green alga *Chlamydomonas reinhardtii*. Values correspond to 1 L culture. Cultures were sealed at about 24 h after suspension of the cells in a sulfur-free medium. Immediately prior to sealing of the cultures, FCCP was added to the medium (concentration range from 0-5 µM). Culture characteristics, at t=0 h, were Chl=9 µM, Cell/mL=$3x10^6$.

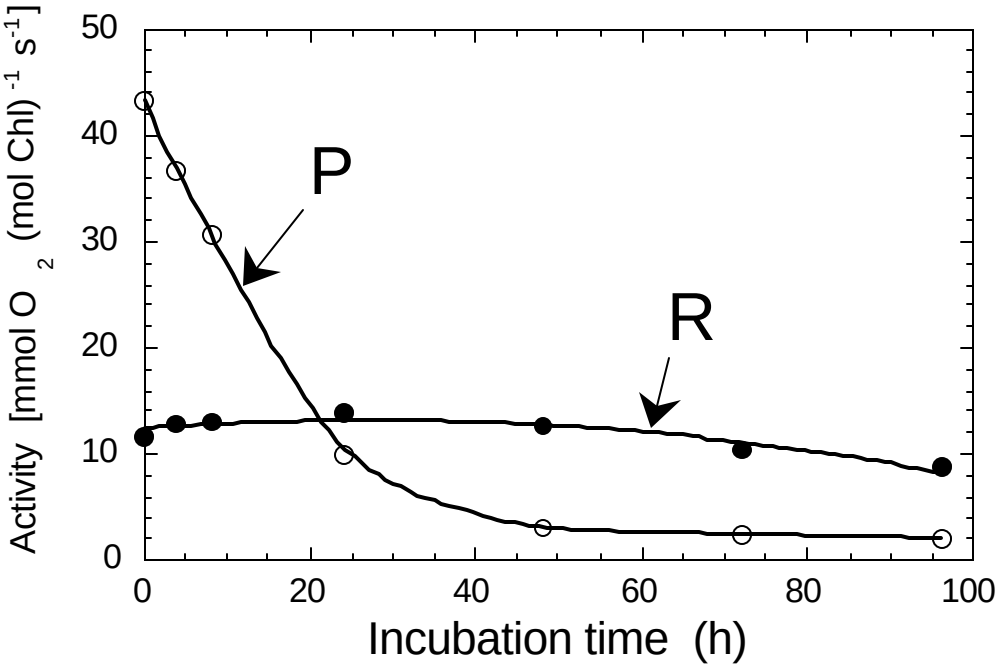

Figure 9. Absolute activity of oxygenic photosynthesis (P) and respiration (R) in *Chlamydomonas reinhardtii* strain CC-124 suspended in a medium devoid of sulfur (S). Cells were first grown on Tris-Acetate-Phosphate (TAP) medium. Incubation under S-deprived conditions started at 0 h. The rate of cellular respiration (R) was recorded in the dark, followed by measurement of the light-saturated rate of photosynthesis (P). Rates of photosynthesis were corrected for the rate of dark respiration. Cultures at 0 h contained 2.2 x 10^6 cells ml^{-1}.

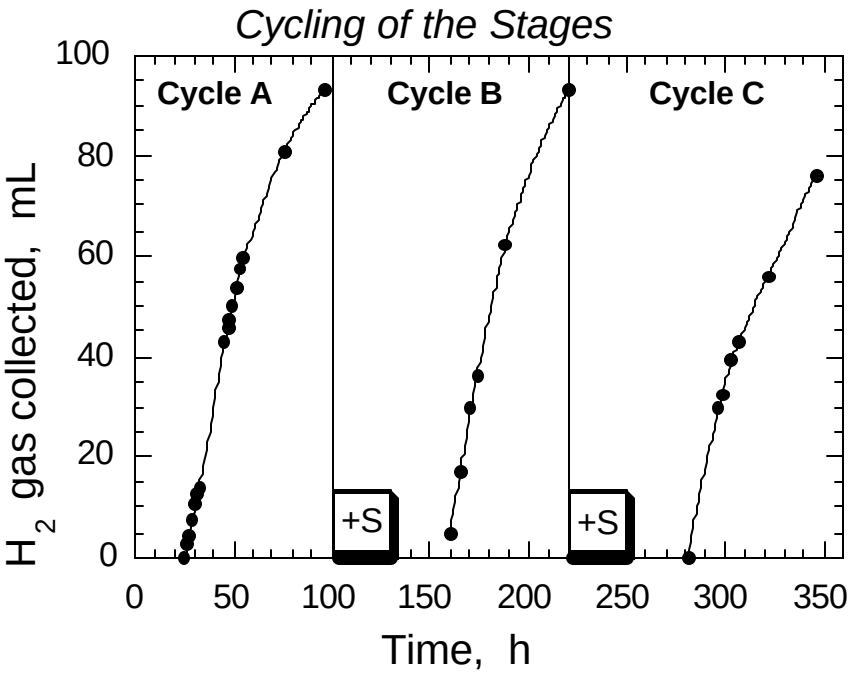

Figure 10. Cycling of Stage 1 → Stage 2 oxygenic photosynthesis and H_2 production in *C. reinhardtii*. Cells were suspended in a Roux bottle (850 ml volume) and grown in S-replete media until they reached a density of 2.4 x 10^6 cells ml^{-1}. They were deprived of inorganic S at t = 0 h and the culture was sealed at t = 24 h. Following H_2-production in cycle A, the culture was made replete with S (added as sulfate salts in the growth medium to a final concentration of 0.9 mM) strictly during the 100-130 h period. Following H_2-production in cycle B, the Stage 1 → Stage 2 process was repeated in cycle C (220-350 h).

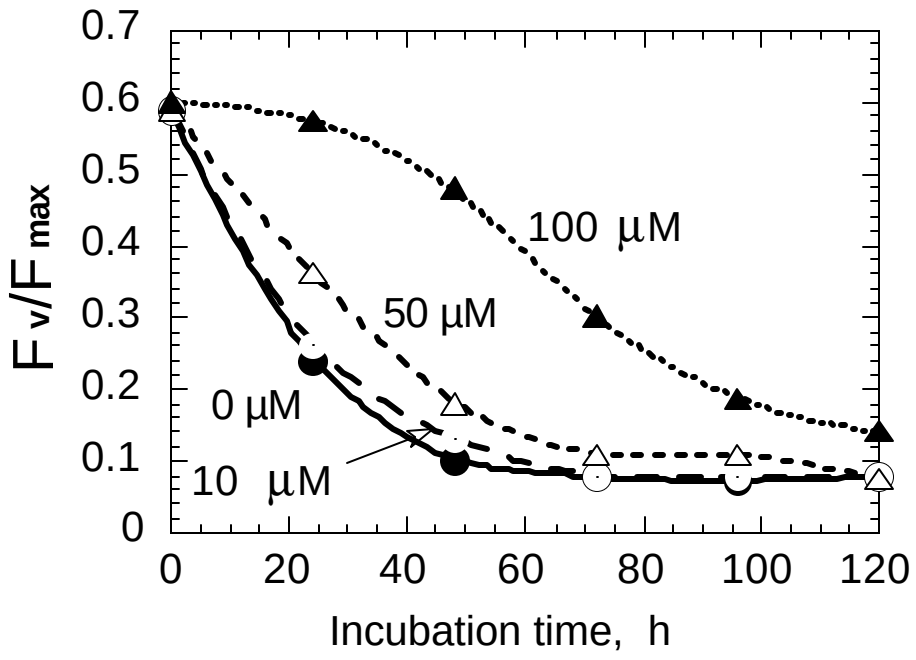

Fig. 11. Time-course of the PSII primary photochemical efficiency (Fv/Fmax ratio) in *Chlamydomonas reinhardtii* suspended in a medium of variable inorganic sulfur concentration. Cells were first grown on TAP medium. Incubation under different sulfate concentration (0, 10, 50 and 100 µM) conditions started at 0 h. Symbols used: solid circles = 0 µM; open circles = 10 µM; open triangles = 50 µM; solid triangles = 100 µM sulfate added at zero hours.

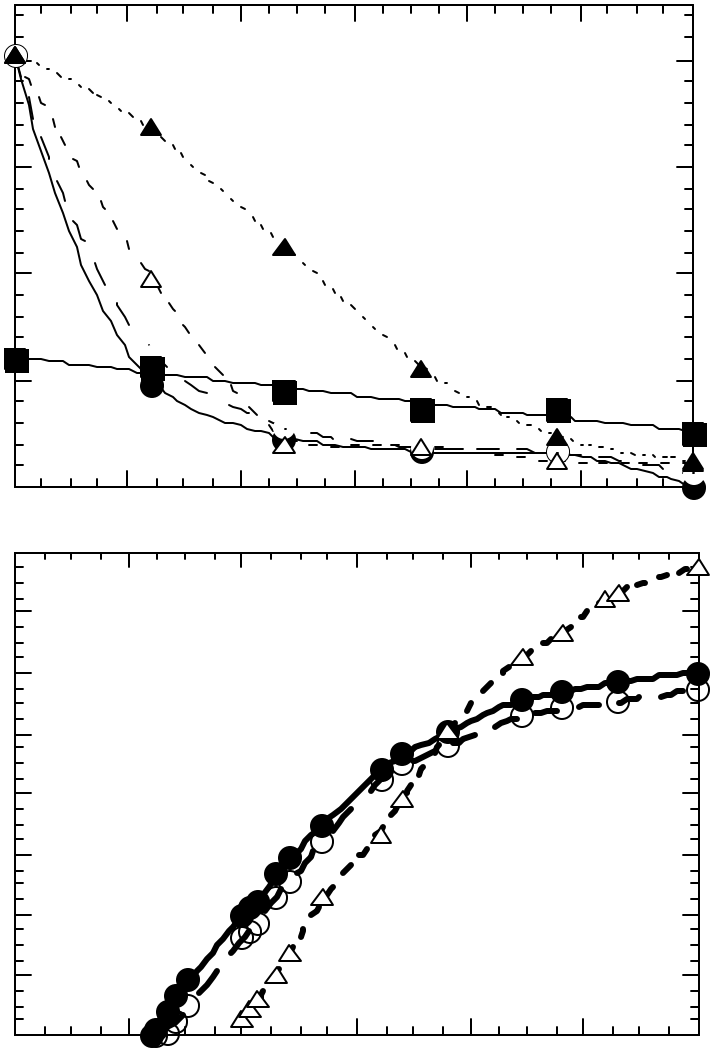

Fig. 12A,B. (**A**) Absolute activity of oxygenic photosynthesis (P) and respiration (R) in *C. reinhardtii* suspended in media of variable inorganic sulfur concentration (0, 10, 50 and 100 μM). The rate of cellular respiration (R) was recorded in the dark, followed by measurement of the light-saturated rate of photosynthesis (P). Cultures at 0 h contained 2.2×10^6 cell ml^{-1}. (**B**) H_2 gas production and accumulation by *C. reinhardtill* cells suspended in media of variable inorganic sulfur concentration. Gases were collected in an inverted burette and measured from the volume of water displacement. Symbols used: solid circles = 0 μM; open circles = 10 μM; open triangles = 50 μM; solid triangles = 100 μM sulfate added at zero hours.

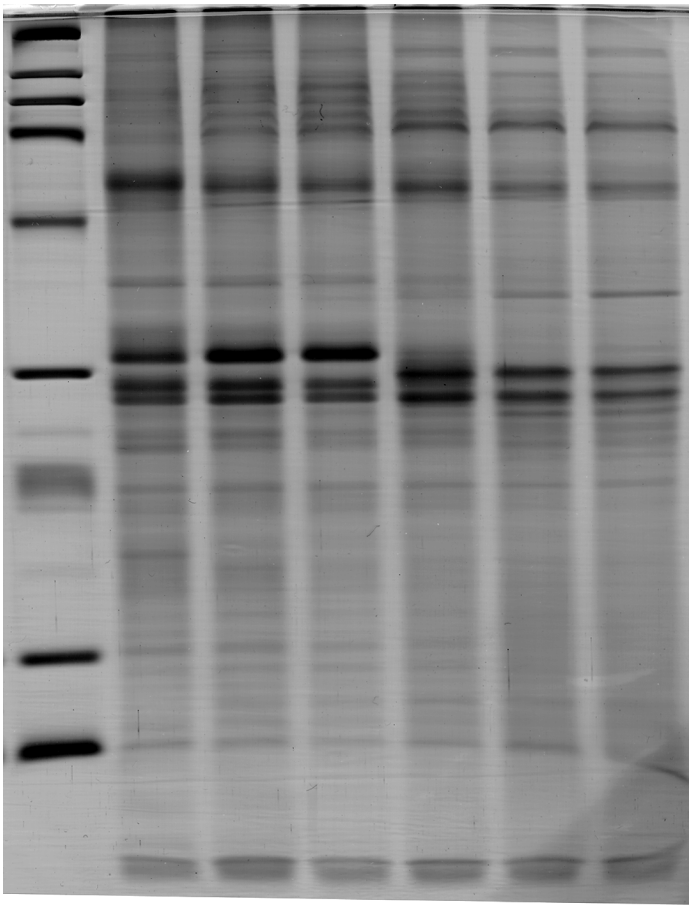

Fig. 13. SDS-PAGE profile of cellular proteins from control (0 h) and S-deprived *C. reinhardtii* (24-120 h). Gels were stained with Coomassie brilliant blue for protein visualization. Note the declining amounts of Rubisco and the altered composition of the LHC II.

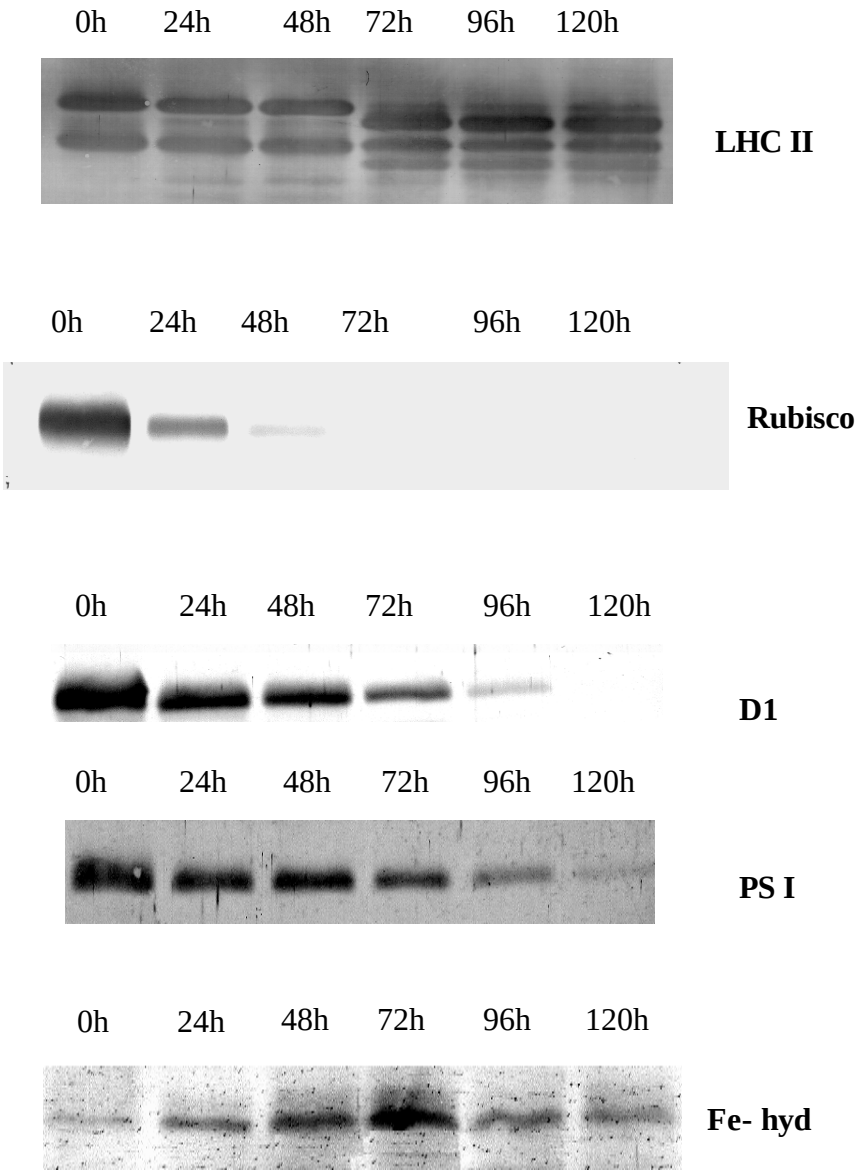

Fig. 14. Western blot analysis of photosynthetic proteins from *C. reinhardtii*. Nitrocellulose filters were probed with polyclonal antibodies that were specific against the LHC II, Rubisco, D1, *psaA/psaB*, or the hydrogenase. Note the precipitous loss of Rubisco and the transient increase and subsequent gradual decline in the amount of the hydrogenase.

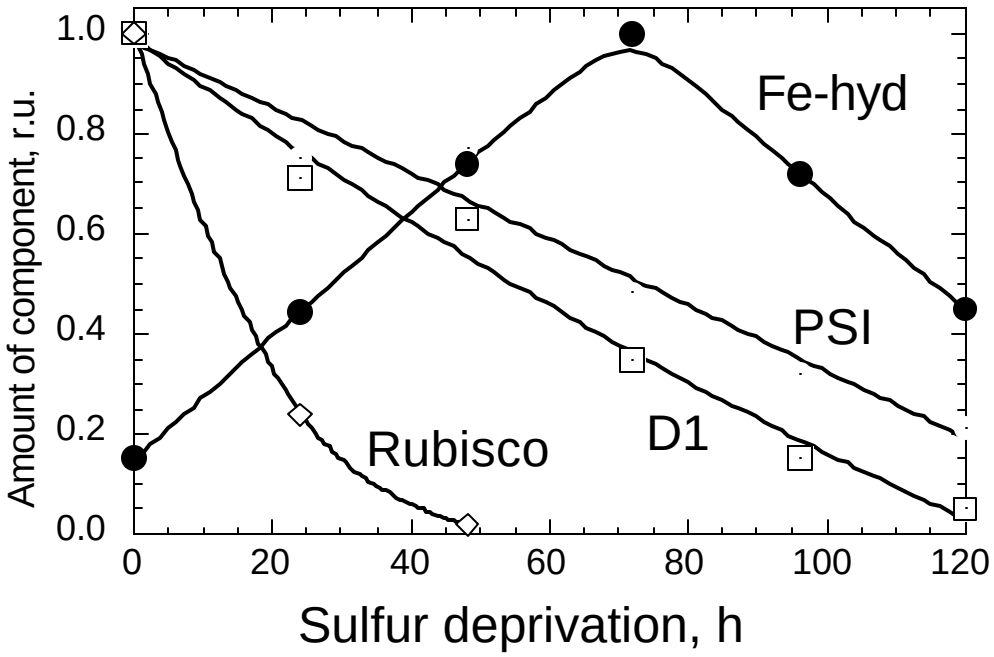

Fig. 15. Quantitation of the relative amounts of Rubisco, D1 and *psaA/psaB* proteins in *C. reinhardtii* as a function of time in S-deprivation. Note the precipitous drop in the amount of Rubisco and the more gradual decline in the PSII (D1) and PSI *(psaA/psaB)* reaction center proteins. (**B**) Time course of hydrogenase induction in *C. reinhardtii* as a function of time in S-deprivation. Note the significant differences in the induction and subsequent gradual decline of the hydrogenase between the two different samples.

Conclusions, Recommendations and Benefits to California

Conclusions

- The research streamlined conditions for H_2-production under S-deprivation. Thus, improvements in the onset of H_2-production upon S-deprivation were achieved. Improvements in the yield were modest, on the order og 10-15%.
- Reversible application of the switch (presence/absence of S) permitted the algae to alternate between O_2 production and H_2 production (temporal separation of the two processes), thus bypassing the incompatibility and mutually exclusive nature of the O_2 and H_2 producing reactions. This method will permit the *ad infinitum* albeit intermittent production of H_2 by a green alga culture.
- Interplay between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway is essential for this light-mediated H_2-production process.
- The release of H_2 gas serves to sustain baseline levels of chloroplast and mitochondrial electron transport for the generation of ATP, which is needed for the survival of the organism under sulfur-deprivation stress conditions.
- Upon further refinement, the "Two-Stage Photosynthesis and H_2-Production" process may serve in the generation of H_2 gas for the fuel and chemical industries. Except for the required yield increase, there are no absolute technical barrier for the application of this technology in the commercial production of H_2 gas.

Recommendations

The "Two-Stage Photosynthesis and H_2-Production" process brings together two normally separate fields: Energy and Biotechnology. The successful development and employment of this method will boost California's competitiveness in the fields of Energy, Agriculture and Chemistry.

It is thus recommended that every effort be made to advance this technology to its fullest potential

Benefits to California

Both small-scale (industrial and commercial) and larger (utility) solar energy conversion plants (photobioreactors) can be envisioned utilizing the Two-Stage Photosynthesis and H_2-Production process. Remote photobioreactors could be installed as modules in arid areas where sunlight is plentiful and alternative uses of land are minimal. Such a process of H_2 gas production would be sustainable, environmentally friendly and economically attractive compared to most other alternatives.

In addition to H_2, a valuable and clean fuel, the Two-Stage Photosynthesis and H_2-Production process will generate green algal biomass as a significant "Value-Added Bioproduct" that will enhance the economics and competitiveness of the process. In sum, the process will find application in the:

- Generation of a valuable, clean and renewable fuel.
- Global warming mitigation and reduction in the level of atmospheric polution.
- Production of biomass from which "High-value Bioproducts and Biochemicals" could be extracted.

Glossary

Chl: chlorophyll -- DCMU: dichlorophenyl dimethyl urea -- FCCP: Carbonyl Cyanide p-Trifluoromethoxyphenyl-hydrazone -- LHC: light harvesting complex -- PMSF: phenymethylsulfonylfluoride -- PSII: photosystem II -- PSI: photosystem I -- PAGE: polyacrylamide gel electrophoresis -- SDS: sodium dodecyl sulfate

References

Adams MWW (1990) The structure and mechanism of iron-hydrogenases. Biochim. Biophys. Acta 1020: 115-145

Davies JP, Yildiz F, Grossman AR (1996) *Sac1*, a putative regulator that is critical for survival of *Chlamydomonas reinhardtii* during sulfur deprivation. The EMBO Journal 15: 2150-2159

Ferreira RMB, Teixeira ARN (1992) Sulfur starvation in *Lemna* leads to degradation of Ribulose-bisphosphate carboxylase without plant death . J Biol Chem 267: 7253-7257

Florin L, Tsokoglou A, Happe T (2001) A novel type of Fe-hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetical electron transport chain. J. Biol. Chem. 276, 6125-6132.

Gaffron H (1939) Reduction of CO_2 with H_2 in green plants. Nature, 143, 204-205.

Gaffron H, Rubin J (1942) Fermentative and photochemical production of hydrogen in algae. J. Gen. Physiol. 26: 219-240

Gfeller RP, Gibbs M (1984) Fermentative metabolism of *Chlamydomonas reinhardtii* I. Analysis of fermentative products from starch in dark-light. Plant Physiol. 75, 212-218.

Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E and Melis A (2000) Microalgae: a green source of renewable H_2. Trends in Biotechnology 18: 506-511Ghirardi ML, Togasaki RK, Seibert M (1997) Oxygen sensitivity of algal H_2-production. Appl. Biochem. Biotech. 63: 141-151

Gibbs M, Gfeller RP, Chen C (1986) Fermentative Metabolism of *Chlamydomonas reinhardtii*. III Photoassimilation of acetate. Plant Physiol. 82, 160-166.

Greenbaum E (1982) Photosynthetic hydrogen and oxygen production: kinetic studies. Science 196: 879-880

Greenbaum E (1988) Energetic efficiency of hydrogen photoevolution by algal water-splitting. Biophys. J. 54: 365-368

Happe T, Mosler B, Naber JD (1994) Induction, localization and metal content of hydrogenase in the green alga Chlamydomonas reinhardtii. Eur J Biochem 222: 769-774

Happe T, Naber JD (1993) Isolation, characterization and N-terminal amino acid sequence of hydrogenase from the green alga *Chlamydomonas reinhardtii*. Eur. J. Biochem. 214: 475-481

Hell R (1997) Molecular physiology of plant sulfur metabolism. Planta 202: 138-148

Kirk JTO (1994) Light and Photosynthesis in Aquatic Ecosystems (2nd Edition). Cambridge University Press, Cambridge, England

Kitajima M and Butler WL (1975) Quenching of Chl fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochim. Biophys. Acta 376: 105-115

Ley AC, Mauzerall DC (1982) Absolute absorption cross sections for photosystem II and the minimum quantum requirement for photosynthesis in *Chlorella vulgaris*. Biochim Biophys Acta 680: 95-106

Mattoo AK, Edelman M (1987) Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32 kDa herbicide-binding protein. Proc Natl Acad Sci USA 84: 1497-1501

Melis A (1999) Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage *in vivo?* Trends in Plant Science 4: 130-135

Melis A, Neidhardt J, Benemann JR (1999) *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. J. appl. Phycol. 10: 515-525

Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol. 122: 127-136

Meyer J, Gagnon J (1991) Primary structure of hydrogenase I from *Clostridium pasterianum*. Biochemistry 30: 9697-9704

Neale PJ and Melis A (1989) Salinity-stress enhances photoinhibition of photosynthesis in *Chlamydomonas reinhardtii*. J. Plant Physiol. 134: 619-622

Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC (1998) X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 angstrom resolution. Science 282: 1853-1858

Roessler PG, Lien S (1984) Activation and *de novo* synthesis of hydrogenase in *Chlamydomonas*. Plant Physiol. 76: 1086-1089

Schlegel HG and Schneider K (1978) In, Hydrogenases - Their Catalytic Activity, Structure and Function (Schlegel HG and Schneider K, eds), Erich Goltze KG, Göttingen, pp. 15-44

Voordouw G, Strang JD, Wilson FR (1989) Organization of the genes encoding [Fe] hydrogenase in *Desulfovibrio vulgaris*. J. Bacteriol. 171: 3881-3889

Wykoff DD, Davies JP, Melis A, Grossman AR (1998) The regulation of photosynthetic electron-transport during nutrient deprivation in *Chlamydomonas reinhardtii*. Plant Physiol. 117: 129-139

Zhang L, Happe T, Melis A (2001) Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta in press.

Zhang L, Niyogi KK, Baroli I, Nemson JA, Grossman A and Melis A (1997) DNA insertional mutagenesis for the elucidation of a photosystem-II repair process in the green alga *Chlamydomonas reinhardtii*. Photosynth. Res. 53: 173-184