

IEA Agreement on the Production and Utilisation of Hydrogen

Annex 15

Table of Contents

1. Summary
2. Introduction
3. Annex 15
 - 3.1. Scope
 - 3.2. Goals
 - 3.3. Participation
 - 3.4. Effort
4. Program of work
 - 4.1. Objectives
 - 4.2. Approach
 - 4.3. Subtasks
 - 4.3.1. Subtask A
 - 4.3.2. Subtask B
 - 4.3.3. Subtask C
 - 4.3.4. Subtask D
5. Achievements
 - 5.1. General
 - 5.2. Subtask specific
 - 5.3. Subtasks
 - 5.3.1. Subtask A
 - 5.3.2. Subtask B
 - 5.3.3. Subtask C
 - 5.3.4. Subtask D
 - 5.4. Specific Scientific exchanges within the partners of Annex 15
6. Conclusions
7. Outlook
8. Appendix
 - 8.1. Expert Meetings
 - 8.2. References/Published papers within Annex 15
 - 8.3. Meetings/conferences

Photobiological Production of Hydrogen “Annex 15” of the IEA

Hydrogen Program

1. Summary

Annex 15 (Task 15), Photobiological Production of Hydrogen, has been an internationally collaborative research effort conducted over 5 years under the guidance and coordination of the International Energy Agency (IEA) Hydrogen Implementing Agreement (HIA).

Biological hydrogen production, the production of H₂ by microorganisms, has been an active field of basic and applied research for over two decades. Realization of practical processes for photobiological hydrogen production from water using solar energy would result in a major, novel biological source of sustainable and renewable energy, without greenhouse gas emissions or environmental pollution. However, development of such practical processes requires significant scientific and technological advances, and relatively long-term (>10 yr) basic and applied R&D.

Task 15 of the IEA Agreement on the Production and Utilisation of Hydrogen dealt specifically with "biophotolysis", i.e. the biological production of hydrogen from water and sunlight using microalgal photosynthesis. The overall objective of Task 15 over five years was to advance the basic and early-stage applied science in this area with the main objective to develop hydrogen production by microalgae (both green algae and cyanobacteria) emphasizing on early-stage applied research on biophotolysis processes with intermediate CO₂ fixation.

Task 15 was approved at the ExCom meeting in May 1999, started officially July 1, 1999, and was originally scheduled for five years (3 + 2 years; years four and five confirmed at the ExCom meeting in March 2002). Task 15 ended with the last ExpertsMeeting held in June-July 2004 in Japan.

The participants in Task 15 were Canada (years 3-5), Japan, Norway, Sweden, The Netherlands (years 3-5), UK (years 4-5), and The United States. In addition, specific observers from Hungary and Portugal frequently participated in the meetings and many other scientists were directly involved in the more open scientific activities (e.g. BioHydrogen 2002 and BioHydrogen 2004). During the period, 1999-2004, Annex 15 arranged 10 highly successful, specific ExpertsMeetings.

The work in Task 15 was divided into four Subtasks; (A) Light-driven Hydrogen Production by Microalgae; (B) Maximizing Photosynthetic Efficiencies; (C) Hydrogen Fermentations; and (D); Improve Photobioreactor Systems for Hydrogen Production.

Major achievements are: (1) A novel sustainable photobiological production of molecular hydrogen upon a reversible inactivation of the oxygen evolution in the green alga *Chlamydomonas reinhardtii* (Subtask A), (2) Identification of accessory genes and gene products necessary for the photoproduction of H₂ in *Chlamydomonas reinhardtii* (Subtask A), (3)

Identification and characterization of *tlal*, a novel gene involved in the regulation of the Chl antenna size in photosynthesis in *C. reinhardtii* (Subtask B), (4) The generation of 11.6 mol of H₂ per mol of glucose-6-phosphate using enzymes of the oxidative pentose phosphate cycle coupled to a hydrogenase purified from *Pyrococcus furiosus* (Subtask C), and (5) The establishment of both smaller and larger Photobioreactors (Subtask D).

In addition, several specific scientific exchanges occurred within Annex 15 and numerous scientific publications were published within the framework of Photobiological hydrogen production.

2. Introduction

Since its inception in 1974 following the first oil crisis, the International Energy Agency (IEA) has operated as an autonomous body within the framework of the Organisation for Economic Cooperation and Development (OECD). The purpose of the IEA is to bring together a broad range of experts in specific technology areas to address energy-related challenges in a collaborative manner with a longer-term global view, and to share the resulting benefits.

The hydrogen program, known as the Hydrogen Implementing Agreement (HIA), of the IEA was established in 1977. This was one of many international IEA energy cooperation programs, which were grouped into fossil fuels, renewable energies (including hydrogen), efficient energy end-use and fusion power. The HIA itself specifically set out to advance hydrogen production, storage and end-use technologies, and to accelerate hydrogen's acceptance and widespread utilisation.

The HIA can look back to an impressive portfolio of fundamental work conducted since 1977. As illustrated in Table 1, the HIA has been undertaking collaborative, precommercial Research, Development and Demonstration (RD&D) programs through twenty two specific Annexes (or Tasks).

Table 1: List of current and completed Annexes (or Tasks) by the IEA-HIA.

Past and Present IEA-HIA Annexes

Annex-1 Thermochemical Production, 1977 – 1988
Annex-2 High-Temperature Reactors, 1977 – 1979
Annex-3 Assessment of Potential Future Markets, 1977 – 1980
Annex-4 Electrolytic Production, 1979 – 1988
Annex-5 Solid-Oxide Water Electrolysis, 1979 – 1983
Annex-6 Photocatalytic Water Electrolysis, 1979 – 1988
Annex-7 Storage, Conversion and Safety, 1983 – 1992
Annex-8 Technical and Economic Assessment of Hydrogen, 1986 – 1990
Annex-9 Hydrogen Production, 1988 – 1993
Annex-10 Photoproduction of Hydrogen, 1995 – 1998
Annex-11 Integrated Systems, 1995 – 1998
Annex-12 Metal Hydrides for Hydrogen Storage, 1995 – 2000

Annex-13 Design and Optimisation of Integrated Systems, 1999 – 2001
Annex 15 Photoelectrolytic Production of Hydrogen, 1999 – 2004
Annex 15 Photobiological Production of Hydrogen, 1999 – 2004
Annex-16 Hydrogen from Carbon-Containing Materials, 2002 – 2004
Annex-17 Solid and Liquid State Hydrogen Storage Materials, 2001 – 2005
Annex-18 Integrated Systems Evaluations, 2004 – 2006
Annex-19 Hydrogen Safety, 2004-2009
Annex-20 Hydrogen from Waterphotolysis, 2004-2009
Annex-21 Biohydrogen, 2005-2008
Annex-22 Fundamental and Applied Hydrogen Storage Materials Development, 2006-2009
Annex-23 Small-Scale Reformers for On-site Hydrogen Supply, 2006-2009
Annex-24 Wind Energy and Hydrogen Integration, 2006-2009
Annex-25 High Temperature Production of Hydrogen, 2006-2009

Since 1995, the IEA-HIA pioneered the joint scientific investigation of direct, biological solar-to-hydrogen production routes based on fundamental research efforts in biophotolysis of water in Annex-10 followed by Annex 15. Collaborative, pre-commercial RD&D in biological based solar hydrogen continues to be of core interest to the IEA-HIA program (see e.g. Annex-21).

3. Annex 15

3.1. Scope

Biological hydrogen production, the production of H₂ by microorganisms, has been an active field of basic and applied research for over two decades, with significant applied R&D programs supported in Europe, Japan and the USA. Realization of practical processes for photobiological hydrogen production from water using solar energy would result in a major, novel biological source of sustainable and renewable energy, without greenhouse gas emissions or environmental pollution. However, development of such practical processes requires significant scientific and technological advances, and relatively long-term (>10 yr) basic and applied R&D.

Photobiological hydrogen production was a component of the prior Task 10 of the IEA Hydrogen Agreement, and evolved into the independent Task 15. This effort covered research areas and needs at the interface of basic and applied R&D which were of mutual interest to the countries and researchers participating in the IEA Hydrogen Agreement. Task 15 provided for the establishment of collaborative research projects among participating countries in a coordinated program.

Task 15 dealt specifically with "biophotolysis", i.e. the biological production of hydrogen from water and sunlight using microalgal photosynthesis. An earlier IEA-HIA Task 10 Report [Benemann 1998] concluded that, in theory, photobiological reactions could achieve close to a 10% solar energy conversion efficiency. The overall objective of Task 15 over five years was to advance the basic and early-stage applied science in this area. This process uses microalgae, either green algae or cyanobacteria, to fix CO₂ into carbohydrates, which are used by the algae to generate H₂ gas, first in the dark by fermentations and then in the light through photosynthesis-coupled reactions. The analysis earlier published by the IEA-HIA suggested that the goal of a 10% conversion efficiency, although ambitious, is potentially approachable in the long-term.

Task 15 was approved at the ExCom meeting in May 1999, started officially July 1, 1999, and was originally scheduled for five years (3 + 2 years; years four and five were confirmed at the ExCom meeting in March 2002). Task 15 ended with the last Experts Meeting held in June-July 2004 in Japan.

3.2. Goals

The main objective of Task 15 was to develop hydrogen production by microalgae (both green algae and cyanobacteria) emphasizing on early-stage applied research on biophotolysis processes with intermediate CO₂ fixation. This research will help to provide the advances required to achieve the practical efficiencies and cost goals of biological hydrogen production. The Task investigated microalgal hydrogen metabolism, both in the dark and in the light, as well as the mechanisms that would allow the photosynthetic processes and hydrogen evolution reactions to achieve their maximum possible efficiencies. In addition, subsidiary metabolic processes required investigations, such as the efficient accumulation of large amounts of carbohydrates, the regulation of the photosynthetic processes and the recycling of the algal cells after hydrogen evolution is completed. Complex underlying genetic mechanisms and biochemical pathways are involved in these physiological processes and were important parts of the research efforts of Task 15.

3.3. Participation

The participants in Task 15 were Canada (years 3-5), Japan, Norway, Sweden, The Netherlands (years 3-5), UK (years 4-5), and The United States. In addition, specific observers from Hungary and Portugal frequently participated in the meetings and many other scientists were directly involved in the more open scientific activities (e.g. BioHydrogen 2002 and BioHydrogen 2004).

The following research groups were officially active in Annex 15:

Canada: Université de Montréal (UM)

Japan: National Institute of Advanced Industrial Science and Technology (AIST)

Norway: Norwegian Institute for Water Research (NIVA)

Sweden: Uppsala University (UpU)

The Netherlands: Energy Research Centre of the Netherlands (ECN)

UK: University of Glamorgan (UG) & Heriot-Watt University (W-WU)

The United States: The National Renewable Energy Laboratories (NREL)

3.4. Efforts

Overall, the combined level of efforts invested by the seven participating research groups over the 5-year duration of Annex 15 amounted to an equivalent of approximately 25 “man-years”.

4. Program of Work

Allowing for the necessary flexibility in the conduct of fundamental R&D work, Annex 15 has

set out in July 1999 with the following objectives, task approach and subtask work program.

4.1. Objectives

The main objective of Task 15 was to develop hydrogen production by microalgae (both green algae and cyanobacteria) emphasizing on early-stage applied research on biophotolysis processes with intermediate CO₂ fixation. This research will help to provide the advances required to achieve the practical efficiencies and cost goals of biological hydrogen production. The Task investigated microalgal hydrogen metabolism, both in the dark and in the light, as well as the mechanisms that would allow the photosynthetic processes and hydrogen evolution reactions to achieve their maximum possible efficiencies. In addition, subsidiary metabolic processes required investigations, such as the efficient accumulation of large amounts of carbohydrates, the regulation of the photosynthetic processes and the recycling of the algal cells after hydrogen evolution is completed. Complex underlying genetic mechanisms and biochemical pathways are involved in these physiological processes and were important parts of the research efforts of Task 15.

4.2. Approach

The work in Task 15 was divided into four Subtasks; (A) Light-driven Hydrogen Production by Microalgae; (B) Maximizing Photosynthetic Efficiencies; (C) Hydrogen Fermentations; and (D); Improve Photobioreactor Systems for Hydrogen Production.

4.3. Subtasks

Subtask A: Light-driven Hydrogen Production by Microalgae

Light-driven hydrogen evolution mediated by hydrogenase(s) was discovered in green algae over fifty years ago, and subjected to extensive investigations over the following decades. However, there are still many fundamental and applied issues that must be addressed before this type of reaction can be considered for practical applications:

- The electron transport pathways coupling stored products with hydrogenase activity and involvement of the photosynthetic system(s), directly (through light-driven electron transport) or/and indirectly (through metabolic energy generation).
- The activation of pre-existing and biosynthesis of new hydrogenase enzymes, their regulation and genetics, in selected microalgae.
- The down-regulation of the oxygen-evolving component of the photosynthetic process, to avoid oxygen production and concomitant inhibition of hydrogenase function during the light-driven hydrogen production phase.

These objectives require a fundamental understanding of the genetics, biochemistry and physiology of hydrogenase functions, including the metabolism and factors affecting growth of microalgae. This research requires the application of modern and advanced tools of molecular

biotechnology and microbial physiology, techniques already available at leading laboratories in the participating countries.

Subtask B: Maximizing Photosynthetic Efficiencies

Photosynthesis can achieve relatively high solar conversion efficiencies, but only at low light intensity. At full sunlight, efficiencies drastically decline. The reason is the large amounts of so-called light-harvesting pigments, which capture more photons at full sunlight than the photosynthetic apparatus can actually handle. These excess photons are thus wasted, with their energy released as heat or fluorescence, even causing damage to the photosynthetic apparatus. Reducing antenna sizes is a method for increasing photosynthetic efficiencies, and this is a central R&D need in photobiological hydrogen production. Specific activities within this subtask were to:

- Develop green algae and cyanobacteria with considerable reduced light-gathering pigment contents in both photosystems using molecular genetic techniques.
- Demonstrate that such organisms can be used in CO₂ fixation, carbohydrate storage and H₂ production with greatly enhanced overall conversion efficiencies at high light intensities.
- Use photosynthetic bacteria as laboratory model systems to demonstrate increased photosynthetic efficiencies in pigment-reduced mutants of single photosystem microbes.

Subtask C: Hydrogen Fermentations

After accumulation of carbohydrates, and activation of their inducible hydrogenase, a fermentation process is initiated in which storage carbohydrates are converted to hydrogen and a number of fermentation products, including acetate, glycerol, and other excreted substrates. Such fermentations have been reported in both green algae and cyanobacteria, but require further study. At present, typical hydrogen yields from storage carbohydrates in the algae are less than 10%, based on a stoichiometry of 12 H₂/mole glucose. A goal of a 30% yield or higher is required and could be achieved through application of the modern methods of metabolic engineering to redirect metabolic reactions. Specific activities included:

- Investigate yields of anaerobic fermentations as a function of both genetic and environmental factors when using different green algae, cyanobacteria and photosynthetic bacteria.
- Carry out fundamental research using model systems such as *Escherichia coli*, and apply the tools of metabolic engineering to demonstrate improved H₂ production from glucose and waste waters in relevant systems.
- Apply the techniques developed in the fermentative studies to photosynthetic bacteria, cyanobacteria and green algae and study the utilization of excreted metabolites (e.g. acetic acid) in hydrogen production (both in the dark and in the light-driven hydrogen production stage).

Subtask D: Improve Photobioreactor Systems for Hydrogen Production

A major objective of applied R&D in photobiological hydrogen production has been the development of suitable photobioreactor systems. Development of such systems will serve as an intermediate step in the scale-up of hydrogen production from the laboratory scale to the commercial sector. A large number of different concepts and designs have been proposed and tested. However, there is a lack of engineering research for practical devices. As part of this international collaboration, R&D in photobioreactor engineering and operations was included under Subtask D:

- Development of mathematical models for photobioreactors adapted to hydrogen production, including mass transfer, hydrodynamic, and heat balance calculations.
- Development of methods for measurement of the major photobioreactor performance parameters, including hydraulic (dispersion coefficients), gas transfer coefficients, sunlight interception and H₂ losses.
- Comparative evaluations of alternative photobioreactor designs, including side-by-side comparisons and testing for biological hydrogen production.
- Experiments in pilot plants for determination of the H₂ production capacity.

5. Achievements

The following provides a brief overview of the main research that have been covered in a collaboratively manner by the seven research groups of Annex 15 over the past 5 years (June 1999 – July 2004). These efforts have not only been building on but did progress beyond the achievements of the previous Annex 10 (1995 – 1998) in a most significant manner.

General progress within Annex 15

The most important general of Annex 15 activity was held 2002 as the International conference *BioHydrogen 2002*. The aims of Biohydrogen 2002 were to summarise the state-of-the-art of biological hydrogen production, evaluate current progress on early-stage applied science in this area of research and development, and to identify promising research directions for the future.

Biohydrogen 2002 coincided with the end of the first 3 years of Annex 15 of the IEA Hydrogen Agreement. This most successful international conference, held under auspices of IEA Hydrogen Implementing Agreement Annex 15 *Photobiological Hydrogen Production*, and EU COST 8.41 *Biological and Biochemical Diversity of Hydrogen Metabolism* in Ede, the Netherlands, April 21-24, 2002, attracted about 150 scientists. In addition to a final announcement leaflet and a book of abstracts/program the financial contribution from the Hydrogen agreement and the countries participating in Annex 15 made it possible to publish the Proceedings (45 double refereed papers) as a Hardbound book of a Special Issue (2002, volume 27, # 11/12, November/December) of the International Journal of Hydrogen Energy (IJHE), see Figure 1. All participants of BioHydrogen 2002 received a complementary hardbound copy of these proceedings. As a consequence, the scientific advancements within Annex 15 was not only presented and discussed within the smaller group but also spread to a much larger audience.



PERGAMON

International Journal of Hydrogen Energy 27 (2002) 1123–1124

International Journal of
**HYDROGEN
ENERGY**

www.elsevier.com/locate/ijhydene

Editorial Biohydrogen 2002

This special issue contains full papers of both oral and poster presentations of the international symposium 'Biohydrogen 2002' which was held in Ede, The Netherlands, April 21–24, 2002. The symposium focused on biological hydrogen production. The aims of Biohydrogen 2002 are to summarize the state-of-the-art of biological hydrogen production, evaluate current progress on early-stage applied science in this area of research and development, and to identify promising research directions for the future.

BioHydrogen 2002 was organized jointly between the Dutch Biological Hydrogen Foundation, the International Energy Agency Hydrogen Implementing Agreement, specifically Annex 15 Photobiological Hydrogen Production, and COST 8.41, Biological and Biochemical Diversity of Hydrogen Metabolism, of the European Union.

One year ago, Peter Lindblad from IEA asked if we, the Dutch Biological Hydrogen Foundation, could host this conference. We aimed at 75 participants and hoped for 100, but Biohydrogen 2002 drew a stunning 150 participants, indicating an increased attention for this subject.

Hydrogen produced from renewable resources such as water, organic wastes or biomass, either biologically or photobiologically, is termed "biohydrogen". This is still a small area of research. Why is that? So far efficiencies obtained are low, productivity is low and costs are high compared to alternative technologies. This is not necessarily an obstacle that cannot be overcome: many new technologies start(ed) similarly.

Today we easily talk about sustainable energy. Sustainability will gain importance because of global warming, in general, and its impacts like the rise of the sea level, and the dying of coral reefs.

So far none of the alternative energy sources is ideal. Presently, there are several research programs on sustainable hydrogen. The definition of sustainability varies per program. Even hydrogen produced from fossil fuels, like natural gas is considered as sustainable because the CO₂ production is done at central locations which makes it possible to store it in deep reservoirs. Since the use of hydrogen as a fuel, in e.g., fuel cells, does not result in CO₂ production, the hydrogen is considered sustainable. In our opinion sustainable energy can only come from renewable sources (i.e., sun, water, wind, biomass).

Biological hydrogen production processes, also involve the production of CO₂. However, this CO₂ is released

from biomass, in which it was recently taken up, which is in contrast to fossil fuels, where the carbon dioxide has taken millions of years to build up, while the release only takes decades. So the duration of the carbon cycle is very important in view of sustainability, which biohydrogen truly is.

The organization committee of the international conference 'Biohydrogen 2002' acknowledges with gratitude the following organizations: Samsung Engineering (Korea), ECN, Energy research Centre of the Netherlands, and NOVEM, Netherlands Agency for Energy & the Environment.

In addition, the Guest Editors are indebted to the following people, who served as the editorial board for the special issue by reviewing the submitted manuscripts: S. Albracht (University of Amsterdam, Amsterdam, The Netherlands); Y. Asada (Nihon University, Chiba-ken, Japan); H. Barten (Novem, Utrecht, The Netherlands); M. Ghirardi (National Renewable Energy Laboratory, Golden, USA); W.R. Hagen (Delft University of Technology, Delft, The Netherlands); P. Hallenbeck (Université de Montréal, Montréal, Canada); T. Happe (Bonn University, Bonn, Germany); T. Källqvist (Norwegian Institute for Water Research, Oslo, Norway); M.-S. Kim (Korea Institute of Energy Research, Dae-jeon, Korea); E. Koukios (National Technical University of Athens, Athens, Greece); K.L. Kovács (University of Szeged, Szeged, Hungary); D.B. Levin (University of Victoria, Victoria, Canada); A. Melis (University of California at Berkeley, Berkeley, USA); M. Seibert (National Renewable Energy Laboratory, Golden, USA); O. Skulberg (Norwegian Institute for Water Research, Oslo, Norway); A.J.M. Stams (Wageningen University, Wageningen, The Netherlands); P.M. Vignais (UMR CEA CNRS, France); P.C. Wright (Heriott-Watt University, Edinburgh, UK); E. van Zessen (Energy research Centre of the Netherlands, Petten, The Netherlands). With the help of so many, we have been able to turn the international conference 'Biohydrogen 2002' into a high-quality event, ultimately resulting in this special issue of the International Journal of Hydrogen Energy.

It was a pleasure for us to welcome so many scientists and researchers to this truly international conference. I consider us a bit like pioneers. Our challenge is to do research in order to overcome the obstacles just mentioned, and make the biological hydrogen production techniques more efficient and less costly. The conference has been a lively

platform, on which ideas and initiatives were exchanged, and it provided us energy to continue and expand our work on biohydrogen.

Marcel G.J. Janssen
*Food and Bioprocess Engineering group
 Wageningen University, Bomenweg 2
 6703 HD Wageningen, The Netherlands*

Peter Lindblad
*Department of Physiological Botany
 EBC, Uppsala University, Villavägen 6
 SE-752 36 Uppsala, Sweden*

Ed W.J. van Niel
*Department of Bioconversion
 Agrotechnological Research Institute
 Bornsesteeg 59, 6708 PD Wageningen, The Netherlands*

J. Hans Reith
*Energy research Centre of the Netherlands (ECN)
 Westerduinweg 3, 1755 LE, Petten, The Netherlands*

René H. Wijffels
*Food and Bioprocess Engineering group
 Wageningen University, Bomenweg 2
 6703 HD Wageningen, The Netherlands*

Figure 1. Editorial, Biohydrogen 2002 *International Journal of Hydrogen Energy* (2002) 27: 1123-1124

During 2000 the scientific content of Annex 15 was presented and discussed at international conferences. The purpose of these presentations was to announce Annex 15, and its scientific content, to a larger audience.

- 10th Canadian Hydrogen Conference (Quebec; May 2000).

Lindblad, P., Asada, Y., Benemann, J., Hallenbeck, P., Melis, A., Miyake, J., Seibert, M. and Skulberg, O. 2000. IEA Hydrogen Agreement, Task 15: *Photobiological Hydrogen Production - An International Collaboration*. Proceedings 10th Canadian Hydrogen Conference. Pages: 731-733. Editors: Bose, T.K and Benard, P. ISBN 0-9696869-5-1.

- 13th World Hydrogen Energy Conference (Beijing; June 2000).

Lindblad, P., Asada, Y., Benemann, J., Hallenbeck, P., Melis, A., Miyake, J., Seibert, M. and Skulberg, O. 2000. IEA Hydrogen Agreement, Task 15: *Photobiological Hydrogen Production - An International Collaboration*. Hydrogen Energy Progress XIII. Volume 1: 56-59. Editors: Mao, Z.Q. and Veziroglu, T.N.

- 4th Asia-Pacific Conference on Algal Biotechnology (HongKong; July 2000).

During 2001 Annex 15 was presented and discussed at the 12th International Congress on Photosynthesis, Brisbane (Australia) August 18-23 (2001).

In addition, a most wanted book, **Biohydrogen II** (PERGAMON Press, ISBN: 0-08-043947-0, 284 pages), was published 2001 with contributions from leading international experts cover the breadth of Biohydrogen R&D, production to genetic engineering and molecular biology. This volume was designed to be an invaluable resource for researchers and other professionals who wish to obtain an overview of the present status of Biohydrogen R&D.

Subtask specific progresses within Annex 15

Subtask A: Light-driven Hydrogen Production by Microalgae

A most novel sustainable photobiological production of molecular hydrogen upon a reversible inactivation of the oxygen evolution in the green alga *Chlamydomonas reinhardtii* has been demonstrated (Melis et al 2000). In this organism, a two-stage H₂ production method circumvents the severe O₂ sensitivity of the reversible hydrogenase by temporally separating photosynthetic O₂ evolution and carbon accumulation (stage 1) from the consumption of cellular metabolites and concomitant H₂ production (stage 2). A transition from stage 1 to stage 2 is effected upon S deprivation of the culture, which reversibly inactivates photosystem II (PSII) and O₂ evolution. Under these conditions, oxidative respiration by the cells in the light depleted O₂ and causes anaerobiosis in the culture, which is necessary and sufficient for the induction of a reversible hydrogenase. Subsequently, sustained cellular H₂ gas production is observed in the light but not in the dark, see also Figure 2.

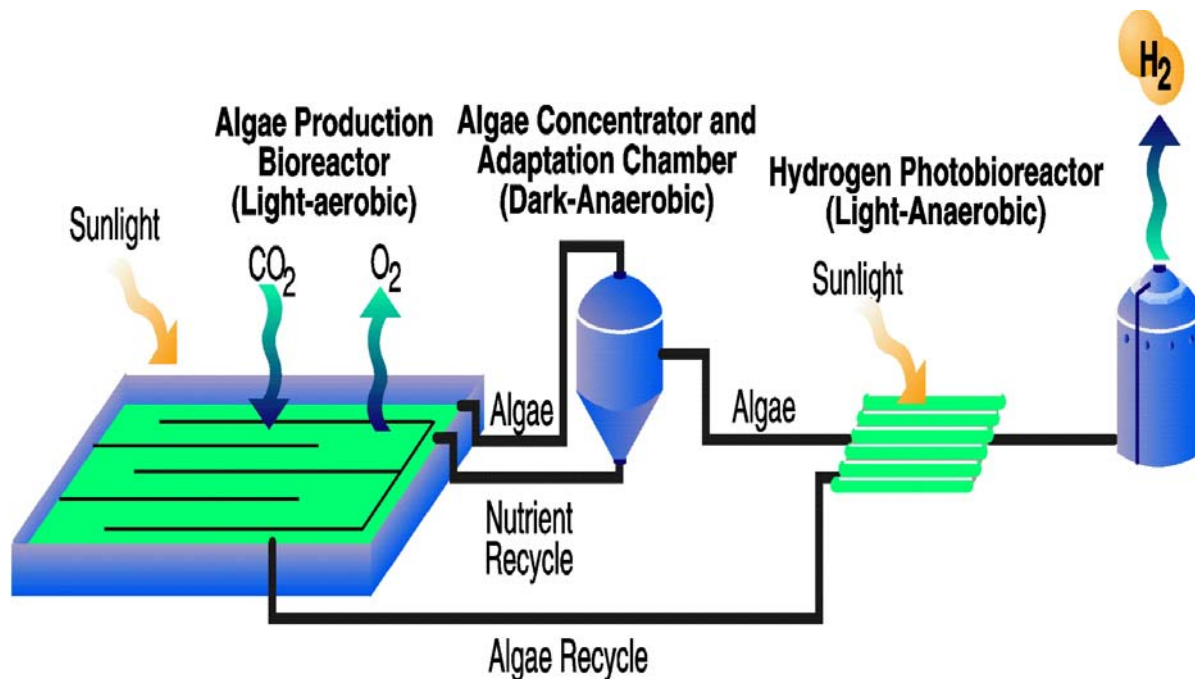


Figure 2. Outline of two-stage system for green algal based H₂-production. In the open stage 1, the green algae carry out complete photosynthesis and fix CO₂ whereas in the closed stage 2, the cells, under anaerobic conditions, produce H₂.

A heterologous expression of a clostridial hydrogenase has been achieved in the cyanobacterium *Synechococcus* PCC7942 (Asada et al 2000). The *Clostridium pasteurianum* hydrogenase I was expressed in *Synechococcus* PCC7942. The hydrogenase gene was cloned downstream of a strong promoter, previously isolated from *Synechococcus* PCC7942, with a cat gene as a reporter gene. Expression of clostridial hydrogenase was confirmed by Western and Northern blot analyses in both *Synechococcus* and *Escherichia coli*, whereas in vivo/in vitro measurements and activity staining of soluble proteins separated on non-denaturing polyacrylamide gels revealed functional expression of the clostridial hydrogenase in the cyanobacterial cells only.

The present knowledge about cyanobacterial hydrogenases, and their potential as photobiological producers of molecular hydrogen, has been reviewed (Lindblad and Tamagnini 2000, 2001). The diversity of cyanobacterial hydrogenases was examined using a molecular approach (Tamagnini et al 2000). Filamentous strains from a broad range of sources were screened for the presence of *hup* (uptake hydrogenase), *xisC* (recombinase responsible for the rearrangement within *hupL*), and *hox* (bidirectional hydrogenase) genes. As expected, an uptake hydrogenase seems to be present in all N₂-fixing cyanobacteria. On the other hand, no evidence was found for the presence of a conventional bidirectional enzyme in several strains. The natural molecular variation of hydrogenases in cyanobacteria is a field to explore, both to understand the physiological functions of the respective enzymes, and to identify a genetic background to be used when constructing a strain for photobiological H₂ production in a bioreactor. Initial bioreactor experiments, using wildtype and genetically modified strains have been presented. E.g. a mutant with the gene encoding the uptake hydrogenase inactivated evolves molecular hydrogen at at least similar rates as sulfur deprived green algae, see Figure 3. Moreover, a method for examining "real competition experiments" between different strains, e.g. wildtype and a genetically modified mutant has been developed.

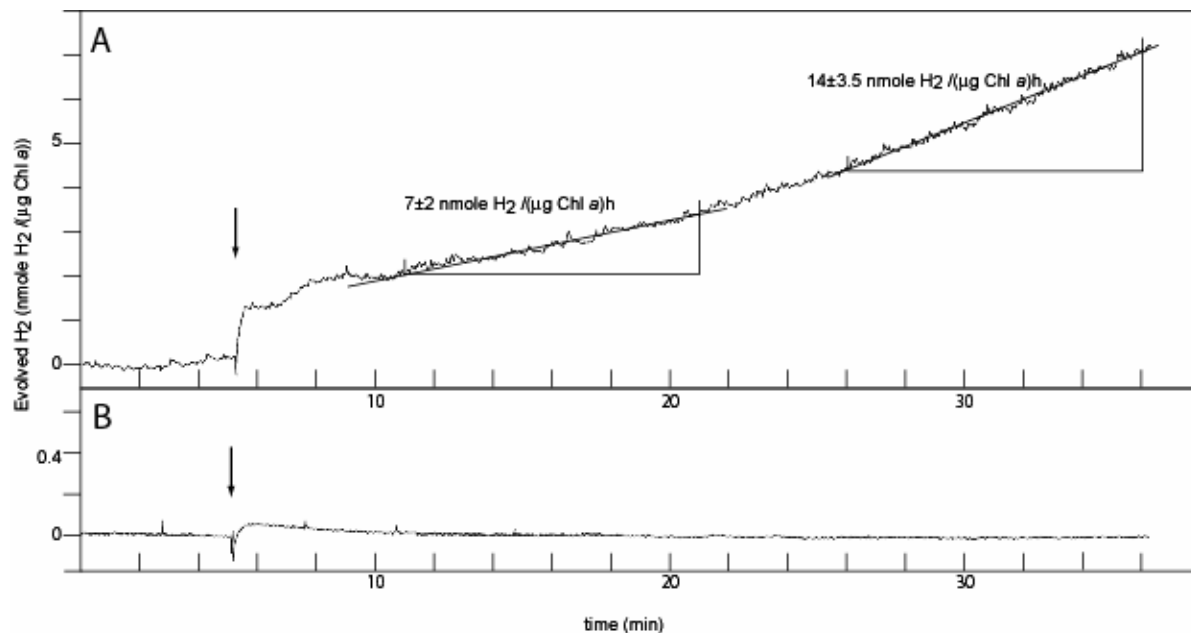


Figure 3. Wild-type (B) and a genetically modified mutant lacking a functional uptake hydrogenase (A) of the filamentous cyanobacterium *Nostoc punctiforme*. At the zero the cells were added to the H₂-electrode and after 5 minutes the light (solar energy, arrow) was applied to the cells. Note the absence of H₂ leaving in the culture (eventhough it is produced, but immediately consumed) in the wildtype, and the instant production by the genetically modified cells.

Maturation of [NiFe]-hydrogenases requires the action of several groups of accessory genes. Homologues of one group of these genes, the so called *hyp* genes, putatively encoding proteins participating in the formation of an active uptake hydrogenase in the filamentous, heterocyst-

forming cyanobacterium *Nostoc* PCC 73102, have been identified and characterised (Hansel et al 2001).

In Norway, an operating test system to study the hydrogen production and screening of strains of green algae for suitable candidates for H₂ production has been established. Experimental work with various strains has been performed to gain experience on the adaptation process necessary for triggering H₂ evolution. For this purpose both sulphur deprivation and addition of reducing agents have been applied to achieve sustained anaerobic conditions in illuminated cultures. In other experiments, evolution of H₂ in the dark after degassing the cultures with nitrogen has been studied. The initial results indicate large variation in the response on sulphur deprivation, and hydrogen production has so far only been observed in strains that have previously been reported to produce hydrogen by photolysis, e.g. *Chlamydomonas reinhardtii*. Lower yields of H₂ were obtained from cultures of *C. reinhardtii* and *Chlorella fusca*, incubated in the dark.

During 2002, a new algal H₂ project concept was developed at Oak Ridge National Laboratory, USA (Lee/Greenbaum Lab (ORNL), Designer Alga for Efficient and Robust Production of Hydrogen). This new project concept is based on a novel approach that we have recently invented for photobiological production of H₂ by water splitting (2001 ORNL Invention Disclosure ID 0981). In this approach, a “designer alga” for efficient and robust H₂ production is created by genetic insertion of hydrogenase promoter-programmed polypeptide proton channels in photosynthetic thylakoid membranes. This designer alga can also be integrated with the benefits of an O₂-tolerant hydrogenase that will be created by NREL (USA) and a smaller chlorophyll antenna size that will be created by UC Berkeley (see Subtask B).

An algal system for the continuous photoproduction of bulk H₂ gas from water was demonstrated at a laboratory scale, and projections of the cost of the H₂ that is produced by this system was lowered significantly (NREL). In addition, a spectroscopic remote-sensing system has been developed to monitor the H₂-production viability of an algal culture in the above system. Finally, the first ‘engineered’ algal hydrogenase with enhanced tolerance to O₂ inhibition was generated. With additional improvements, use of modified organisms might lead to the development of an aerobic hydrogen-producing system.

In UK, work continued on a range of cyanobacteria for potential H₂ production. The non-heterocystous marine cyanobacterium *Lyngbya majuscula* was examined in 5 different geometries of 2-5 litre bioreactors to obtain additional biomass, with a near 20 fold improvement. The genes encoding cyanobacterial hydrogenases, *hupSL* and *hox* genes, have been examined in 12 environmental examples of filamentous non-heterocystous cyanobacteria. Work has been focused on *Anabaena sphaerica* and in addition to genetic/molecular information it has been grown successfully at scales of up to 25 litres.

Upon exposure to CO, the photosynthetic bacterium *Rubrivivax gelatinosus* CBS induces a water-gas shift or CO shift pathway according to the equation: $\text{CO} + \text{H}_2\text{O} \leftrightarrow \text{CO}_2 + \text{H}_2$. This CO shift reaction occurs in darkness with an equilibrium constant greater than 10⁵ at 30 °C, therefore, is ideal for scale-up H₂ production. In order to commercialize this process for prolonged H₂ production, two approaches to gain fundamental understanding of this reaction has been examined (NREL). After screening a transposon-insertional mutant library, the molecular biology

approach resulted in the identification of two mutants unable to shift CO. Sequencing DNA flanking the transposon revealed three putative genes involved in the CO shift reaction; *cooS* (encoding CO dehydrogenase), *cooF* (encoding an iron-sulfur protein), and *cooH* (encoding an evolving hydrogenase), all showing high level of homology (approximately 65% identical) with their respective counterparts in *Rhodospirillum rubrum*. Sequencing of a genomic library further revealed neighboring genes with putative function in nickel insertion for CO dehydrogenase (*cooC*), and a *hypB*-like gene with a putative function in nickel processing for the hydrogenase. Work is underway to obtain more complete sequence of the genes encoding the CO shift pathway. Using a physiological approach, three lines of evidence supporting that CO shift reaction yields energy were obtained. Significant cell growth was observed when CO served as the only carbon substrate in darkness, indirectly implying energy conservation during CO shift. Based on stimulatory effect of a proton ionophore CCCP on CO shift rate, its inhibition by an ATP synthase inhibitor DCCD, and the reversal of the inhibition when DCCD was added along with CCCP, it was concluded that CO shift reaction is coupled to a proton-translocating, chemiosmotic mechanism yielding energy. Direct ATP measurement using a luciferase/luciferin-based bioluminescence assay detected ATP formation when cell suspension was exposed to CO, but not its argon gas control. This finding has significant implication in the economics of biological H₂ production. ATP generated during CO shift in darkness can be used to support cell division, which will produce new enzymes/proteins when existing ones are turned over thereby prolonging H₂ production from CO. Since light input is not required, bioreactor design and operation is simplified resulted in an improved H₂ economy.

Seven research groups headed from Japan are working together with the aim to develop a biomolecular device to produce hydrogen gas from water by light energy. The complexes/proteins of photosystem I and II from the thermophilic cyanobacterium *Synechococcus elongatus* and purified hydrogenase from *Thiocapsa roseopersicina* are handled to form the device by molecular engineering techniques such as Langmuir-Blodgett film. An efficient electron transfer within the components and stable immobilization were also examined. During 2002 an international workshop was held (Kyoto; October 11 to 12, 2002) and initial reports were presented at BioHydrogen 2002.

Using molecular engineering of green algal hydrogenases and a goal to improve the O₂ tolerance of native algal hydrogenases in order to photoproduce H₂ under aerobic conditions *C. reinhardtii* was transcribed with a DNA construct containing a hydrogenase gene, modified by site-directed mutagenesis. The first successful transformant was recovered, and they exhibited an order of magnitude improvement in O₂ tolerance as the result of a single amino acid change.

In order to utilize unique photosynthetic bacteria to quantitatively shift, in darkness, a crude synthesis gas from biomass containing carbon monoxide into additional hydrogen, the mechanism of the biological water-gas shift pathway to optimize the CO shift process, and thereby maximize H₂ production, was examined. As a result, partial sequences of nine genes responsible for CO oxidation, electron transport and H₂ production in the CO shift process have been obtained. The CO shift reaction yields energy in darkness in the form of ATP, which can be used to support the dark CO shift bioreactor during long-term operation without solar input.

Two major break-thoughts occurred during 2004 within this subtask;

(a) Accessory genes necessary for the photoproduction of H₂ in *Chlamydomonas reinhardtii* have been identified by screening randomly inserted mutants unable to produce H₂. One of the identified mutants, denoted hydEF-1, was incapable of assembling an active [Fe] hydrogenase. Although the hydEF-1 mutant transcribes both hydrogenase genes and accumulates full-length hydrogenase protein, H₂ production activity is not observed. The HydEF protein contains two unique domains that are homologous to two distinct prokaryotic proteins, HydE and HydF, which are found exclusively in organisms containing [Fe] hydrogenase. In the *C. reinhardtii* genome, the HydEF gene is adjacent to another hydrogenase-related gene, HydG. All organisms with [Fe] hydrogenase and sequenced genomes contain homologues of HydE, HydF, and HydG, which, prior to the published study (Posewitz et al 2004a), were of unknown function. Within several prokaryotic genomes HydE, HydF, and HydG are found in putative operons with [Fe] hydrogenase structural genes. Both HydE and HydG belong to the emerging radical S-adenosylmethionine (commonly designated "Radical SAM") superfamily of proteins. They demonstrated that HydEF and HydG function in the assembly of [Fe] hydrogenase. Northern blot analysis indicated that mRNA transcripts for both the HydEF gene and the HydG gene are anaerobically induced concomitantly with the two *C. reinhardtii* [Fe] hydrogenase genes, HydA1 and HydA2. Complementation of the bx;*1C. reinhardtii* hydEF-1 mutant with genomic DNA corresponding to a functional copy of the HydEF gene restored the hydrogenase activity. Moreover, co-expression of the *C. reinhardtii* HydEF, HydG, and HydA1 genes in *Escherichia coli* resulted in the formation of an active HydA1 enzyme. This major finding represented the first report on the nature of the accessory genes required for the maturation of an active [Fe] hydrogenase.

(2) In a second report, based on work performed in the US, NA insertional transformants of *Chlamydomonas reinhardtii* were screened chemochromically for attenuated H₂ production. One mutant, displaying low H₂ gas photoproduction, has a nonfunctional copy of a gene that shows high homology to the family of isoamylase genes found in several photosynthetic organisms (Posewitz et al 2004b). DNA gel blotting and gene complementation were used to link this isoamylase gene to previously characterized non-tagged sta7 mutants. This mutant was therefore denoted sta7-10. In *C. reinhardtii*, the STA7 isoamylase gene is important for the accumulation of crystalline starch, and the sta7-10 mutant reported here contains <3% of the glucose found in insoluble starch when compared with wild-type control cells. Hydrogen photoproduction rates, induced after several hours of dark, anaerobic treatment, were attenuated in sta7 mutants. RNA gel blot analysis indicated that the mRNA transcripts for both the HydA1 and HydA2 [Fe]-hydrogenase genes are expressed in the sta7-10 mutant at greater than wild-type levels 0.5 h after anaerobic induction. However, after 1.5 h, transcript levels of both HydA1 and HydA2 began to decline rapidly and reach nearly undetectable levels after 7 h. In wildtype cells, the hydrogenase transcripts accumulated more slowly, reach a plateau after 4 h of anaerobic treatment, and maintained the same level of expression for >7 h under anaerobic incubation. Complementation of mutant cells with genomic DNA corresponding to the STA7 gene restored both the starch accumulation and H₂ production phenotypes. The results indicate that STA7 and starch metabolism play an important role in *C. reinhardtii* H₂ photoproduction. Moreover, the results indicate that mere anaerobiosis is not sufficient to maintain hydrogenase gene expression without the underlying physiology, an important aspect of which is starch metabolism.

Subtask B: Maximizing Photosynthetic Efficiencies

Photosynthesis can achieve relatively high solar conversion efficiencies, but only at low light intensity. At full sunlight, efficiencies drastically decline. The reason is the large amounts of so-called light-harvesting pigments, which capture more photons at full sunlight than the photosynthetic apparatus can actually handle. These excess photons are thus wasted, with their energy released as heat or fluorescence, even causing damage to the photosynthetic apparatus. Reducing antenna sizes is a method for increasing photosynthetic efficiencies, and this is a central R&D need in photobiological hydrogen production.

The goal of the research, mainly carried out in the USA, was to maximize the solar conversion efficiency and H₂ production of photosynthetic organisms by minimizing, or “truncating” the chlorophyll antenna size of photosynthesis, see Figure 4.

Regulation of the Chl antenna size

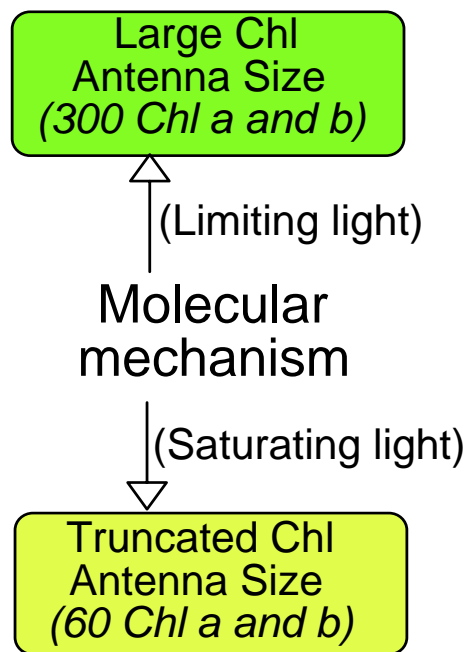


Figure 4. Schematic overview of Large versus Truncated Chl Antenna size in relation to Light condition.

The approach and objectives can be summarized as:

- i) A molecular genetic approach with the green alga *Chlamydomonas reinhardtii*.
- ii) Identify and characterize genes that confer a “truncated Chl antenna size” in green algae.

Work focused on the identification of: (i) Genes for pigment biosynthesis (Chl *b*, Lutein), and (ii) Genes for the regulation of the Chl antenna size.

tla1 has been identified and characterized, the first-time isolation of a gene for the regulation of the Chl antenna size in photosynthesis. Figure 5 shows a greenhouse experiment in which growth and productivity (O₂ bubbling) in WT and *tla1* are compared. Note the higher cell density, lower amount of Chl and greater bubbling of the *tla1* relative to the WT.

Cultures in the Greenhouse



| <u>Parameter</u> | <u>WT</u> | <u><i>tla1</i></u> |
|--------------------------------|-----------|--------------------|
| Cell/mL (x10 ⁶) | 6.36 | 10.0 |
| [Chl] (uM) | 25.6 | 15.4 |

Figure 5. A greenhouse experiment in which growth and productivity (O₂ bubbling) in WT and *tla1* are compared. Note the higher cell density, lower amount of Chl and greater bubbling of the *tla1*-mutant relative to the WT.

Photosynthesis can achieve relatively high solar conversion efficiencies, but only at low light intensity. At full sunlight, efficiencies drastically decline. The reason is the large amounts of so-called light-harvesting pigments, which capture more photons at full sunlight than the photosynthetic apparatus can actually handle. These excess photons are thus wasted, with their energy released as heat or fluorescence, even causing damage to the photosynthetic apparatus.

Reducing antenna sizes is a method for increasing photosynthetic efficiencies, and this is a central R&D need in photobiological hydrogen production. The goal of the present research, mainly carried out in the USA, is to maximize the solar conversion efficiency and H₂ production of photosynthetic organisms by minimizing, or “truncating” the chlorophyll antenna size of photosynthesis.

In research with the green alga *Chlamydomonas reinhardtii*, the molecular mechanism for the regulation of the chlorophyll antenna size of photosynthesis was investigated and a detailed model for the signal transduction pathway was presented (Melis Lab (UC Berkeley, USA), Chlorophyll Antenna Size Adjustments). A novel gene that confers a "truncated Chl antenna size" in green algae (*Tla1*) has been identified and characterized. *Tla1* (GenBank Accession numbers AF534570, AF534571) is the first known gene that is involved in the signal transduction pathway leading to regulation of the Chl antenna size of photosynthesis. This advance represents the first-time cloning and functional characterization of a Chl antenna size regulatory gene in photosynthesis.

Photosynthesis can achieve relatively high solar conversion efficiencies, but only at low light intensity. At full sunlight, efficiencies drastically decline. The reason is the large amounts of so-called light-harvesting pigments, which capture more photons at full sunlight than the photosynthetic apparatus can actually handle. These excess photons are thus wasted, with their energy released as heat or fluorescence, even causing damage to the photosynthetic apparatus. Reducing antenna sizes is a method for increasing photosynthetic efficiencies, and this is a central R&D need in photobiological hydrogen production. The goal of the present research, mainly carried out in the USA, is to maximize the solar conversion efficiency and H₂ production of photosynthetic organisms by minimizing, or “truncating” the chlorophyll antenna size of photosynthesis.

The overall goals are to decrease PSII from 230 to 37 Chl mol (100 ⇒ 16%) and PSI from 240 to 95 Chl molecules (100 ⇒ 40%). Current results are a PSII Chl Antenna Size at 50% (40% in the Chl *b*-less) and a PSI Chl Antenna Size at 67% .

The dependence and importance on carbon source on the photosynthetic apparatus organization and function in wild type and a Chl *b*-less mutant of the green alga *Chlamydomonas reinhardtii* has been examined (Polle et al 2000).

Subtask C: Hydrogen Fermentations

Although in theory the amount of hydrogen that could be generated from renewable sources of energy such as cellulose (a polymer of glucose) is vast, only 16-24% of the maximum stoichiometric yield of hydrogen from glucose is typically achieved by biological methods. However, an amazing 11.6 mol of H₂ generated per mol of glucose-6-phosphate has been demonstrated using enzymes of the oxidative pentose phosphate cycle coupled to a hydrogenase purified from *Pyrococcus furiosus* (Woodward et al 2000), a real breakthrough!

In Canada, a team was organized to develop, using manipulation of microbial physiology, genetic engineering and advanced bioprocess engineering, an optimal system for the biological production of hydrogen through fermentation.

Fermentative processes, using either biomass obtained in a first stage light conversion process or perhaps more attractively, various waste streams, present an interesting yet largely unexplored avenue for the biological production of hydrogen, see Figure 6. Much is presently known about the molecular biology and biochemistry of the hydrogen producing enzymes, reductant generating systems, and physiology of many hydrogen producing organisms. The enormous potential of metabolic engineering for redirecting electron flux to hydrogen production in various microorganisms remains to be exploited. Very little is presently known about the currently attainable yields or the possibilities for improving these yields in the future. These questions were addressed in the research activities.

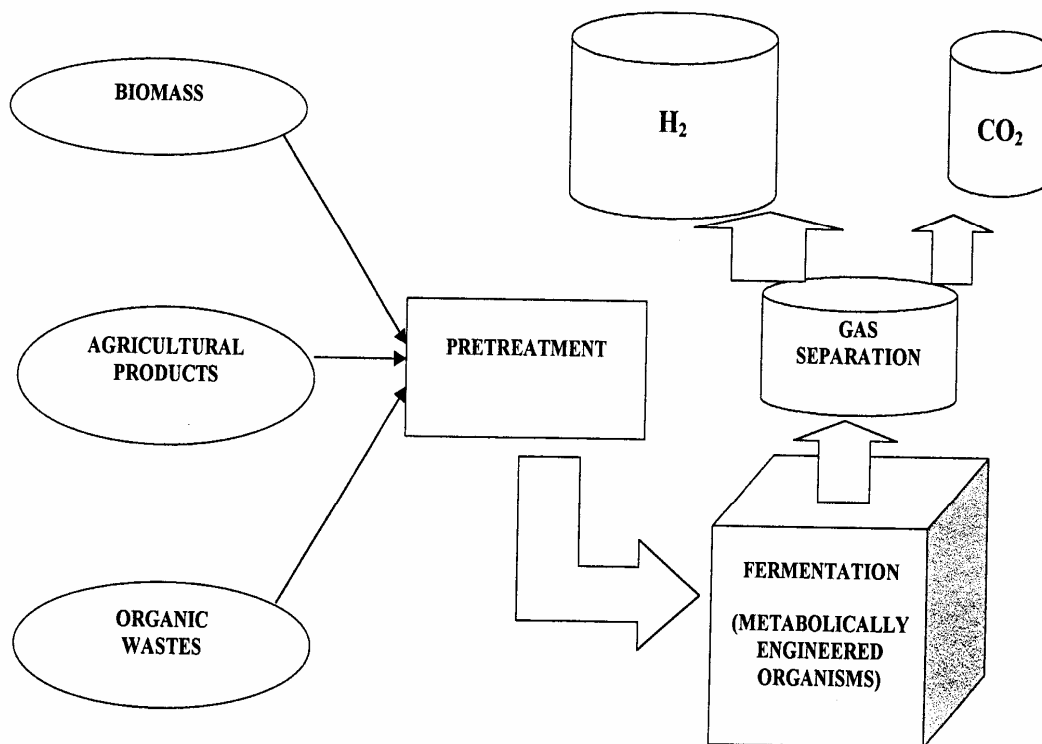


Figure 6. Overview of the fermentation process producing H₂ from biomass and/or organic wastes.

The program was multi-disciplinary involving researchers with expertise in recombinant DNA technology, biochemistry and microbiology, and chemical and bioprocess engineering. The research program, in which various avenues was used to generate strains capable of high levels of

hydrogen production, will involve several distinct phases over five years. Finally, a conceptual system in which biohydrogen production is integrated with energy production (Fuel Cell) systems was subjected to an energy balance analysis.

In the Netherlands there were two projects on Biological hydrogen production. Both projects aimed at the fermentative conversion of biomass (energy crops and organic waste streams) to hydrogen, employing extreme thermophilic and photoheterotrophic microorganisms. The research focussed on the optimisation of hydrogen fermentation processes and concomitant technological production methodologies.

Extreme thermophilic microorganisms ferment sugars to hydrogen and acetate (stage 1). With suspended cultures 83% of the maximal theoretical conversion ($3.3 \text{ H}_2/\text{glucose}$) were obtained. The hydrogen production rates are similar to the fastest producers reported in the literature. Several extreme thermophiles are relatively tolerant to partial hydrogen pressures up to $1\text{-}2 \cdot 10^4$ Pa, making the thermofermentation process technologically feasible. For the process design it also has to be considered that the microorganisms may have a relatively low tolerance for the by-product of fermentation, sodium acetate.

In the photoheterotrophic fermentation (stage 2) hydrogen must be produced from acetic acid. In this second year of the project we focused solely on optimizing and modeling of photoheterotrophic growth and hydrogen production. The influence of three operational parameters on biomass growth and hydrogen production was investigated. The following parameters were taken into account: acetic acid concentration; molar ratio of acetate to glutamate; light intensity (photon flux density). In addition, results from additional batch experiments were used to make carbon and nitrogen balances.

The yield of hydrogen on acetate ($Y_{\text{H}_2,\text{Ac}}$) was highest at a low ratio of acetate to glutamate ($C_{\text{Ac}}/C_{\text{Glu}}$). At a ratio of 5, $Y_{\text{H}_2,\text{Ac}}$ is between 37 and 43 %. At a $C_{\text{Ac}}/C_{\text{Glu}}$ of 10 or higher, glutamate appeared to be limiting biomass growth and hydrogen production. In additional experiments it was found that all nitrogen (N), consumed as glutamate, is retrieved as biomass-N. These results support the hypothesis that hydrogen production from acetic acid is coupled to biomass growth.

The amount of carbon (C) consumed, on the other hand, could not be accounted for completely. Estimated balances of hydrogen (H) and oxygen (O) showed these elements also disappeared. The only explanation was the excretion of a dissolved product. And indeed, in additional experiments, a considerable increase in the chemical oxygen demand of the culture supernatant was observed.

The influence of the light intensity was very small in the range of 270 to 620 $\mu\text{mol PAR m}^{-2} \text{ s}^{-1}$. It seems that a light intensity of 270 $\mu\text{mol PAR m}^{-2} \text{ s}^{-1}$ ($= 163 \text{ W m}^{-2}$, 400 – 950 nm) is sufficient to support maximal growth and hydrogen production.

In short,

1. For 2002 a production of Sweet Sorghum has been determined at 125 tons wet weight/ha (\equiv 30 tons dry matter/ha). The yield of sucrose (dominant sugar in the juice) was about 13.6 tons/ha.

2. The enzymatic hydrolysis of potato steam peels was successful with respect to yield of hydrolysis, and readiness of thermophiles to ferment the hydrolysate. The use of the by-product, which has been increased in protein content, as a fodder is promising, only neutralisation for acidity should be carried out with KOH instead of NaOH.
3. The overall effect of oxygen on hydrogen production by the extreme thermophiles, *Thermotoga elfii*, *T. lettingae*, *T. neapolitana*, and *Caldicellulosiruptor saccharolyticus*, is negative. The sensitivity to oxygen is different for each organism and decreases according to *C. saccharolyticus*>*T. elfii*>*T. neapolitana*. *C. saccharolyticus* is completely inhibited by 5% O₂, while *T. neapolitana* can take O₂ concentrations up to 10%. In short, addition of oxygen has no positive effect on hydrogen production by these thermophiles.
4. *T. elfii* ferments glucose simultaneously with other sugars such as xylose, arabinose, and cellobiose. In contrast, *C. saccharolyticus* growing on sugar mixtures prefers other sugars (such as cellobiose, sucrose, xylose, arabinose, ribose) over glucose. Only at low concentrations of pentose (5 mM) catabolic repression by glucose was observed.
5. The inhibition of hydrogen production and growth of *C. saccharolyticus* by sugars and acetate is caused by the osmotic potential. Hydrogen inhibits its own production, resulting in a metabolic shift towards lactate formation and reductive acetate pathway. This is possibly regulated via the internal redox potential. The external redox potential probably does not affect these metabolic shifts to a great extent.
6. A mathematical model describing growth and hydrogen production by *C. saccharolyticus* was developed, including substrate and product inhibition kinetics, cell lysis kinetics, and the effect of temperature.
7. In principle it is possible to use CO₂ instead of N₂ as stripping gas to drive out H₂ from the liquid phase. It is thereby important to keep the pH between 6.7 and 7.0. At higher pH the amount of bicarbonate will increase dramatically and hence will negatively influence the hydrogen production because it strengthens the osmotic potential of the culture. In a successful demonstration with a duration of about 6 h, H₂ was continuously stripped with CO₂ from an active continuous culture, the gas was subsequently washed with NaOH to fixate CO₂, and the remaining H₂ was used in a PEM fuel cell to produce electricity to drive a small ventilator.
8. A 380 L stainless steel trickling filter reactor has been built and is already more than a half year successfully producing H₂ from sucrose using *C. saccharolyticus*. The fermentation takes place at 70°C and 0.5 bar in a working volume of 80 L. Improvements in the configuration of the reactor and of the process conditions increased the production of H₂ from 100 to 320 L/day. The *C. saccharolyticus* culture has shown to be of a robust nature since it easily recommenced H₂ production after a regular reactor shutdown caused by failing peripheral equipment.
9. Due to consultancy with a Dutch fuel cell company it appears that the gas produced in the fermentations does not need an efficient purification procedure for the application of hydrogen in fuel cells. This will not only simplify the BHP plant and reduce production costs, but will also allow for a higher flexibility in BHP configurations.

10. Several options for gas purification have been studied. It is possible to separate CO₂ in high quantities and of high quality. This makes it economically attractive to sequester this gas.

A scenario was made with 8-10 small farms generating raw H₂ gas from fermentation of Sweet Sorghum juice. The by-product stream (bagasse) has not been included. There are 3 transport possibilities to consider, i.e. that of the solids, the juice or the H₂ gas. Production costs should include everything and remain below 1000 Euro/ha. Presently the price of H₂ is 1 Euro/kg. A rough estimate is that it may go to 2 Euro/kg. An important factor is that about 30% of the bagasse already covers the energy required to drive the biohydrogen process.

In addition, work has been performed at Wageningen univ using photoheterotrophic fermentation where hydrogen is produced from organic acids, predominantly acetate. The acetate is produced in a thermophilic dark fermentation. The objective was the development of the photoheterotrophic process and further optimization of its performance.

Subtask D: Improve Photobioreactor Systems for Hydrogen Production

In the beginning of Annex 15 only limited progress was been made within this subtask. After some years photobioreactors with both green algae and cyanobacteria were up and running and initial experiments were performed examining hydrogen production and evolution, gas transfer coefficients, sunlight interception, solar conversion efficiencies and H₂ losses. A small-scale photobioreactor was established to monitor the competition between wildtype and genetically modified cyanobacteria, see Figure 7.

Nanoscale bioreactors made from liposomes that contain enzymes encapsulated in a lipid bilayer can potentially be used for the enzymatic production of hydrogen from glucose. This process was being studied in detail within the US Biohydrogen program (Evans Lab (ORNL), Production of Hydrogen from Glucose). The enzymes and the cofactor NADP⁺ were retained at high local concentration inside the liposomes, while hydrogen gas readily diffuses through the lipid membrane. The transport of glucose into liposomes is facilitated by synthetic carriers based on boronic acids. Hydrogen production by a liposome-based bioreactor constructed by the encapsulation of glucose dehydrogenase from *Thermoplasma acidophilum* and *P. furiosus* hydrogenase with NADP⁺ was demonstrated using tertbutylphenyl boronic acid as the glucose carrier.

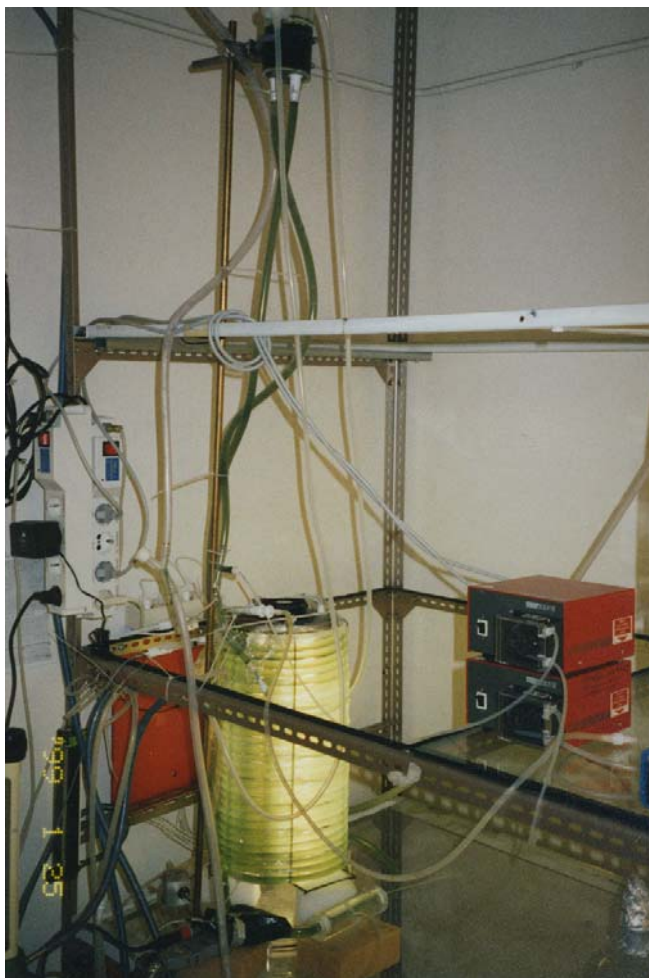


Figure 7. Small scale photobioreactor developed with in the frame-work of Annex 15 and used to both determine the specific H₂-evolution rates by genetically modified cyanobacteria under different environmental conditions and to perform direct competition experiments between wildtype and genetically modified cyanobacteria "on-line".

Within the use of green algal based hydrogen production, a specific Continuous Algal Hydrogen Photoproduction System was design and evaluated by DOE/NREL. The goal was to lower the cost of the sulfur-deprived algal H₂ photoproduction system by eliminating centrifugation and producing H₂ continuously. A two-reactor, continuous production system was established and improved, the H₂ photoproduction was demonstrated for 480 hours and the costs calculated. The current cost is etimated to be below \$200/kg of H₂ (down from \$670 in 2001), see Figure 8. This should be compared with the long-term target (2010) to produce H₂ continuously for at least 1500 hours at a cost of \$30/kg of H₂.

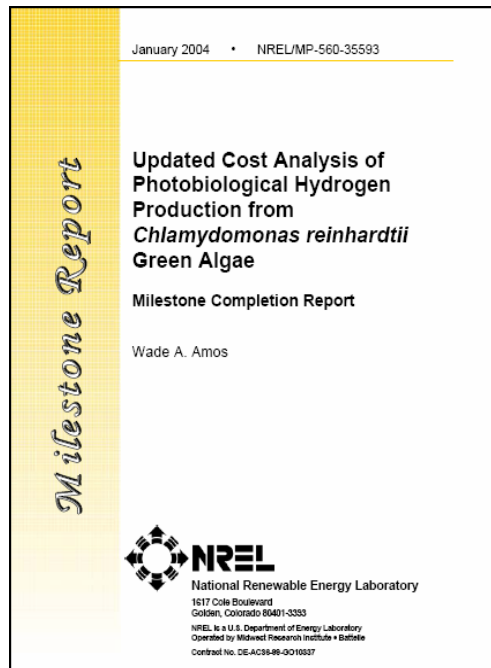


Figure 8. A cost analysis of photobiological hydrogen production from the green algae *Chlamydomonas* performed within the frame-work of Annex 15.

Specific Scientific exchanges within the partners of Annex 15

Year 2000; Marc Forestier (postdoc in the lab of Mike Seibert; NREL, USA) visited and worked in the lab of the OA for a week proceeding the Hydrogenases 2000 and the Experts-meetings in Potsdam, Germany (2000). The subject was green alga hydrogenases and specifically the identification and characterization of the molecular information needed to synthesize a functional hydrogenase.

During July 2001: Röbbbe Wünschiers (PostDoc in Uppsala, Sweden) visited and worked in the laboratory of Michael Seibert at NREL (Golden, CO, USA) for one week as well as visited and discussed with Anastasios Melis at UC-Berkeley (CA, USA).

During 2002 two specific exchanges occurred;

(i) PhDstudents Skjånes and Gjelland from NIVA (Norway) visited and worked in the lab of Professor Lindblad in Uppsala (Sweden), May 23 to 26, 2002. The exchange was financially supported by the respective IEA/Annex 15 budgets and the scientific purpose was to set and to work with a H₂-electrode.

(ii) Professor Schultz (Christian-Albrechts-Universitaet, Kiel, Germany) visited the Algal Hydrogen Laboratory at NREL from August 29 to September 11, 2002, under auspices of IEA/COST (through funding support and facilities provided by the U.S. and airfare covered, personally, by Prof Schultz) in order to use the automated photobioreactor systems at NREL to test the hydrogen-production efficiency of a number of algal strains that has been collected and

are putative high producers of hydrogen. NREL has unique equipment for simultaneously comparing a number of new strains with the NREL control strain in a controlled and statistically valid manner.

6. Conclusions

Some of the major highlights achieved throughout the 5 years of collaborative R&D in Annex 15 can be summarised as follows (provided in no order of priority and including important contributions by some of the above mentioned Annex 15 observer groups):

6.1. Subtask specific progresses within Annex 15

Subtask A: Light-driven Hydrogen Production by Microalgae

1. A novel sustainable photobiological production of molecular hydrogen upon a reversible inactivation of the oxygen evolution in the green alga *Chlamydomonas reinhardtii* has been demonstrated. In this organism, a two-stage H₂ production method circumvents the severe O₂ sensitivity of the reversible hydrogenase by temporally separating photosynthetic O₂ evolution and carbon accumulation (stage 1) from the consumption of cellular metabolites and concomitant H₂ production (stage 2).
2. A heterologous expression of a clostridial hydrogenase has been achieved in the cyanobacterium *Synechococcus* PCC7942. The *Clostridium pasteurianum* hydrogenase I was expressed in the cyanobacterium *Synechococcus* PCC7942. Expression of clostridial hydrogenase was confirmed by Western and Northern blot analyses in both *Synechococcus* and *Escherichia coli*, whereas *in vivo/in vitro* measurements and activity staining of soluble proteins separated on non-denaturing polyacrylamide gels revealed functional expression of the clostridial hydrogenase in the cyanobacterial cells only.
3. The knowledge about cyanobacterial hydrogenases, and their potential as photobiological producers of molecular hydrogen, has been reviewed extensively.
4. Accessory genes and gene products necessary for the photoproduction of H₂ in *Chlamydomonas reinhardtii* have been identified. The so called HyDEF protein contains two unique domains that are homologous to two distinct prokaryotic proteins, HydE and HydF, which are found exclusively in organisms containing [Fe] hydrogenase. In the *C. reinhardtii* genome, the HyDEF gene is adjacent to another hydrogenase-related gene, HydG. All organisms with [Fe] hydrogenase and sequenced genomes contain homologues of HydE, HydF, and HydG, which, prior to the published study all were of unknown function. Within several prokaryotic genomes HydE, HydF, and HydG are found in putative operons with [Fe] hydrogenase structural genes.
5. A mutation in a novel gene that shows high homology to the family of isoamylase genes found in several photosynthetic organisms, *sta7*, resulted in higher hydrogen photoproduction rates. The results demonstrated that STA7 and starch metabolism play a most important role in *C. reinhardtii* H₂ photoproduction. Moreover, the results indicate that mere anaerobiosis is not

sufficient to maintain hydrogenase gene expression without the underlying physiology, an important aspect of which is starch metabolism.

Subtask B: Maximizing Photosynthetic Efficiencies

6. *tlal*, a novel gene involved in the regulation of the Chl antenna size in photosynthesis has been identified and characterized in *C. reinhardtii*. This is the first genetic explanation of the regulation of the size of a Chl antenna.

Subtask C: Hydrogen Fermentations

7. Although in theory the amount of hydrogen that could be generated from renewable sources of energy such as cellulose (a polymer of glucose) is vast, only 16-24% of the maximum stoichiometric yield of hydrogen from glucose is typically achieved using biological methods. However, an amazing 11.6 mol of H₂ generated per mol of glucose-6-phosphate has been demonstrated using enzymes of the oxidative pentose phosphate cycle coupled to a hydrogenase purified from *Pyrococcus furiosus*, a real breakthrough!

Subtask D: Improve Photobioreactor Systems for Hydrogen Production

9. A small-scale photobioreactor has been established to monitor the competition between wildtype and genetically modified cyanobacteria.

10. A two-reactor, continuous green algal based H₂ production system, for 480 hours, has been established lowering the cost from \$670/kg of H₂ in 2001 to below \$200 with a long term target (year 2010) of a continuous production of H₂ for at least 1500 hours at \$30 per kg H₂.

11. Several specific scientific exchanges occurred between the partners of Annex 15 and numerous scientific publications were published within the frame of Photobiological hydrogen production.

7. Outlook

The fundamental aspects of Photobiological Production of Hydrogen, and the more applied potential use as future producers of renewable H₂ from sun and water, are receiving increased international attention. At the same time significant progress is made in e.g. the understanding of the molecular regulation of the genes encoding both the enzymes as well as the accessory proteins needed for the correct assembly of an active hydrogenase. In the last few years some transcription factors directly involved in the regulation of specific hydrogenases have been identified. Moreover, the first steps to use isolated components from cyanobacteria, green algae and other microorganisms in order to create a functional H₂ producing unit are being taken. With the increasing scientific community and public interest in clean and renewable energy sources, and consequent funding opportunities, rapid progress will be made in the fundamental understanding of the regulation and maturation of hydrogenases at both genetic and protein levels. Unique and unexpected results in the transcriptional regulation will emerge during the coming years. Moreover, the more applied aspects will be high-lighted with progress in

generating genetically modified strains with an increased capacity for renewable H₂ from sun and water. The possibilities and challenges within synthetic biology, including the use of isolated proteins and parts, will be explored aiming at creating both cyanobacteria and green algae with a high potential for H₂ production as well as functional *in vitro* systems.

8. Appendix

8.1. ExpertsMeetings (1999-2004)

1999

June 22-23 (1999) the National Institute for Advanced Interdisciplinary Research (NIAIR) of Japan arranged BioHydrogen '99 *An approach to Environmentally Acceptable Technology* in Tsukuba (Japan) with about 40 participants. During this workshop a Task 15 ExpertsMeeting with national experts from Japan, USA, and Sweden was held and the new Annex *Photobiological hydrogen production* discussed. Moreover, the Operating Agent presented IEA, the Hydrogen Agreement, and specifically Annex 15 during the workshop. About 20 contributions/chapters (several written by national experts participating in Task 15) was edited (Editor: Miyake, Jun; national expert of Japan) and published as "Biohydrogen 99" by Elsevier during 2000.

2000

a) March 20-21, at NREL, Colorado (US) with 15 participants (including observers from Hungary, Italy and The Netherlands).

b) August 11, at Potsdam, Germany, following the "6th International Conference on the Molecular Biology of Hydrogenases" (Hydrogenases 2000; August 5-10, 2000) with 10 participants (including observers from UK, Hungary, The Netherlands, Portugal and Italy).

2001

a) March 26-27, in Porto (Portugal) with 8 active participants.

b) September 7-12, in Szeged, Hungary. This meeting was organized as a joint workshop, with the title *BioHydrogen*, between the IEA Hydrogen Agreement Annex 15 and the European COST Action 8.41 (*Biological and Biochemical Diversity of Hydrogen Metabolism*) with, in total, 31 participants.

2002

a) April 22 in Ede, The Netherlands. An ExpertsMeeting was held during BioHydrogen 2002. The OA used the opportunity and invited all scientists from "Annex 15 countries" to participate. About 40 came and discussed the present Annex and a continuation/extension of Annex 15, the slight change/addition in the work program, subtask leaders and future common activities. As a result many more scientists in the respective countries were aware of Annex 15, what is being

done, possibilities etc and hopefully much more national discussions occurred in the following years.

(b) October 3-4 in Montreal, Canada. In this more "traditional ExpertsMeeting", with 7 participant, the progress etc in the respective participating countries and laboratories were reviewed, a general plan for years four and five agreed on, and possible interactions with other IEA relevant activities discussed.

2003

a) April 10-11 in Uppsala, Sweden, with 6 participants. In addition to a more "traditional ExpertsMeeting" a discussion about a putative new Annex after the completion of Annex 15 resulted in the following possible subtasks: (A) Light-dependent processes, (B) Light independent processes, and (C) Systems Integration.

(b) September 21-23 in Kyoto, Japan. This ExpertsMeeting was organized as an integrated part of the *Marine Biotechnology Conference 2003* (6th International Marine Biotechnology Conference & 5th Asia Pacific Marine Biotechnology Conference) with approximately 750 participants. The Annex 15 meeting consisted of an "open part", 6 oral presentations in a single *BioHydrogen* session and 12 posters, and a "closed part" (approximately 1.5 days) where we discussed Annex 15 specific matters (all Annex 15 countries, except UK, were represented in the meeting, in addition several countries interested to join a tentative new annex were present).

2004

The major IEA Hydrogen Annex 15 ExpertsMeeting was held within the 15th World Hydrogen Energy Conference (15WHEC, Yokohama, Japan; June 27 - July 2, 2004). It was organized by Professors Igarashi, Matsunaga, Tanisho and Dr Miyake and attracted 68 participants.

The overall program of the "BioHydrogen part" within 15WHEC was

June 29, 2004; 6 + 5 oral presentations

June 30, 2004; Plenary lecture *Biological Hydrogen Production* (more than 1000 persons attended the presentation), IEA H2 Annex meeting (3 h), 6 +5 oral presentations

July 1, 2004; IEA H2 Annex meetin (5 h), IEA BioHydrogen Mixer

June 29 – July 1, 2004; 62 posters

8.2. Scientific Publications / References

Akkerman I, Janssen M, Rocha J, Wijffels RH. 2002. Photobiological hydrogen production: photochemical efficiency and bioreactor design. *International Journal of Hydrogen Energy* 27: 1195-1208.

Asada Y, Koike Y, Schnackenberg J, Miyake M, Uemura I, Miyake J. 2000. Heterologous expression of clostridial hydrogenase in the cyanobacterium *Synechococcus* PCC7942. *Biochimica et Biophysica Acta* 1490: 269-278.

Axelsson R, Lindblad P. 2002. Transcriptional Regulation of *Nostoc* Hydrogenases: Effects of Hydrogen, Oxygen and Nickel. *Applied and Environmental Microbiology* 68: 444-447.

Barbosa MJ, Janssen M, Ham N, Tramper J, Wijffels RH. 2003. Microalgae cultivation in air-lift reactors: Modeling biomass yield and growth rate as a function of mixing frequency. *Biotechnology and Bioengineering* 82: 170-179.

Chénier I, Dubé F, Hallenbeck PC. 2000. Mutations permitting *fdhF* (Formate Dehydrogenase H) expression in a *selD* mutant of *Salmonella typhimurium*. *Current Microbiology* 41: 39-44.

Claassen PAM, Budde MAW, van der Wal FJ, Kádár Zs, van Noorden GE, de Vrije T. 2002. Biological hydrogen production from biomass by thermophilic bacteria. In: Palz W, Spitzer J, Maniatis K, Kwant K, Helm P, Grassi A, editors. Proc 12th Eur Conf Technol Exhibition Biomass for Energy, Industry & Climate Protection, Amsterdam, p. 529-532.

De Vrije T, de Haas GG, Tan GB, Keijsers ERP, Claassen PAM. 2002. Pretreatment of *Miscanthus* for hydrogen production by *Thermotoga elfii*. *International Journal of Hydrogen Energy* 27: 1381-1390.

Elam CC, Gregoire Padro CE, Sandrock G, Luzz A, Lindblad P, Fjermstad-Hagen E. 2003. Realizing the hydrogen future: the International Energy Agency's effort to advance hydrogen energy technologies. *International Journal of Hydrogen Energy* 28: 601-607.

Flynn T, ML Ghirardi, M Seibert. 2002. Accumulation of Multiple O₂-Tolerant Phenotypes in H₂-Producing Strains of *Chlamydomonas reinhardtii* by Sequential Application of Chemical Mutagenesis and Selection. *International Journal of Hydrogen Energy* 27: 1421-1430.

Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A. 2000. Microalgae: a green source of renewable H₂. *Trends in Biotechnology* 18: 506-511.

Hallenbeck PC, JR Benemann 2002. Biological hydrogen production; fundamentals and limiting processes. *International Journal of Hydrogen Energy* 27: 1185-1193.

Hallenbeck, P.C. 2001. Integration of hydrogen evolving systems with cellular metabolism: the molecular biology and biochemistry of electron transport factors and associated reductases. In: Biohydrogen II, An approach to environmentally acceptable technology. Pages 171-184. Editors: Miyake, J., Matsunaga, T. and San Pietro, A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.

Hallenbeck, P.C., Viger, J.-F. 2000. Biocatalysis and Bacterial Cells; Problems and Prospects for Biohydrogen. Proceedings 10th Canadian Hydrogen Conference. Pages: 173-182. Editors: Bose, T.K, Benard, P. ISBN 0-9696869-5-1.

Hallenbeck, Patrick C. 2001. Biohydrogen Production through Fermentation: Future Prospects and Studies with a Model System. Proceedings of the 11th Canadian Hydrogen Conference, 406-412.

Hansel, A., Axelsson, R., Lindberg, P., Troshina, O., Wünschiers, R. and Lindblad, P. 2001. Cloning and characterization of a *hyp* gene cluster in the filamentous cyanobacterium *Nostoc* sp. strain PCC 73102. *FEMS Microbiology Letters* 201: 59-64.

Hejazi MA, Wijffels RH. 2003. Effect of light intensity on beta-carotene production and extraction by *Dunaliella salina* in two-phase bioreactors. *Biomolecular Engineering* 20: 171-175.

Hoekema S, Bijmans M, Janssen M, Tramper J, Wijffels RH. 2002. A pneumatically agitated flat-panel photobioreactor with gas re-circulation: anaerobic photoheterotrophic cultivation of a purple non-sulfur bacterium. *International Journal of Hydrogen Energy* 27: 1331-1338.

Janssen M, Tramper J, Mur LR, Wijffels RH. 2003. Enclosed outdoor photobioreactors: Light regime, photosynthetic efficiency, scale-up, and future prospects. *Biotechnology and Bioengineering* 81: 193-210.

Jin ES, Yokthongwattana K, Polle JEW, Melis A. 2003. Role of the reversible xanthophyll cycle in the photosystem II damage and repair cycle in *Dunaliella salina*. *Plant Physiology* 132: 352-364.

- Kosourov S, AA Tsygankov, M Seibert, M L Ghirardi. 2002. Sustained Hydrogen Photoproduction by *Chlamydomonas reinhardtii*--Effects of Culture Parameters. *Biotechnology and Bioengineering* 78: 731-740.
- Kosourov S, Seibert M, Ghirardi ML. 2003. Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H₂-producing *Chlamydomonas reinhardtii* cultures. *Plant and Cell Physiology* 44: 146-155.
- Kovács KL, Fodor B, Kovács ÁT, Csanadi G, Maróti G, Balogh J, Arvani S, Rákhely G. 2002. Hydrogenases, accessory genes and the regulation of [NiFe] hydrogenase biosynthesis in *Thiocapsa roseopersicina*. *International Journal of Hydrogen Energy* 27: 1463-1469.
- Laurinavichene TV, IV Tolstygina, RR Galiulina, ML Ghirardi, M Seibert, AA Tsygankov. 2002. Different methods to deprive *Chlamydomonas reinhardtii* cultures of sulfur for subsequent hydrogen photoproduction. *International Journal of Hydrogen Energy* 27: 1245-1249.
- Lee, JW, L Mets, E Greenbaum. 2002. Improvement of photosynthetic efficiency at high light intensity through reduction of chlorophyll antenna size. *Applied Biochemistry and Biotechnology* 98: 37-48.
- Lindberg P, Hansel A, Lindblad P. 2000. *hupS* and *hupL* constitute a transcription unit in the cyanobacterium *Nostoc* sp. PCC 73102. *Archives of Microbiology* 174: 129-133.
- Lindberg P, Schütz K, Happe T, Lindblad P. 2002. A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. *International Journal of Hydrogen Energy* 27: 1291-1296.
- Lindberg, P., Lindblad, P. and Cournac, L. 2004. Gas exchange in the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133 and its hydrogenase deficient mutant strain NHM5. *Applied and Environmental Microbiology* 70: 2137-2145.
- Lindblad P, Christensson K, Lindberg P, Pinto F, Tsygankov A. 2002. Photoproduction of H₂ by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: from laboratory experiments to outdoor culture. *International Journal of Hydrogen Energy* 27: 1271-1281.
- Lindblad P. 2003. Hydrogen in Biological Systems. In: *Hydrogen in Materials & Vacuum Systems*; pages 11-20. Editor: Rao Myeni R, Chattopadhyay S. American Institute of Physics, USA. ISBN 0-7354-0137-3.
- Lindblad P. 1999. Cyanobacterial H₂-metabolism: Knowledge and potential/strategies for a photobiotechnological production of H₂. *Biotechnology Aplicada* 16: 141-144.
- Lindblad, P., Tamagnini, P. 2000. Cyanobacterial Hydrogenases and Biohydrogen: Present Status and Future Potential. *Hydrogen Energy Progress XIII*. Volume 1: 382-389. Editors: Mao, Z.Q., Veziroglu, T.N.
- Lindblad, P., Tamagnini, P. 2001. Cyanobacterial Hydrogenases and Biohydrogen: Present Status and Future Potential. In: *Biohydrogen II, An approach to environmentally acceptable technology*. Pages 143-169. Editors: Miyake, J., Matsunaga, T., San Pietro, A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.
- Lindblad, P., Tamagnini, P. 2000. Cyanobacterial Hydrogenases and Biohydrogen: Present Status and Future Potential. *Proceedings 10th Canadian Hydrogen Conference*. Pages: 166-172. Editors: Bose, T.K., Benard, P. ISBN 0-9696869-5-1.
- Lindblad, P., Asada, Y., Benemann, J., Hallenbeck, P., Melis, A., Miyake, J., Seibert, M., Skulberg, O. 2000. IEA Hydrogen Agreement, Task 15: *Photobiological Hydrogen Production - An International Collaboration*. *Hydrogen Energy Progress XIII*. Volume 1: 56-59. Editors: Mao, Z.Q., Veziroglu, T.N.
- Lindblad P, Asada Y, Benemann J, Hallenbeck P, Melis A, Miyake J, Seibert M, Skulberg O. 2000. IEA Hydrogen Agreement, Task 15: *Photobiological Hydrogen Production - An International Collaboration*. *Proceedings 10th Canadian Hydrogen Conference*. Pages: 731-733. Editors: Bose TK, Benard P. ISBN 0-9696869-5-1.

- Maness PC, PF Weaver. 2002. Hydrogen production from a carbon-monoxide oxidation pathway in *Rubrivivax gelatinosus*. *International Journal of Hydrogen Energy* 27: 1407-1411.
- Maness PC, S Smolinski, AC Dillon, MJ Heben, PF Weaver. 2002. Characterization of the oxygen tolerance of a hydrogenase linked to a carbon monoxide oxidation pathway in *Rubrivivax gelatinosus*. *Applied and Environmental Microbiology* 68: 2633-2636.
- Masuda T, Polle JEW, Melis A. 2002. Biosynthesis and distribution of chlorophyll among the photosystems during recovery of the green alga *Dunaliella salina* from irradiance stress. *Plant Physiology* 128: 603-614.
- Masuda T, Tanaka A, Melis A. 2003. Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll a oxygenase (CAO) and Lhcb gene expression. *Plant Molecular Biology* 51: 757-771.
- 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 Physiology* 122: 127-136.
- Miyake MJ, Schnackenberg C, Nakamura Y, Asada Y, Miyake J. 2001. Molecular Handling of Hydrogenase. In: *Biohydrogen II, An approach to environmentally acceptable technology*. Pages 205-222. Editors: Miyake J, Matsunaga T, San Pietro A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.
- Palmfeldt J, Paese M, Hahn-Hagerdal B, Van Niel EWJ. 2004. The pool of ADP and ATP regulates anaerobic product formation in resting cells of *Lactococcus lactis*. *Applied and Environmental Microbiology* 70: 5477-5484.
- Pinto F, Troshina O, Lindblad P. 2002. A brief look at three decades of hydrogen evolution by cyanobacteria. *International Journal of Hydrogen Energy* 27: 1209-1215.
- Polle JEW, Benemann JR, Tanaka A, Melis A. 2000. Photosynthetic apparatus organization and function in wild type and a Chl *b*-less mutant of *Chlamydomonas reinhardtii*. Dependence on carbon source. *Planta* 211: 335-344.
- Polle JEW, Kanakagiri S, Benemann JR, Melis A. 2001. Maximizing photosynthetic efficiencies and hydrogen production in microalga cultures. In: *BioHydrogen II* (J Miyake, T Matsunaga, A San Pietro, eds), Pergamon/Elsevier Science, Kidlington, Oxford, U.K., pp. 111-130.
- Polle JEW, Kanakagiri SD, Melis A. 2003. *ta1*, a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size. *Planta* 217: 49-59.
- Polle JEW, Niyogi KK, Melis A. 2001. Absence of lutein, violaxanthin and neoxanthin affects the functional chlorophyll antenna size of photosystem-II but not that of photosystem-I in the green alga *Chlamydomonas reinhardtii*. *Plant Cell Physiology* 42: 482-491.
- Posewitz MC, King PW, Smolinski SL, Zhang LP, Seibert M, Ghirardi ML. 2004a. Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. *Journal of Biological Chemistry* 279: 25711-25720.
- Posewitz MC, Smolinski SL, Kanakagiri S, Melis A, Seibert M, Ghirardi ML. 2004b. Hydrogen photoproduction is attenuated by disruption of an isoamylase gene in *Chlamydomonas reinhardtii*. *Plant Cell* 16: 2151-2163.
- Qian D-J, Wenk S, Nakamura C, Wakayama T, Zorin N, Miyake J. 2002. Photoinduced hydrogen evolution by use of porphyrin, EDTA, viologens and hydrogenase in solutions and Langmuir-Blodgett films. *International Journal of Hydrogen Energy* 27: 1481-1487.
- Rocha, J.S., M.J. Barbosa, R.H. Wijffels. 2001. Hydrogen production by photosynthetic bacteria: culture media, yield and efficiencies. In: *Biohydrogen II, An approach to environmentally acceptable technology*. Pages 3-32. Editors: Miyake J, Matsunaga T, San Pietro A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.

San Pietro A, Benemann JR. 2001. Biohydrogen: an opportunity for international cooperation. In: Biohydrogen II, An approach to environmentally acceptable technology. Pages 93-102. Editors: Miyake, J., Matsunaga, T. and San Pietro, A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.

Schütz K, Happe T, Troshina O, Lindblad P, Leitao E, Oliveira P, Tamagnini P. 2004. Cyanobacterial H₂ production – a comparative analysis. *Planta* 218: 350-359.

Seibert M, DK Benson, TM Flynn. 2002. "System for Rapid Biohydrogen Phenotypic Screening of Microorganism using a Chemochromic Sensor," U.S. Patent # 6,448,068.

Seibert M, Wasielewski MR. 2003. The isolated Photosystem II reaction center: first attempts to directly measure the kinetics of primary charge separation. *Photosynthesis Research* 76: 263-268.

Seibert M, Flynn T, Ghirardi ML. 2001. Strategies for improving oxygen tolerance of algal hydrogen production. In: Biohydrogen II, An approach to environmentally acceptable technology. Pages 67-80. Editors: Miyake J, Matsunaga T, San Pietro A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.

Serebryakova LT, Sheremetieva ME, Lindblad P. 2000. H₂-uptake and evolution in the unicellular cyanobacterium *Chroococcidiopsis thermalis* CALU 758. *Plant Physiology and Biochemistry* 38: 525-530.

Sheremetieva ME, Troshina OT, Serebryakova LT, Lindblad, P. 2002. Identification of *hox* genes and analysis of their transcription in the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 growing under nitrate-limiting conditions. *FEMS Microbiology Letters* 214: 229-233.

Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers R, Lindblad P. 2002. Hydrogenases and Hydrogen Metabolism of Cyanobacteria. *Microbiology and Molecular Biology Reviews* 66: 1-20.

Tamagnini P, Costa JL, Almeida L, Oliveira MJ, Salema P, Lindblad P. 2000. Diversity of cyanobacterial hydrogenases, a molecular approach. *Current Microbiology* 40: 356-361.

Troshina O, Serebryakova LT, Sheremetieva ME, Lindblad P. 2002. Production of H₂ by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *International Journal of Hydrogen Energy* 27: 1283-1289.

Tsygankov AA, S Kosourov, M Seibert, ML Ghirardi. 2002. Hydrogen Photoproduction under Continuous Illumination by Sulfur-Deprived, Synchronous *Chlamydomonas reinhardtii* Cultures. *International Journal of Hydrogen Energy* 27: 1239-1244.

Wakayama, T., J. Miyake. 2001. Hydrogen from Biomass. In: Biohydrogen II, An approach to environmentally acceptable technology. Pages 41-52. Editors: Miyake, J., Matsunaga, T. and San Pietro, A. Pergamon/Elsevier, Oxford, UK. ISBN 0-08-043947-0.

Van Groenestijn JW, Hazewinkel JHO, Nienoord M, Bussmann PJT. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. *International Journal of Hydrogen Energy* 27: 1141-1147.

Van Niel EWJ, Budde MAW, de Haas GG, van der Wal FJ, Claassen PAM, Stams AJM. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. *International Journal of Hydrogen Energy* 27: 1391-1398.

Wenk S, Qian D-J, Wakayama T, Nakamura C, Zorin N, Roegner M, Miyake J. 2002. Biomolecular device for photoinduced hydrogen production. *International Journal of Hydrogen Energy* 27: 1489-1493.

Woodward J, Orr, M, Cordray K, Greenbaum E. 2000. Enzymatic Production of Biohydrogen. *Nature* 405: 1014-1015.

Wünschiers R, Lindblad P. 2002. Hydrogen in Education – A Biological Approach. International Journal of Hydrogen Energy 27: 1131-1140.

Wünschiers R, Bahtur M, Lindblad P. 2003. Presence and expression of hydrogenase specific C-terminal endopeptidases in cyanobacteria. BMC Microbiology 3: 8 (on-line journal, 12 pages; <http://www.biomedcentral.com/1471-2180/3/8>).

Wünschiers R, Lindblad P. 2003. Light-Dependent Hydrogen Uptake and Generation by Cyanobacteria. In: Handbook of Photochemistry and Photobiology. Volume 4, Chapter 10; pages 295-328. Editor: Nalwa HS. American Scientific Publishers, CA, USA. ISBN 1-58883-025-X.

Yakunin AF, Hallenbeck PC. 2000. Regulation of nitrogenase activity in *Rhodobacter capsulatus* under dark microoxic conditions. Archives of Microbiology 173: 366-372.

8.3. Meetings/Conferences

Barrios-Llerena M, A.M. Burja, P. Tamagnini, P.C. Wright. 2002. Hydrogenases and *Anabaena sphaerica*. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

Burja, A.M., M. Barrios-Llerena, P. Tamagnini, P.C. Wright. 2002. Engineering of large scale, continuous H₂ production by cyanobacteria, BioHydrogen 2002, Ede, The Netherlands, 21-24 April, 2002.

Burja, A.M., MT. Bustard, P. Tamagnini and P.C. Wright. 2001. Specific Amplification of 16S rRNA gene segments from cyanobacteria and microalgae by PCR, COST 841 Workshop, Reading UK, March 2001.

Burja, A.M., P. Tamagnini, P.C. Wright. 2001. *Lyngbya majuscula* Bioprocess Intensification – Creating a Cell Factory, Society of Industrial Microbiology 2001 Annual Meeting, July 29 - August 2, 2001, Adam's Mark Hotel, St. Louis, USA.

Burja, A.M., P. Tamagnini, P.C. Wright. 2002. Molecular identification techniques for cyanobacteria, 5th European Workshop on the Molecular Biology of Cyanobacteria, Stockholm, Sweden, June 9-12, 2002.

Claassen, P.A.M. Utilisation of biomass for dark hydrogen fermentation. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

Ghirardi, M.L. 2002. Identification of *Chlamydomonas reinhardtii* Genes for the Photoproduction of Hydrogen. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

Hallenbeck P.C. 2002. Biological hydrogen production; fundamentals and limiting processes. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

Hallenbeck P.C. 2002. Biological hydrogen production; limiting processes, presented at Biohydrogen in Kyoto, Kyoto, Japan, October, 2002.

Hallenbeck, P.C. 2001. Biohydrogen Production through Fermentation: Future Prospects and Studies with a Model System. 01-06-17. 11th Canadian Hydrogen Conference; Victoria, Canada.

Kanakagiri S, Polle J, Melis A. 2000. A small chlorophyll antenna size (*sca1*) mutant of *Chlamydomonas reinhardtii* shows reduced levels of chlorophyll and higher photosynthetic productivity. In Abstracts and Program of the Annual Meeting of the American Society of Plant Physiologists (San Diego, July 15-19, 2000), Abstract 783, p. 159.

Kanakagiri S, Polle JEW, Melis A. 2002- Tenth International Conference on the Cell & Molecular Biology of *Chlamydomonas*. University of British Columbia, Vancouver. June 11-16, 2002.

- Kovács, K. 2002. Hydrogenases, accessory genes and the regulation of [NiFe] hydrogenase biosynthesis in *Thiocapsa roseopersicina*. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.
- Lee, Greenbaum. 2002. A new oxygen sensitivity in photosynthetic H₂ production, presented at 24th Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, April 28-May 1, 2002.
- Lee, J. W., L. Mets, D. Xu, B. Evans, J. Zhou, E. Greenbaum, L. Trowbridge, W. Steele. 2002. Development of efficient and robust algal H₂-production systems, a new project proposal presented at DOE H₂ Program Review meeting. Denver, CO, USA. May 6-10, 2002.
- Lee, James W. 2002. Overcoming nation's roadblocks to H₂ production: Creation of designer alga for efficient and robust production of H₂, presented at DOE Headquarters, Washington, DC. USA. November 1, 2002.
- Leitão, E., F. Oxelfelt, P Oliveira, A.M. Burja, P.C. Wright, P. Tamagnini. 2002. Genes Related to Hydrogenases in Non-heterocystous cyanobacteria. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.
- Lindblad P. 2002. Cyanobacterial Hydrogenases. Recent Advances in the Understanding of the Bidirectional Enzyme. COST 8.41 WG 3 Genetics of hydrogenases. Cercedeilla, Spain. October 7, 2002.
- Lindblad P. 2002. Hydrogen in Biological Systems. International Workshop on Hydrogen in Materials & Vacuum Systems. Newport News, USA. November 12, 2002.
- Lindblad P. 2002. The potential of using cyanobacteria as producers of molecular hydrogen. International Symposium on Hydrogen and Methane Fermentation of Organic Wastes. Tokyo, Japan. March 6, 2002.
- Lindblad P. 2002. The Potential of Using Cyanobacteria as Producers of Molecular Hydrogen. NEDO International project evaluation workshop, Osaka, Japan. October 11, 2002.
- Lindblad, P. 2001. Biohydrogen: International collaboration and Status & potential of using cyanobacteria. 01-08-22. 12th International Congress on Photosynthesis; Brisbane, Australia.
- Lindblad, P. 2001. Cyanobacterial hydrogenases and biohydrogen: Present status and future potential & IEA H₂Agreement Task 15: *Photobiological Hydrogen Production* - An international collaboration. 01-06-19. 11th Canadian Hydrogen Conference; Victoria, Canada.
- Lindblad, P. 2001. Cyanobacterial hydrogenases. 01-06-07. ESF Summer School *Cyanobacterial N₂ Fixation: Physiology and Molecular Biology*; Sevilla, Spain.
- Lindblad, P. 2001. Photoproduction of H₂ by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: From laboratory experiments to outdoor culture. 01-09-09. Biohydrogen, Joint workshop IEA Hydrogen Annex 15 & COST 8.41; Szeged, Hungary.
- Maness, P.C. 2002. Characterization of the carbon-monoxide oxidation pathway in the photosynthetic bacterium *Rubrivivax gelatinosus*. Asilomar CA, USA. 11th Western Photosynthesis Conference, Jan 3-6, 2002.
- Maness, P.C. 2002. The production of H₂ from a CO-oxidation reaction in the photosynthetic bacterium *Rubrivivax gelatinosus* CBS. Honorarium lecture, Washington State University, Pullman WA. March 2002.
- Masuda T, Tanaka A, Melis A. 2001. Annual Meeting of the American Society of Plant Biologists. Providence, Rhode Island, July 21-25, 2001.
- Melis A, Polle JEW, Kanakagiri S, Masuda T. 2001. International Photosynthesis Congress, Australia, Aug. 15-18, 2001.

- Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. Single-Organism, Two-Stage Biophotolysis and H₂ Production in Green Algae. 2000 AAAS Annual Meeting and Science Innovation Exposition. Washington, D.C., Pages A27-A28.
- Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. Sustained photosynthetic hydrogen gas production upon reversible inactivation of oxygen evolution in *Chlamydomonas reinhardtii*. Abstracts in *Chlamy 2000*, Ninth International Conference on the Cell and Molecular Biology of Chlamydomonas. 21-26 May 2000, Noordwijkerhout, The Netherlands. Page 31 (Abstract # 20).
- Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. Single-organism two-stage photosynthesis and hydrogen production in green algae. In Abstracts and Program of the Annual Meeting of the American Society of Plant Physiologists (San Diego, July 15-19, 2000), Abstract 796, p. 161.
- Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. Two-stage photosynthesis and hydrogen production in green algae. In Abstracts and Program of the Annual Meeting of the American Society of Plant Physiologists (San Diego, July 15-19, 2000), Abstract 16001, p. 13.
- Melis A. 2002. Green algae hydrogen production: Progress, problems, and prospects. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.
- Melis A. 2002. Green algae hydrogen production: Progress, problems, and prospects. British Royal Society Meeting, London. March 13-15, 2002.
- Polle J, Kanakagiri S, Melis A. 2000. The stability/organization of PSII is affected in a nuclear mutant of *Chlamydomonas reinhardtii*. In Abstracts and Program of the Annual Meeting of the American Society of Plant Physiologists (San Diego, July 15-19, 2000), Abstract 805, p. 163.
- Polle JEW, Kanakagiri S, Melis A. 2000. DNA insertional mutagenesis of *Chlamydomonas reinhardtii* aberrant in photosystem chlorophyll antenna size. Abstracts in *Chlamy 2000*, Ninth International Conference on the Cell and Molecular Biology of Chlamydomonas. 21-26 May 2000, Noordwijkerhout, The Netherlands. Page 32 (Abstract # 22).
- Polle JEW, Kanakagiri S, Jin E-S, Melis A. 2002. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.
- Polle JEW, Niyogi KK, Melis A. 2001. Annual Meeting of the American Society of Plant Biologists. Providence, Rhode Island, July 21-25, 2001.
- Seibert, M. 2002. Photosystem II, the Engine for Algal Hydrogen Production. Seminar presented at ORNL, USA.
- Seibert M. 2002. Regulation of Photosystem II Activity, H₂ Production, and O₂-Consumption Pathways in Sulfur-deprived *Chlamydomonas reinhardtii* cultures. BioHydrogen 2002, April 21-24, Ede, The Netherlands.
- Sellstedt, A., Lundberg, C., Johansson, L. Sandström, G. 2001. Nickel affects localization of hydrogenase in *Frankia* 12th International Meeting on Frankia and Actinorhizal Plants. June 17th-21st, 2001. Carry-le-Rouet, France.
- Tamagnini P., A.M. Burja, P.C. Wright. 2002. The *hupSL* intergenic region in cyanobacteria. 5th European Workshop on the Molecular Biology of Cyanobacteria, Stockholm, Sweden, June 9-12, 2002.
- Tamagnini, P., A.M. Burja, E. Leitão, P.C. Wright. 2001. *hup* and *hox* genes in the filamentous non-heterocystous cyanobacterium, *Lyngbya majuscula* CCAP 1446/4 – Potential for large scale production, COST 841 Workshop, Reading UK, March 2001.
- Tóth A. 2002. Expression and activity of hydrogenases in *Thermococcus litoralis* grown under various conditions. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

van Groenestijn JW. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

van Niel EWJ. 2002. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biohydrogen 2002, 21-24 April, Ede, The Netherlands.

van Niel EWJ. 2002. Towards a process with extreme thermophiles for optimal H₂ production from sugars. Lund University, Department of Applied Microbiology, 23 September 2002, Lund, Sweden.

Wright PC, Bustard MT, Tamagnini P, Burja AM, Mohammed BT. 2001. Integrated Bioremediation and Biofuels Generation: Characterisation of Enzymatic Reactions within Extremophiles, *American Society of Microbiology*. May 20-24th, 2001 Orlando, Florida, USA.