An Approach to Environmentally Acceptable Technology

BIOHYDROGEN II

Edited by

Jun Miyake
Tadashi Matsunaga
Anthony San Pietro

PERGAMON

BIOHYDROGEN II

An Approach to Environmentally Acceptable Technology

Elsevier Science Internet Homepage

http://www.elsevier.nl (Europe)
http://www.elsevier.com (America)
http://www.elsevier.co.jp (Asia)

Consult the Elsevier Science homepage for full catalogue information on all books, journals and electronic products and services.

Related Journals

Free specimen copy gladly sent on request. Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, UK

International Journal of Hydrogen Energy
http://www.elsevier.com/locate/ijhydene

Renewable Energy
http://www.elsevier.com/locate/renene

Renewable and Sustainable Energy Reviews
http://www.elsevier.com/locate/rser

To Contact the Publisher

Elsevier Science welcomes enquiries concerning publishing proposals: books, journal special issues, conference proceedings, etc. All formats and media can be considered. Should you have a publishing proposal you wish to discuss, please contact, without obligation, the publisher responsible for Elsevier's renewable energy programme:

Tony Roche
Publishing Editor, Renewable Energy
Elsevier Science Ltd
The Boulevard, Langford Lane
Kidlington, Oxford
OX5 1GB, UK

Phone: +44 1865 843887
Fax: +44 1865 843920
E.mail: t.roche@elsevier.co.uk

General enquiries, including placing orders, should be directed to Elsevier's Regional Sales Offices - please access the Elsevier Science homepage for full contact details (homepage details at the top of this page).

BIOHYDROGEN II

An Approach to Environmentally Acceptable Technology

Edited by

Jun Miyake,
Tissue Engineering Research Center,
AIST/METI, Tsukuba, Ibaraki, Japan

Tadashi Matsunaga,
Tokyo University of Agriculture and Technology,
Koganei, Tokyo, Japan

Anthony San Pietro,
Indiana University, Bloomington, Indiana, USA

2001
PERGAMON
An Imprint of Elsevier Science

Amsterdam - London - New York - Oxford - Paris - Shannon - Tokyo

ELSEVIER SCIENCE Ltd
The Boulevard, Langford Lane
Kidlington, Oxford OX5 1GB, UK

First edition 2001

Library of Congress Cataloging in Publication Data
A catalog record from the Library of Congress has been applied for.

British Library Cataloguing in Publication Data

Biohydrogen II
1.Hydrogen - Biotechnology
I.Miyake, Jun II.Matsumura, Tadashi III.San Pietro,
Anthony, 1922-
665.8'1

ISBN: 0-08-043947-0

♾ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).
Printed in The Netherlands.

Dedicated to the late Professor David O. Hall

The late Professor David O. Hall
A firm advocate and believer in 'Biohydrogen' as an energy resource in the New Millennium.

In the final quarter of the 20th century, no other individual would have devoted so much energy and enthusiasm as Professor David Hall did towards promoting the benefits in the use of renewable energy resources in general, and hydrogen energy in particular, for a cleaner environment for us all to live in.

David Hall had an outstanding academic career in King's College, which spanned 35 years. He was a world authority in the production and utilisation of plant biomass as an alternative to fossil-based energy sources and built up an internationally active network of 'Biomass Users'. He was a member of the Royal Society Interdisciplinary Committee on Environmental Research, Coordinator of an United Nations Environment Programme (UNEP) on 'Bioproductivity and Photosynthesis', Project Leader for European Commission Research on Photobiology, Biomass Expert for the EEC, member of SCOPE Advisory Committee, Cofounder and Chairman of UK Solar Energy Society, member of IEA Agreement on the Production and Utilisation of Hydrogen and many other Scientific Organisations.

David's interest in Biohydrogen started in the 1970s following the 'Arab Oil Embargo' and the resultant world energy crisis. Solar energy harvest and conversion to storable electrical (eg. photovoltaic) or chemical (eg. H_2) energy was considered as the environmentally acceptable, alternative, although probably an economically unfavorable, process. There was a flurry of research, world-wide, on hydrogenases and on photosynthetic organisms which can effect solar energy bioconversion producing H_2. In the late 1980's a Biohydrogen Programme was initiated by the Hawaii Natural Energy Research Institute with the cooperation of the USA, Japan, the UK (D. Hall) and other nations with the specific objective of developing sustainable biological H_2 Production Systems.

David was a prolific writer of scientific articles and books. His thirst for knowledge was unquenchable. He took two sabbaticals, one in 1978 in Marseilles to work on hydrogenases in sulphate reducers and the second in 1991 in Princeton (Dept. of Energy & Environment). I spent 30 years with David Hall. He was a warm-hearted, friendly person with a charming smile, ever-willing to help his students and colleagues; 20 students took doctorate under his guidance. David Hall will be remembered and missed by those interested in Photosynthesis, Hydrogen Energy, Clean Environment, Global Climate Preservation and other 'green' issues. He was also a generous benefactor readily supporting various individuals and educational charities around the world.

David Oakley Hall, Scientist, born 14 November 1935 in East London, South Africa;
died 22 August 1999 in London, UK.

Krishna Rao.

This Page Intentionally Left Blank

PREFACE

Our continued, and ever increasing, reliance on carbonaceous fossil fuels is the major contributor to the World's environmental problems, such as the "Greenhouse Effect", because of the potential for global climate change from fossil fuel CO_2 emissions. Even a more conservative use of carbonaceous fossil fuels, a strategy employed during the oil embargo of the 1970s, will merely diminish the rate of environmental deterioration but will not contain it at the *status quo*. The need for, and use of, a renewable, sustainable and non-polluting energy source is the only pathway to containment of environmental deterioration.

As has been noted elsewhere and in many publications, hydrogen is an almost ideal fuel and its use would result in an improvement in the environment due to decreased air pollution. It is the element of greatest abundance in the universe; however, its production from renewable resources remains a major challenge.

Hydrogen produced from renewable resources such as water, organic wastes or biomass, either biologically or photobiologically, is termed "**Biohydrogen**". Unfortunately, there are presently no practical **Biohydrogen** processes despite the multi-million dollar expenditures in Japan (by MITI and RITE/NEDO), in the United States (by the US Department of Energy), in Germany (by the German Government) and elsewhere. However, several approaches, such as Indirect Biophotolysis or Dark Fermentations of Organic Wastes, could be developed in the mid- to long-term.

The NAIR-sponsored "Workshop on Biohydrogen 99" was convened in Tsukuba, Japan, in June 1999. The purpose of the workshop was to evaluate the current status of **Biohydrogen** research worldwide and to consider future research directions. The international scope of the workshop is reflected in participation by forty-two attendees from nine countries.

The papers presented herein enhance and expand upon presentations made at the "Workshop on Biohydrogen 99" (http://www.aist.go.jp/NAIR/miyake/news/h2/program.html). Contributions from leading international experts cover the breadth of **Biohydrogen** R&D from production to genetic engineering and molecular biology. It is our hope and expectation that this volume will prove useful both to researchers as well as to interested persons who wish to obtain an overview of **Biohydrogen** R&D.

It is with utmost respect and admiration that we dedicate this book to the memory of Professor David Hall.

Jun Miyake
Tadashi Matsunaga
Anthony San Pietro

January 2001

This Page Intentionally Left Blank

CONTENTS

I. Hydrogen Production

II. Hydrogen World

III. Biochemical and Metabolic Systems

IV. Molecular Biology

V. Genetic Engineering

VI. Photobioreactors

This Page Intentionally Left Blank

I. Hydrogen Production

This Page Intentionally Left Blank

HYDROGEN PRODUCTION BY PHOTOSYNTHETIC BACTERIA: CULTURE MEDIA, YIELDS AND EFFICIENCIES

J. S. Rocha, M. J. Barbosa and R. H. Wijffels

Food and Bioprocess Engineering Group,
Wageningen University the Netherlands

ABSTRACT

Hydrogen is recognized nowadays as the fuel of the future. For its large scale use the production of large quantities of economic hydrogen is essential. Probably, the cheapest hydrogen will not be produced from a renewable source in the near future. However, biological hydrogen production, as an environmental-friendly resource of energy, must be developed in order to be an alternative technology, so that it can be used when other goals become more important than price.

Hydrogen can be produced by photo-fermentation with photosynthetic bacteria, carried out with suitable nutrients, under anaerobic conditions, in the absence of nitrogen gas, with illumination and with stressful concentrations of nitrogen sources. The main enzymes involved are nitrogenase and hydrogenase. Valuable by-products can also be formed. These advantages put together with waste treatment give this system potential at a short term. The design of photobioreactors with efficient light transfer is still lacking. The optimal production of hydrogen by photosynthetic bacteria, to be economically attractive, has to take into account several parameters, such as the productivity, the light efficiency and the yield on carbon source. Some literature data are promising, but not yet enough to forecast the real future of the biological hydrogen production.

The main bottleneck of biological hydrogen production seems not to be the hydrogen yield itself (it is easy to reach 70-80% of the theoretical yield) but light efficiency, because of the cost of large areas to capture sufficient light. The calculated light efficiencies are, in general, lower than desirable. Some values are promising (7-9%) but they were obtained under very peculiar circumstances, namely on a very small scale or with low irradiation intensities. These values suggest that the limitation may not be biological but physical.

KEYWORDS: Hydrogen production, photosynthetic bacteria, photobioreactor, light energy conversion

INTRODUCTION

Hydrogen is recognised nowadays as the fuel of the future. Taking into account the exponential development, from the first to the third world, the related consumption of fossil fuels will stress two kind of problems, the loss of petroleum and coal resources on the one hand and the unacceptable level of pollution for life on the other hand. The main advantage of hydrogen is as a clean and efficient fuel. However, before it can be used at large scale many problems related with storage, transportation and security must be solved. The large scale use of hydrogen will be, in a near future, in fuel-cell powered vehicles. The present-day race between the biggest car-manufacturing companies is not to develop a fuel-cell car but to lower its cost. Low and zero emission vehicles will be protected by law in a few years, in States like California, and this poses a great challenge to car manufacturers (The Economist, 1999 a and b). Assured of hydrogen's future, the question is how to provide the necessary quantity of economic and renewable hydrogen. These two parts of the same question may be in contradiction because the cheapest way to produce hydrogen will not probably be, in the short term, from a renewable source of energy. The current global situation on the inventory of fossil fuel and the extent of environmental pollution is perhaps not yet severe enough to create much demand for the development of hydrogen biotechnology (Nandi and Sengupta, 1998). Even so, alternative technology and know-how must be available in order to be used when prices change or when other goals speak louder than price. That is the case of biological hydrogen production.

The first evidence that phototrophic bacteria could produce hydrogen appeared in 1949 (Gest and Kamen, 1949) but, the use of hydrogen as an alternative fuel and its production from a renewable source was not taken seriously until the first energy crisis of the 1970's. Unfortunately, there was a lessening of the enthusiasm in the following decade, when the price and availability of fossil fuels became more favourable, with some exceptions related to political instability of some oil producing countries. Thus, it was only in the 1990's that the energy potential of hydrogen began to be discussed by statesmen of the most influential countries of the world. However, the main goal was then quite different because environmental protection was more important than stable fuel prices and guarantees for industrial development. That concern induced several problems such as air pollution, global climate changes and the greenhouse effect. Hydrogen, as a clean fuel (it only produces water when burned with oxygen) was seen as a solution for different problems provided it could be obtained from the most available resources in the world (water and sun light) by microorganisms. Microalgae (green alga and cyanobacteria) are unique organisms with the ability to produce hydrogen by water photolysis with light as the energy source (Hall *et al.*, 1995), but with very low conversion efficiencies. Beside these photosynthetic organisms there are also phototrophic bacteria able to produce hydrogen from organic substrates, as well as other anaerobic bacteria which can drive dark fermentative hydrogen production from low-cost substrates and wastes (Zajic *et al.*, 1978). The last are quite interesting and provide the possibility to associate energy production with the reduction of organic pollutants; they deserve our best attention. These perspectives, with a double positive effect, were recently supported by the governments of United States of America (Benemann, 1996 and 1998) and Japan (Mitsugi, 1998) in long term programs. From a strictly economic point of view, it is difficult to expect in the next few decades that hydrogen produced by biological

ways can compete with synthesis hydrogen. However, from a global point of view, where waste treatment and the production of by-products are also taken into account, the scenario can be quite different. Some uses of biological hydrogen, without the need of further purification such as in the enrichment of biogas to be used in internal combustion engines or in feed fuel cells, are very attractive, in a short term strictly energetic point of view.

WHY PHOTOTROPHIC BACTERIA?

Microalgae are photoautotrophic organisms because they can use light as the energy source and the carbon dioxide as carbon source. Some bacteria are termed photoheterotrophic microorganisms because in spite of their ability of using light as the energy source, they need organic carbon as the carbon source. Within this last group there are bacteria which are able to grow anaerobically and it has been observed that almost all anoxygenic phototrophic bacteria can produce hydrogen from reduced substrates such as organic acids (purple non-sulphur bacteria) or from reduced sulphur compounds (green and purple sulphur bacteria) (Warthmann *et al.*, 1993). Most of these bacteria are nitrogen fixing microorganisms and the enzyme nitrogenase catalyses the reduction of molecular nitrogen (N_2) to ammonia (NH_3). However, the simultaneous evolution of hydrogen along with nitrogen reduction seems to be an inherent property of the nitrogenase (Hall *et al.*, 1995). So, under conditions of nitrogen limitation, nitrogenase is induced which, in the absence of nitrogen gas, reduces protons to hydrogen (H_2). This process is light-driven and ATP-dependent (Warthmann *et al.*, 1993). These energy-consuming reactions are schematically represented as:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

The restrictions of nitrogenase activity for H_2 production by phototrophic bacteria are sufficient to explain why reactors must operate anaerobically, in the absence of nitrogen gas, with illumination and with stressfull concentrations of nitrogen sources. Nitrogenase is highly sensitive to oxygen and other oxygen species, such as superoxide and singlet oxygen, which inhibit nitrogenase as well as photosystem components (Hall *et al.*, 1995). Nitrogenase is also inhibited by ammonium ions and nitrogen gas, although some nitrogen sources, that usually inhibit nitrogenase activity, can also prolong hydrogen production when carefully supplied (Miyake *et al.*, 1982). Photofermentations, the conversion of organic substrates by nitrogen fixing photosynthetic bacteria, achieve high H_2 yields but solar conversion efficiencies are low. Reasons for low photosynthetic efficiencies include the high energy demand for nitrogenase catalysed hydrogen evolution in these bacteria, and the relatively low light intensities at which these bacteria operate which prevent the efficient use of high light intensities. The high energy requirement of nitrogenase suggests that biohydrogen processes must be based on another enzyme, namely the "reversible" hydrogenase.

In the hydrogen metabolism of phototrophic bacteria and microalgae, there are two other enzymes involved and they are both hydrogenases. One of them is designated as "reversible" hydrogenase and it is presumed to catalyse H_2 evolution or uptake depending on the redox status of the culture (Hall *et al.*, 1995). The H_2 production activity of this soluble enzyme *in vivo* is questionable because the enzyme itself is apparently constitutive but is not active under normal photosynthetic and aerobic conditions (Benemann *et al.*, 1980). The other hydrogenase is

generally a membrane-bound enzyme and is designated as "uptake" hydrogenase, with its main function being the consumption of hydrogen. Phototrophic microorganisms can produce small amounts of hydrogen in nature when particular conditions are available, such as a microaerobic environment, partial vacuum, argon or CO_2 enriched atmosphere and adequate nutritional status. However, this is always a secondary parasite reaction in cell catabolism. In order to compensate for this waste, cells can oxidise the H_2 produced using the "uptake" hydrogenase. At least with cyanobacteria, it was suggested a link exists between hydrogenase and nitrogenase; nitrogenase activity is induced and, consequently, the capacity for N_2 fixation is increased in cultures growing in the presence of H_2 gas. This evidence can also be important in the use of cyanobacteria as agricultural fertilisers (Tel-Or *et al.*, 1977). The net hydrogen production by phototrophic microorganisms is the overall balance of the activity of the three enzymes. Thus, the research approach is to use physiological, biochemical and molecular biological techniques for modifying the H_2 metabolism activities towards an enhanced photoevolution of hydrogen (Rao and Hall, 1996).

The main enzyme in hydrogen production is nitrogenase. In fact, studies with *Rhodopseudomonas palustris* have shown that the rate of hydrogen production was almost directly proportional to nitrogenase activity but no relationship was seen between hydrogen production and hydrogenase activity. Further, mutant strains which were deficient in nitrogenase activity were incapable of forming hydrogen (Kim *et al.*, 1980).

Hydrogenases from prokaryotic microorganisms are well-known and presently more than 30 microbial hydrogenases have been characterised. Most of bacterial hydrogenases have nickel as co-factor and the knowledge of a few crystal structures is very recent. Hydrogenases can also be found in eukaryotic organisms, such as algae and cyanobacteria, but, in this case, the information is not very abundant. Under anaerobic conditions, algae are able to catalyse either the light dependent uptake of hydrogen (photoreduction of CO_2) or the evolution of molecular hydrogen upon illumination (photoproduction of hydrogen). Since nitrogenase is missing in green algae, hydrogen must be produced by a hydrogenase *i.e.*, under photosynthetic conditions where oxygen is produced (Schulz, 1996). However, hydrogenases of green algae are extremely sensitive to molecular oxygen and are completely inactive under aerobic conditions; this is the reason why phototrophic bacteria offer a greater potential for biological hydrogen production.

BY-PRODUCTS

As stated above, one way to overcome the economic restrictions of biological hydrogen production by photosynthetic bacteria is to associate this process with waste water treatment. Another way is the simultaneous production of by-products which increase the added value of the overall process. One of these by-products is, obviously, the biomass itself. Cells from photosynthetic bacteria are not only rich in high quality protein (with a great potential as a supplementary protein source) but also contain significantly large amounts of carotenoid pigments, biological co-factors and vitamins (of the B group) (Kobayashi and Kurata, 1978; Vrati, 1984). Protein of *Rhodopseudomonas capsulata* was shown to be rich in essential and sulphur amino acids with a high lysine content (5.4%) (Vrati and Verma, 1983). Proteins from microorganisms, popularly known as single cell protein (SCP), are usually poor in methionine content, but this is not the case of SCP obtained from photosynthetic bacteria, whose methionine contents are superior to soybean and meat proteins (cited by Vrati, 1984). SCP from photosynthetic bacteria have lower amounts of lysine compared to the proteins of animal origin,

but comparable amounts of lysine to FAO reference protein; this means they can be used as a complementary diet of animals because cereals are poor in their lysine content (Vrati, 1984). The use of photosynthetic bacteria can be of significant importance in pisciculture, in the poultry industry and in horticulture. The last application is related to the ability of photosynthetic bacteria for nitrogen fixation and carbon dioxide assimilation (which also make them hopeful protein producer). Kobayashi and Kurata (1978) have reported that the addition of 0.01% fresh photosynthetic bacterial cells (*Rhodopseudomonas capsulata*) to hen feed increased the egg laying rate by 10% and improved the quality of the egg yolk. Other advantages of photosynthetic bacteria are: (i) they are not pathogenic or toxic (this is not the case with cyanobacteria); (ii) the contamination by other microorganisms is easy to be controlled because they can grow in the absence of oxygen; and (iii) they can use raw materials free of cost, which can be utilised to purify some industrial liquid wastes. Although the superior quality of amino acid composition of SCP from photosynthetic bacteria enhances its promise as feed and fertiliser, cell productivity of photosynthetic bacteria is far lower than that of aerobic heterotrophic microorganisms and its usefulness has not been very encouraging. Studies on the growth parameters in pure or mixed cultures, the use of other agricultural or industrial wastes and the development of different cultivation systems, are quite necessary (Kobayashi and Kurata, 1978; Vrati, 1984).

Bioconversion of organic wastes by photosynthetic bacteria can also produce other valuable chemicals, such as 5-aminolevulinic acid (ALA). ALA can be applied to agricultural fields as a herbicide or a growth-promoting factor and enhancer of salt tolerance for plants, as well as in the medical field as a cancer treatment and as a medication. ALA was produced by *Rhodobacter sphaeroides* (ALA formation is light dependent) from a volatile fatty acids medium prepared from the effluent of the anaerobic digestion of swine waste. This strain is able to produce up to 4-9 mM ALA extracellularly, under anaerobic light conditions, in addition to hydrogen production. The addition of levulinic acid (LA), as an inhibitor of ALA dehydratase, and glycine or succinate as precursors of ALA biosynthesis, were also studied (Sasaki *et al.*, 1990; Sasaki, 1998).

Another useful by-product of hydrogen production using photosynthetic bacteria is polyhydroxybutyrate (PHB), which is a biodegradable thermoplastic that is synthesised during unfavourable growth conditions, particularly under stress conditions during the stationary phase of growth, as a storage material. PHB accumulation inside the cells of photosynthetic bacteria, when grown under anaerobic conditions, depends on carbon substrate and nitrogen availability, as well as the pH of the medium. The highest levels of PHB produced by *Rhodobacter sphaeroides* were obtained with acetate, recognised as the substrate best suited for PHB synthesis (Krahn *et al.*, 1996) under both ammonium or nitrogen deprived conditions. A better understanding of PHB synthesis regulation would allow for control of the cultivation process to increase the production of the desired product. PHB has important industrial applications, particularly to construct biodegradable carriers for long term dosages, either in the agriculture field for herbicides and insecticides, or in the medical field for drugs (and also for surgical sutures) (Khatipov *et al.*, 1998; Yigit *et al.*, *in press*).

NATURAL CULTURE MEDIA

A practical application of photosynthetic bacteria for hydrogen production can not utilize expensive synthetic culture media like that used in laboratory experiments. Instead cheap organic substrates must be used, such as residual wastes from the food industry or waste waters with high

level of organic compounds and thereby provide the advantages of both energy production and waste treatment. The major components of waste waters are carbohydrates and proteins but it is well known that photosynthetic bacteria can more effectively use low molecular organic compounds as carbon sources. This problem may be overcome by using two steps: the first one is a dark fermentative hydrogen production driven by anaerobic bacteria – the effluent from which is used in a second step, photo-fermentative hydrogen production driven by photosynthetic bacteria. In both processes the higher concentration of organic matter may cause the volume of hydrogen produced per unit time to be higher, but the yield of organic substrates converted to hydrogen is not so high. The decision must take into account the available concentration of organic residues (it is easy to dilute but not to concentrate) and what is the main goal of the global process: to maximise the hydrogen production or to minimise the organic content of waste waters. The efficiency depends on many factors which include bacterial species, inhibitors, carbon sources and environmental conditions of culture such as pH, temperature, illumination, stirring and reactor type. Two of the most important inhibitors to hydrogen production are oxygen and ammonium ion. They are always present in waste waters: (i) oxygen is freely dissolved from air (although oxygen solubility in water is low, it is enough to support aerobic growth and inhibition of some enzymes); (ii) ammonium ion can be easily obtained from organic nitrogenous substances such as protein and amino acids. Hydrogen evolution by photosynthetic bacteria mainly depends on nitrogenase which is very sensitive to ammonia and oxygen. Hydrogen evolution by anaerobic bacteria mainly depends on hydrogenase that is not so sensitive to ammonia but also very sensitive to oxygen. The oxygen problem in waste waters, where mixed cultures can grow, is not very important because in highly concentrated organic environment, the facultative heterotrophic bacteria can grow very rapidly and consume the dissolved oxygen available. Ammonia can also be consumed by bacteria but it also can be easily released from organic nitrogenous matter. So, the need for an ammonia resistant system is very important, what can probably be done by using mixed cultures. Another important way to obtain a stable and longer term hydrogen production is by removing the hydrogen produced to avoid its consumption by the bacteria themselves (Zhu *et al.*, 1995). The gas produced can be collected and measured in an inverted graduated cylinder filled with saturated NaCl solution, followed by passing through 10% NaOH solution to neutralise CO_2 (Taguchi *et al.*, 1992).

Some non-photosynthetic bacteria can produce hydrogen from different organic substrates, such as *Enterobacter aerogenes* from glucose (Perego *et al.*, 1998) or a *Clostridium beijerinckii* strain that produces hydrogen from glucose and starch (Taguchi *et al.*, 1992). In biological waste water treatment systems, with production of biogas, the main reaction steps (acidogenesis and methanogenis phases) must be separated because of the different growth conditions of the bacteria in each phase. Acidogenic bacteria can be used to produce volatile acids and hydrogen gas, from glucose concentrations below the level at which inhibitory effects occur (Majizat *et al.*, 1997). Another non-photosynthetic bacteria, *Clostridium sp.* (strain n° 2), was found to convert cellulosic materials to hydrogen. These materials were hydrolysates of polyglucose (Avicel) and polyxylose (xylan), obtained with crude cellulase and xylanase preparations, respectively. It was observed that hydrogen production was as efficient as that from glucose and xylose. The attempt to do the direct conversion of cellulosic material to hydrogen, using commercial enzymes and the bacteria in one step, has shown that simultaneous conversion was less efficient. In the future this problem will be overcome by the use of novel hydrogen producing strains with high activities of cellulases and hemicellulases (Taguchi *et al.*, 1995).

Non-photosynthetic bacteria like *Clostridium butyricum* evolve hydrogen from carbohydrates at a high rate, but the yield is limited because they also produce organic acids. On the other hand,

photosynthetic bacteria produce hydrogen from organic acids (but very little from glucose), with high yields, under illumination and in the absence of oxygen and nitrogen gases. A combination of two species makes possible the conversion of carbohydrates to hydrogen. Usually this is done in two separate steps, but hydrogen production from glucose by a co-culture of *Clostridium butyricum* and *Rhodopseudomonas sp.* RV was already reported (Miyake *et al.*, 1984). The major reactions of hydrogen production from glucose in *Clostridium butyricum*, catalysed by hydrogenase without any energy supply, are the following:

$$\text{glucose} \rightarrow 2H_2 + 2CO_2 + \text{butyrate}$$
$$\text{glucose} + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2\ \text{acetate}$$

The conversion of butyrate or acetate to hydrogen are energetically unfavourable reactions:

$$C_4H_8O_{2liq} + 6H_2O_{liq} \rightarrow 4CO_{2gas} + 10H_{2gas}, \quad \Delta G° = 223.3\ kJ$$
$$C_2H_4O_{2liq} + 2H_2O_{liq} \rightarrow 2CO_{2gas} + 4H_{2gas}, \quad \Delta G° = 75.2\ kJ$$

These last reactions can be catalysed by nitrogenase, but the high energy cost of nitrogenase limits the rate and the yield of hydrogen production. In photosynthetic bacteria the reactions catalysed by nitrogenase are driven by light energy and can be performed (Miyake *et al.*, 1984).

Different organic substrates from agro- and food-industries have been anaerobically fermented, with the separation of the effluent slurry into two fractions: the solid residue and the liquid supernatant. The solids can be obtained in the form of a dry cake, rich in good quality protein, and be used as animal feed; the supernatant, rich in organic acids, can be refermented by photosynthetic bacteria to produce hydrogen. One of the cheapest and abundant residues is cow dung (Vrati and Verma, 1983). Another kind of abundant residue, able to be submitted to acidogenesis, is selected municipal solid wastes. When a large concentration of easily biodegradable organic matter is present (like in market wastes), with a favourable carbon/nitrogen ratio (C/N > 28), acidogenesis can proceed according to the homolactic fermentation. (Fascetti *et al.*, 1998).

There are cheap waste substrates able to support directly the growth of photosynthetic bacteria and consequently photoproduction of hydrogen, such as: (i) the waste water from a sugar refinery (Bolliger *et al.*, 1985), (ii) the waste water of a distillery (where cane molasses was used as raw material for alcohol fermentation by yeast) (Sasikala *et al.*, 1992), or (iii) waste water from a milk industry (in this case used to replace the carbon source in a standard growth medium, with advantage on hydrogen production; however, the nutrient in waste from the milk factory was not sufficient alone to support the growth of *Rhodobacter sphaeroides*, probably by lacking some essential minerals) (Turkarslan *et al.*, 1998).

Some species of the facultative phototrophic purple non-sulphur bacteria, namely *Rhodopseudomonas palustris*, were recognised to have the capability to grow and produce hydrogen even from unusual organic compounds, that can be used as photosynthetic electron donors and sources of carbon. This is the case for *Rhodopseudomonas sp.*No. 7 (similar to *Rhodopseudomonas palustris*) which is able to utilise as substrates short straight chain primary alcohols, like ethanol, propanol and butanol, under anaerobic-light conditions. Although the relationship between the metabolism of organic compounds and the hydrogen production is still under investigation, several constitutive enzymes for the metabolism of alcohols were already

identified in that strain (Fujii *et al.*, 1983). Another class of strange substrates, available as waste products from many industrial processes, is the aromatic compounds. Different aromatic acids, such as benzoate, p-hydroxybenzoate, cinnamate and D- and L-mandelate , were metabolized by several strains of *Rhodopseudomonas palustris* with hydrogen production, although the hydrogen yields were significantly lower when compared with lactate, acetate or malate as carbon and electron sources. However, the possibility to convert aromatic acids to hydrogen is very promising and further developments should lead to higher yields (Fiβler *et al.*, 1994). The best nitrogen source for hydrogen production was again L-glutamate. When the L-amino acids were used as single carbon and nitrogen sources, some of them led to phototrophic growth of the bacteria (such as alanine, glutamine, leucine, tyrosine or valine), but hydrogen production was never observed under these conditions (Fiβler *et al.*, 1994).

The production of biological hydrogen from expensive organic substrates is not economically viable. On the other hand, the use of cheap organic wastes also have some technical problems associated with collection, storage and transport, because most of the times they are very disperse and not in sufficient quantity to be used locally. A different and very interesting strategy is to produce algal biomass for obtaining organic matter that, after treatment, can be used by the photosynthetic bacteria. This system includes three steps but has the advantage of being a closed process, not dependent of the availability of any component. The first step is the photosynthetic starch accumulation by cultivation of microalgae, which is an aerobic and light driven process with simultaneous CO_2 fixation and O_2 evolution. Green alga can transform water, CO_2 from the atmosphere and solar energy into biomass by photosynthesis. Several strains of these autotrophic organisms can be used to produce microalgal biomass, such as the fresh water green algae *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa*, or the marine green alga *Dunaliella tertiolecta*. The second step is the dark anaerobic fermentation of the algal starch biomass to produce hydrogen and organic compounds. The third step is the conversion of those organic compounds (mainly low molecular weight fatty acids) to produce hydrogen using photosynthetic bacteria. This process is light driven, anaerobic and occurs in the absence of nitrogen gas (Akano *et al.*, 1996; Ike *et al.*, 1997a). The requirement of the second step is as a pretreatment necessary to transform raw algal biomass into suitable substrates for photosynthetic bacteria. This goal can be achieved chemically by heat and/or acid treatment. However, this approach did not lead to high efficiency in hydrogen production because (a) it includes several chemical and physical processes, (b) it is energy consuming, (c) it produces relatively large molecular compounds not usable by photosynthetic bacteria (low yield of fatty acids from the biomass), (d) of the presence of inhibitors and (e) the pH drops in the medium (Ike *et al.*, 1996). A fermentative process of the algal biomass, such as the one that produces lactic acid, which is a preferred substrate for hydrogen production by photosynthetic bacteria, is more advantageous. In lactic acid fermentation from starch, the thermal or enzymic saccharification step is usually indispensable. However, by using one of the starch-hydrolysing lactic acid bacteria, such as *Lactobacillus amylovorus*, that step becomes unnecessary. The production of lactic acid lowers the pH and to avoid the cessation of fermentation a neutraliser must be added; as there are some evidences that the growth of *Rhodobacter sphaeroides* RV is inhibited by calcium, the pH control must be done by using NaOH instead of $CaCO_3$. Other advantages of algal biomass fermentation by *Lactobacillus amylovorus* include: (i) no need to add any nutrients; (ii) no treatment of the fermentate to reduce inhibitory effects on H_2 production, and (iii) no need for thermochemical cell breakage with *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* (Ike *et al.*, 1997a). The last observation suggests that *Lactobacillus amylovorus* may play a role in the degradation of

those algal cells as well as in the digestion of starch. *Dunaliella* is a unicellular green alga without a cell wall and is easily broken; *Chlamydomonas reinhardtii* has a cell wall with different kinds of proteins but without cellulose; *Chlorella* strains are very rigid microalgae and generally indigestible. In the combined biological approach to H_2 production noted above, 4.6 mol of H_2 was obtained per mole of algal starch glucose (starch glucose means that starch was detected as glucose) (Ike *et al.*, 1997a). However, with the halotolerant phototrophic bacteria *Rhodobium marinum*, the yield increased to 7.9 mol H_2 / mol of starch glucose, which is 66% of the theoretical value (Ike *et al.*, 1997b). The main drawback of this strategy for biological hydrogen production, using three steps, is the requirement for large areas to capture sufficient solar energy (Ike *et al.*, 1996).

CULTIVATION REGIME

Hydrogen production by photosynthetic bacteria has been performed mainly in batch cultures and on a small scale. Some references to continuous hydrogen production also involve batch cultures with a periodic supply of fresh medium (replaced by the same volume of culture removed) (Zurrer and Bachofen, 1979) and, sometimes, also with the addition of cells grown overnight in the same medium in order to investigate the effect of a new innoculum (Eroglu *et al.*, 1998).

Truly continuous cultures of photosynthetic bacteria have been grown, most of the times in a single stage chemostat reactor, with a growth limiting nutrient and a constant dilution rate (Zurrer and Bachofen, 1979 and 1982; Kim *et al.*, 1987b). The actual production of H_2 by phototrophic bacteria, such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, in continuous cultures in laboratory scale photobioreactors, is not as high as the theoretically expected value, calculated from hydrogen evolution rates in batch incubation vials. Even when the continuous culture is not limited by light or carbon source, there must be another limitation or inhibiting factor to explain the difference between actual and potential rates of hydrogen photoproduction. A decrease in the H_2 production rate could be due to the presence of accumulated H_2 which somehow results in a kind of product inhibition. Hydrogen production increases with argon flow passing through the reactor but, as a disadvantage, hydrogen and argon must be separated, with an energy expenditure (Tsygankov *et al.*, 1998). Besides this decrease in volumetric rate of H_2 production, compared to the expected value, it is also possible to forecast a further decrease during scaling-up of photobioreactors because of difficulties in providing saturating light intensities. The same problem of light distribution inside the reactor, especially in long light paths and high cell concentrations, explains the discrepancy between the theory and experiments of a chemostat culture. Most of the times, instead of one limiting substrate there is a double limitation (Tsygankov *et al.*, 1998). Double limitation may occur in chemostat cultures, when two nutrients are used for cell culture and within a certain range of ratios between these two nutrients, such as carbon and nitrogen. In chemostat cultures of phototrophic microorganisms light acts as a substrate and a similar state often occurs because light intensity in the culture is not homogeneous and it is decreased by the absorption of cells. The effect of light on the growth and nitrogenase activity of phototrophic bacteria has been studied but the results are controversial. Cell conditions are affected not only by light intensity but also by the light path, and a double limitation by light and other nutrient in different spots of the reactor will be much more frequent than would be desirable (Tsygankov, 1996). Chemostat cultures of phototrophic bacteria enables us to predetermine their growth rate, but not to control the cell density, especially in the case of

light limitation. Turbidostat cultures allow us to investigate the influence of a factor not associated with the medium flow on the growth rate at predetermined biomass concentration, which is more convenient in some cases (Tsygankov and Laurinavichene, 1996). The influence of the degree and mode of light limitation on growth characteristics of turbidostat cultures of *Rhodobacter capsulatus* was investigated with light limitation achieved in two different ways: (a) increasing the steady state biomass concentration at constant incident light intensity or by (b) decreasing the incident light intensity at constant steady state biomass concentration. It was observed that several physiological characteristics of *Rhodobacter capsulatus* depended on both the degree and mode of light limitation of the cultures (Tsygankov and Laurinavichene, 1996).

Photosynthetic bacteria have been also cultivated in different versions of continuous flow stirred tank photobioreactors. *Rhodobacter sphaeroides* RV was studied for hydrogen production in a two-stage chemostat, by which bacterial growth, coupled with nitrogen removal, was accomplished in the first reactor, while in the second reactor only hydrogen production took place. Under light saturation the two stage chemostat increased the hydrogen evolution rate, mainly due to the consumption of the nitrogen source (ammonium) in the first reactor, and without carbon excess to avoid the accumulation of storage materials (Fascetti and Todini, 1995). The accumulation of storage materials, like poly(3-hydroxybutyrate), results in a decrease in the proportion of substrate converted into hydrogen gas, and must be avoided if we want to maximise hydrogen production. In photosynthetic bacteria, after transition to light-anaerobic and nitrogen-deficient conditions, hydrogen evolution starts with expression of nitrogenase activity, but a lag time is always exhibited by cells with consequent waste of substrate.

A cultivation method consisting of three stages (cell growth, nitrogenase derepression and hydrogen production) can improve substrate conversion to molecular hydrogen (Maeda *et al.*, 1998). The cultivation of photosynthetic bacteria for hydrogen production can be performed in two basic methods which include the simultaneous or independent cell growth and hydrogen production periods. The simultaneous method is simpler (although some glutamate must be added to the cultivation medium as a nitrogen source) but the independent method is more commonly employed. In the period of cell growth, in light microaerobic environment, nitrogenase activity is repressed by ammonium ion (if used as nitrogen source) and dissolved oxygen in the medium. The purpose of the three-stage cultivation, with an intermediate nitrogen derepression stage after cell growth, is to reduce the lag time and to increase the conversion efficiency of hydrogen production. It is suggested that the rate-limiting stage in hydrogen evolution from fermentative products is the derepression of nitrogenase activity, which can be done by preincubation with 1 mM succinate (Maeda *et al.*, 1998).

References to production of molecular hydrogen in outdoor cultures, even with a well defined culture medium, are very few, both in batch (Kim *et al.*, 1982) and semi-continuous operation (Kim *et al.*, 1987a). Hydrogen production by outdoor cultures depends both on illumination and temperature of culture medium. Although illumination is the limiting factor (which means that position and inclination of the reactor vessel must be properly chosen), the temperature of the culture medium is also very important. It happens that within 2 to 3 h after sunrise, the sunlight in the morning can not be effectively utilised for hydrogen production because the culture medium is not warm enough. On the other hand, the colder temperature during night might reduce the maintenance energy of the cells, which would result in a higher conversion efficiency (Kim *et al.*, 1982a). In continuous outdoor cultures, the rate of feeding of fresh medium is a problem, because the rate of consumption of the electron donor, and therefore the rate of hydrogen production, varies depending on the strength of illumination and temperature of the cultivation (Kim *et al.*, 1987b).

Continuous hydrogen production was also performed by anaerobic bacteria in the fermentation of glucose (or related compounds), with simultaneous production of fatty acids, which can be used in further photofermentation. One of the strains used, *Clostridium butyricum*, was isolated from soybean-meal obtained from a silo which exploded owing to the accumulation of biologically produced hydrogen (Kataoka *et al.*, 1997). Another example of continuous hydrogen production by *Clostridium sp.* (strain no 2) makes use of a combined system of two reactors: the first one for the continuous enzymatic hydrolysis of Avicel in an aqueous two-phase system (consisting of 10% polyethylene glycol and 5% dextran), and the second one for hydrogen fermentation from the cellulose hydrolysates. One advantage of this combined procedure is the absence of inhibition of the crude cellulase preparation activity by end-products of Avicel hydrolysis, enabling a high saccharification rate of cellulose to be achieved. The critical steps in this approach are the price of commercial enzymes and polymers, such as dextran, the improvement of an effective hydrolysis reactor and the screening of new bacterial strains with highly active cellulases (Taguchi *et al.*, 1996). Although most reports on hydrogen production by anaerobic bacteria have been carried out using pure culture strains, there are also references to the use of anaerobic microflora from sludge compost to produce hydrogen from cellulose (Ueno *et al.*, 1995) or from sugar factory waste water (Ueno *et al.*, 1996).

Hydrogen production by photosynthetic bacteria uses light as the main driving force, which means that the design of efficient photobioreactors is essential. The main design criterion has been to maximise the illumination surface to volume ratio, and the result is a very small culture thickness that is economically not feasible (El-Shishtawy *et al.*, 1997). For pratical purposes the result should not be a very thin culture. When it is possible to guarantee that light is present in the entire reactor, the volumetric productivity can be maximised. However, the efficiency will only be maximised when light is completely absorbed. Various photobioreactors have been proposed for hydrogen production, illuminated either from outside (Sasikala *et al.*, 1992) or from inside the photobioreactor by a bulb at the centre (Stevens *et al.*, 1984; Fascetti and Todini, 1995; Tsygankov *et al.*, 1996). A new type of photobioreactor was presented and includes a tubular immobilised cell layer around an inner cylindrical optical-fibre illumination device, which ensures a homogeneous illumination and a large active surface. The use of optical fibres to guide light from an external source to the reactor medium is a suitable means for suppressing inhibiting thermal effects at the photosynthetic biocatalyst level (Mignot *et al.*, 1989). The main drawbacks are the price of optical fibres and the diffusion resistance of the immobilised cell agar layer, especially when the cell content of the gel is high (Mignot *et al.*, 1989; El-Shishtawy *et al.*, 1997). Another new and efficient reactor for photobiological hydrogen production is the light induced and diffused photobioreactor (El-Shishtawy *et al.*, 1997 and 1998b). The idea of light diffusion is based on the observation that the smaller the light intensity the higher the efficiency of light energy conversion (Miyake and Kawamura, 1987). The rate of hydrogen production by photosynthetic bacteria is proportional to the rate of light energy absorption by the cells. Under optimum light energy distribution, the thinner the culture the higher the possibility of high absorption of light energy by the cells. From an engineering point of view, the productivity per light receiving area is an important parameter (El-Shishtawy *et al.*, 1997). These reactors are not exclusive for hydrogen photoproduction and can also be used in other photoreactions of economic interest. A floating-type photobioreactor was proposed for use on the sea or lake, with the advantage of no energy required for cooling the reactor (which can be necessary on hot days) and the liquid in the reactor can easily be mixed by wave motion (Otsuki *et al.*, 1998). Other different types of photobioreactors incorporating photosynthetic bacteria are noted by Markov(1998) and described by Pulz and Scheibenbogen (1998). An engineering parameter for

design and scale-up of photobioreactors was presented by Ogbonna *et al* (1998) as a key factor for practical production of biohydrogen.

YIELDS AND EFFICIENCIES

The performance of biological hydrogen production by photosynthetic bacteria must be evaluated by several parameters. One of them is the yield coefficient of hydrogen produced relative to the carbon source consumed. Another important parameter is the efficiency of light utilization; taking into account the hydrogen production rate and the hydrogen energy content, as well as the energy of the absorbed light. Although sun light is cheap (in outdoors experiments), the cost of large areas to capture sufficient light can be very high. Neither yield coefficients nor light efficiencies are very abundant in the literature. A search of the recent literature related to laboratory experiments enabled us to select enough information to calculate the values of those parameters. The calculated values of yields and efficiencies were compared to the ones presented by the authors, when possible (*i.e.* when available), which also allowed us to validate our calculations. Yields and efficiencies, both presented by the authors and calculated by ourselves, as well as all the information collected and used for calculations, are presented in Table 1 (parts A and B). Annotations to Tables 1A and 1B makes the description of calculations easier to understand.

TABLE 1A

STRAINS AND CULTURE CONDITIONS

Order number (1)	Strain (2)	Reference (3)	C-source (4)	N-source (5)	Reactor type (6)	$V_{culture}$ (ml) (7)	Cell density ($mg_{dry\ wt}$/ml) (8)	I_{energy} (9)	I_{area} (cm^2) (10)	Lamp (kind / power) (11)
1	*Rhodobacter sphaeroides* RV	El-Shishtawy *et al.* (1998a)	sodium D,L-lactate (72 mM)	sodium glutamate (10 mM)	Batch (roux flasks)	84	0.18-1.04 (660 nm)	155 W/m^2	84	Halogen
2	*Rhodobacter sphaeroides* RV (mutant P3, UV irradiation)	Miyake *et al.* (1998)	sodium lactate (50 mM)	sodium glutamate (10 mM)	Batch (stainless steel)	2.5		65 W/m^2 (850 nm)	1.77	halogen
3 (a)	*Rhodobacter sphaeroides* S	Sasaki (1998)	L-malic acid (4 g/l)	sodium glutamate (0.17 g/l)	Batch (roux bottle) (1.5 l)	1000		10 klux (35 W/m^2)	200 (e=5 cm)	tungsten
(b)			L-malic acid (3.8 g/l)	$(NH_4)_2HPO_4$ (0.13 g/l)						
(c)			acetic acid (3 g/l)	sodium glutamate (0.17 g/l)						
(d)			acetic acid (3 g/l) + propionic acid (3 g/l)	sodium glutamate (0.17 g/l)						
4 (a)	*Rhodobacter sphaeroides* O.U. 001 (DSM 5648)	Eroglu *et al.* (1998)	L-malic acid (7.5 mM)	sodium glutamate (10 mM)	Continuous (intermitant – 100 h) Glass cylinder cyclic 12 h long light/dark periods	400		200 W/m^2	? If l=30 cm d=4 cm A=120 cm^2	tungsten
(b)			"	"	Batch		8.5			
(c)			L-malic acid (30 mM)	Sodium glutamate (2 mM)	"		5.4			

TABLE 1A

STRAINS AND CULTURE CONDITIONS (CONT.)

Order number (1)	Strain (2)	Reference (3)	C-source (4)	N-source (5)	Reactor type (6)	$V_{culture}$ (ml) (7)	Cell density ($mg_{dry\ wt}$/ml) (8)	I_{energy} (9)	I_{area} (cm^2) (10)	Lamp (kind / power) (11)
5	*Rhodobacter sphaeroides* O.U. 001	Turkarslan *et al.* (1998)	malic acid (1 g/l) dil 1/10 with milk waste	sodium glutamate (1.8 g/l) dil 1/10 with milk waste	Batch cylindrical glass column l=31 cm	150	3.03	200 W/m^2	d=2.5 cm A=77.5 cm^2	tungsten 150 W
6	*Rhodobacter sphaeroides* RV	Khatipov *et al.* (1998)	lactate (40 mM)	none	Batch (polystyrene flat culture flasks)	300	1.8	5000 lux (45 W/m^2)	e=3 cm A=100 cm^2 (preculture) culture tubes A=?	tubular tungsten lamps 2x100 W (light from 2 sides)
7 (a)	*Rhodopseudomonas* sp.	Kim *et al.* (1981)	DL-malate (30 mM)	sodium glutamate (5 mM)	Batch (flat bottle)	-		10 000 lux	141 (12.8 x 11)	Incandes-cent bulbs
(b)			DL-lactate (30 mM)	"		700				
8	*Rhodospirillum rubrum*	Zurrer and Bachofen (1979)	lactate (50 mM)	glutamate (15 mM)	Continuous ($D=0.0135h^{-1}$)	1000	3 – 3.5	300 W/m^2	250	tungsten 2 x 100 W
9	M0006 *Rhodospirillaceae* (?)	Matsumoto *et al.* (1998)	D,L-lactic acid (1.34 g/l)	L-glutamic acid (1.25 g/l)	Batch (flat flasks)	70	2.5 – 3.0	10 000 lux	If e=1 cm A=72 cm^2	halogen 300 W
10 (a)	*Rhodovulum sp.* NKPB160471 R (marine)	Yamada *et al.* (1998)	DL-malate		Batch	12 (3x2x2)	3.1	1800 W/m^2	6	xenon
(b)								13 W/m^2		
(c)	H-1 uptake H_2-ase mutant							1800 W/m^2		
(d)								13 W/m^2		

TABLE 1A
STRAINS AND CULTURE CONDITIONS (CONT.)

Order number (1)	Strain (2)	Reference (3)	C-source (4)	N-source (5)	Reactor type (6)	$V_{culture}$ (ml) (7)	Cell density ($mg_{dry\ wt}$/ml) (8)	I_{energy} (9)	I_{area} (cm^2) (10)	Lamp (kind / power) (11)
11	*Rhodobacter sphaeroides* RV	Nakada *et al.* (1995 and 1998)	sodium D,L-lactate (75 mM)	sodium glutamate (10 mM)	Batch (4 compartments)	18.5 (37x0.5) [it must be 32.5 ml]	13	720 W/m^2 (1^{st}) 223W/m^2 (2^{nd}) 72 W/m^2 (3^{rd}) 22 W/m^2 (4^{th})	37.5 (e=0.5 cm)	tungsten 150 W
12	*Rhodopseudomonas palustris* R-1	Otsuki *et al.* (1998)	acetate (800 mg/l) propionate (800 mg/l) butyrate (800 mg/l) ethanol (400 mg/l)		Continuous (triangle roof on moving bed)	6 000		434 W/m^2	1070	24 halogen lamps (top)
13 (a) (b)	*Rhodobacter sphaeroides* RV	Wakayama *et al.* (1998)	sodium lactate (50 mM)	sodium glutamate (10 mM)		700		Max 1 kW/m^2 Integral: 6 – 7 kWh/m^2 Max 1 kW/m^2 Integral: 7 kWh/m^2	150 (e=4.5 cm)	sun light M-26 halogen bulbs(six discrete steps up and down)
14	*Rhodobacter sphaeroides* RV	Ike *et al.* (1997a)	lactic acid (2.7 g/l) (30 mM)	sodium glutamate (10 mM)	Batch (glass tube) (d=2.5 cm)	60		330 W/m^2	If l=12 cm d=2.5 cm A=30 cm^2	tungsten

TABLE 1A
STRAINS AND CULTURE CONDITIONS (CONT.)

15	*Rhodobacter capsulatus* ST410	Ooshima *et al.* (1998)	DL-malate (30 mM)	L-glutamate (7 mM)	Batch tubes	5		6 600 lux (66 W/m^2)	If A=5 cm^2	tungsten
16	*Rhodobacter sphaeroides* RV	Fascetti and Todini (1995)	lactic acid (100 mM)	NH_4Cl (1.7 mM)	Continuous (2-stages chemostat)		0.5	3 000 lux		2 tungsten lamps 100 W inside each reactor
17	*Rhodospirillum rubrum*	Miyake *et al.* (1982)	L-malate (50 mM)	$(NH_4)_2SO_4$ (10 mM) (just for growth)	Batch (bottle)	180		10 000 lux	69.2 (e=2.6 cm)	tungsten 100 W
18	*Rhodospirillum rubrum*	Zurrer and Bachofen (1982)	L-lactate (50 mM)	L-glutamate (15 mM)	Continuous (turbidostat)	1000		400 W/m^2	330 (22x15)	tungsten (3 lamps)
19	*Rhodobacter sphaeroides*	Miyake and Kawamura (1987)	D,L-lactic acid (75 mM)	L-glutamate (10 mM)	Batch (flasks)			50 W/m^2	24 (e=0.5 cm)	xenon 300 W
20	*Rhodobacter sphaeroides* O.U. 001	Sasikala *et al.* (1991)	malate (30 mM)	sodium glutamate (10 mM)	Batch (Warburg flasks)	3		4 000 lux	?	fluorescent lamps
21	*Rhodobacter marinus*	Yamada *et al.* (1996)	sodium malate (6 g/l)		Batch (50 ml glass tubes)		4.5	135 μE/m^2.s	?	halogen lamp and light diffusing optical fibers (LDOFs)
22	*Rhodobacter sphaeroides* RV	El-Shishtawy *et al.* (1997 and 1998b)	sodium D,L-lactate (72 mM)	Sodium glutamate (10 mM)	Batch Induced and diffused photobiorea-ctors (IDPBR)	300		300 W/m^2	20	halogen
23	*Rhodobacter capsulatus*	Tsygankov *et al.* (1998)	lactate (32 mM)	$(NH_4)_2SO_4$ (4 mM)	Chemostat (ammonium limited, D=0.06 h^{-1})	1000 ml	1.0	250 W/m^2	769 (lightpath = 1.3 cm)	incandes-cent lamps

TABLE 1B

HYDROGEN PRODUCTION RATE, YIELDS AND EFFICIENCIES (BOTH PRESENTED BY AUTHORS AND CALCULATED BY OURSELVES)

Order Number (1)	H_2 (ml) (12)	Total time (h) (13)	H_2 (ml ml^{-1}_{cult} h^{-1}) (14)	H_2 (ml $g^{-1}_{dry\ wt}$ h^{-1}) (15)	Yield (H_2/S) (%) (16)	Yield (H_2/S) (calc) (%) (17)	Efficiency of light (%) (18)	Efficiency of light (calc) (%) (19)	Comments
1		175	0.1309			55.6		2.69	autotrophic CO_2 fixation
2			0.0537 (0.6 mmol m^{-3} s^{-1})					3.72	LH1 / LH2 ratio
3 (a)	1 710	90	0.019	80.3		38.3		8.66	ALA production (pH=7, optimum and controlled)
(b)	1 170		0.013	-		27.6		5.92	
(c)	380		0.0042	-		7.6		1.91	
(d)	950		0.0105	33				4.79	
4 (a)	3600	70 d	0.02			-		1.06	100 ml fresh medium + 30 ml overnight grown cells (2.2-3.6 mg/ml)
(b)	52	55	0.0020			11.5		0.11	
(c)	477	287	0.0042			26.5		0.22	
5	85	90	0.0063			-		0.19	with milk waste alone, no growth
6		24	0.1065	1.42 ml/mg		42.8		11.3(*)	PHB accumulation
7 (a)	-	-		80	-	-		-	
(b)	2100	140			74.5	67.0		3.39 (1.88) (*)	
8			0.065	20		64.5		2.76	
9			11.6 ml/h (highest rate)	55		≈ 80		5.14 (2.85) (*)	
10 (a)			27 µmol / (ml.h)	8.8 mmol/ (g.h)			2	2.14	dissolved H_2 electrode
(b)			2.4µmol / (ml.h)	-			26	26	
(c)			34 µmol / (ml.h)	11.2 mmol/ (g.h)			3	2.7	
(d)			3.2 µmol / (ml.h)				35	35	
11		6	0.235 (1st)			12.6	0.9	0.5 (0.9) (1st)	various light penetration depths
			0.210 (2nd)			11.3	2.5	1.5 (2.6) (2nd)	
			0.090 (3rd)			4.8	3.3	2.0 (3.4) (3rd)	
			0.025 (4th)			1.3	3.2	1.8 (3.1) (4th)	
12	72	66 d				-	0.31 (66 d)	0.31	
	250						0.55 (8 d)		

TABLE 1B

HYDROGEN PRODUCTION RATE, YIELDS AND EFFICIENCIES (BOTH PRESENTED BY AUTHORS AND CALCULATED BY OURSELVES) (CONT.)

Order Number (1)	H_2 (ml) (12)	Total time (h) (13)	H_2 (ml ml^{-1}_{cult} h^{-1}) (14)	H_2 (ml $g^{-1}_{dry\ wt}$ h^{-1}) (15)	Yield (H_2/S) (%) (16)	Yield (H_2/S) (calc) (%) (17)	Efficiency of light (%) (18)	Efficiency of light (calc) (%) (19)	Comments
13 (a)	40 l / m^2	12				11.5	2.2	2.02 (7 kWh/m^2)	irradiation period of 12 h
(b)	25 l / m^2					7.3	1.1	2.36 (6 kWh/m^2) 1.3 (7 kWh/m^2)	
14		100	75 μ mol/ml		41.7	41.7		0.36 (*)	fermentate from algal biomass (4.6 mol H_2 / mol starch -glucose)
15			0.1 [2.5 ml/ml, with acetate 30 mM]	140	73 [84]	73 [83]		4.79 (*)	a mutant of the B 100 strain – hydrogenase deficient
16			1.4 – 1.6 l / (l.day)	75	50 - 70	6.7		2.5 (1.4) (*)	light inside both reactors
17		16	0.048			10.2		3.96 (2.2) (*)	
18			0.18			80.4		4.35	
19			1.45 l / (h.m^2) [7.9 l / (h.m^2) at 1000 W / m^2]				7.9 [2.1 at 1000 W / m^2]	9.25 [2.5]	cells in gel
20		24	0.014			7.4		1.1 (0.62) (*)	
21		12		3.75 mmol /(g.h)					immobilised gel cells – total volume 0.7 – 0.8 ml
22			7812 ml / (m^2.h)		62 – 73	46.5	9.23 (maximum value)	8.31	
23			0.080		30	27.9		1.33	

ANNOTATIONS TO TABLES 1A AND 1B:

(1) Order number has no special meaning. It helps the co-ordination between the two parts of the same table.

(2) Almost all the phototrophic bacteria strains used in hydrogen production are from the genera *Rhodobacter* (and the most used species is *R. sphaeroides*). Some species formerly designated as *Rhodopseudomonas* have been classified as *Rhodobacter* in recent classification criteria (information from Jun Miyake). Maybe some strains designated as *Rhodopseudomonas* and *Rhodospirillum* should be re-classified under new criteria, but in this column is presented the name suggested by the authors. Adaptation of strains to particular media or the selection of mutants, maybe is not enough to define new species.

(3) The references selected to be included in this table were only the ones from which it was possible to obtain enough data to calculate (or, in a few cases to reproduce) the yield of hydrogen production (from a known carbon source) and the efficiency of light conversion into hydrogen (from an energetic point of view). Many other references that present the biological hydrogen production from photoheterotrophic bacteria, but do not allow to do those calculations, were not included in this table, although they are referenced elsewhere in this paper.

(4) The main carbon sources presented in literature, in artificial culture media for hydrogen production, are malate and lactate (sodium salt). References to complex media, such as residual waste waters, were not included here because of the missing of reproducibility.

(5) There is agreement that sodium glutamate is the best nitrogen source for hydrogen production when used in stressful concentrations. However, there are also references concerning the use of nitrogen source only in the growing medium, to induce the synthesis of nitrogenase, and after that, its total absence in the hydrogen production medium.

(6) Most of the experiments have been done in batch, using bottles, flat flasks, tubes or columns. Some references to semi-continuous and continuous operations are also included and, in the last case, the dilution rate (D) is presented, when available. In continuous operation, the one stage chemostat is the most often used but there are also references to more than one (two or three) stage chemostat and to turbidostat.

(7) Most of the culture volumes used are below 1 liter (with one exception of 6 l), which means that almost all the experiments have been done, so far, in a small (sometimes very small) scale.

(8) Cell density is usually referred as both the mass of dry weight per unit of volume and the optical density at 660 nm.

(9) Light irradiation is measured, most of the times, in W/m^2 and this was the unit used to calculate light efficiency. In some cases light irradiation was only presented in lux from which it was not possible to calculate the efficiency of light conversion with certainty, because there is not a conversion between lux and W/m^2; this relationship depends on the

kind of light source, as well as on the kind of sensors used. Some references in the literature present light intensities in both units, but the proportion is not always the same. As a matter of fact, the two units express different physical entities. The third unit used to express light intensity is $\mu mol/m^2.s$ (or $\mu E/m^2.s$, because 1 Einstein (E) = 1 mol of photons). In this case, the conversion to W/m^2 can be done using an approximate conversion factor (1 $W/m^2 \approx 4.7$ $\mu mol/m^2.s$) proposed by McCree (1981), cited by Markov *et al.* (1996). In one case (order number 13) light irradiation is expressed, in an integral way, by energy per unit of area.

(10) The area of illuminated culture is not often given and many times it had to be calculated or estimated with the knowledge of the culture volume and the assumption of the lightpath. In some cases the estimated area is very rough, especially with round tubes and bottles or cylinders. In other cases (order number 21) the illuminated area could not be estimated, which did not enable the calculation of light efficiency.

(11) The spectrum of the light emitted by tungsten / halogen lamps is better than the one from fluorescent lamps, taking into account that phototrophic bacteria use infrared light, with absorption maxima at 800 and 850 nm corresponding to the absorption of bacteriochlorophylls.

(12) The hydrogen produced was evaluated taking into account the volume of gas accumulated and the hydrogen content of that gas, at the temperature of the experiments, which is usually in the range 30-33°C.

(13) The total time is more important, for calculations, in batch than in continuous experiments. However, sometimes we do not know if the start of that time is from the inoculation of the hydrogen production medium or from the beginning of the hydrogen production itself.

(14) Hydrogen production is expressed, most of the times, as a volumetric productivity (volume of hydrogen produced at the temperature of the experiments (*e.g.* 30°C) per volume of culture medium and per unit of time). If there is no doubt about the meaning of this productivity in a continuous culture, the same is not true in batch cultures, which are the most often used. In the last case the productivity rate is a function of time and it can be expressed as local or global productivity. Most probably, the authors always give the best results they achieved and, because of that, the presented values must be understood as the major ones. In some references the amount of hydrogen produced was directly expressed in moles instead of volume; in other references the hydrogen production was already referred to the total culture volume or to the total time of the assay; another way of presentation was also per unit of light irradiation area instead of unit of culture volume. When the units of hydrogen production are not the one shown on the top of this column, they are indicated in place.

(15) Hydrogen production rate is sometimes referred as volume (or moles) of hydrogen per unit of biomass dry weight and per unit of time.

(16) Hydrogen yield, as the number of moles of hydrogen produced per mole of nutrient (carbon source) consumed or as a percentage of the maximum value theoretically possible (as presented in this column) is not very often shown by the authors. Although we can only talk

about yields when referred to well defined carbon sources, which are not the most important ones in a practical point of view, it seems that good yields can be achieved and this will not be a bottleneck in the hydrogen production.

(17) Hydrogen yields were calculated taking into account, on one hand, the culture volume and the initial carbon source concentration (or the molar flow of carbon source that comes into the continuous reactor; in this case, nutrient consumption and hydrogen production were both expressed per unit of volume and per unit of time), and on the other hand, the total amount of hydrogen produced in the total culture volume and during the total time of the experiment. This total volume of hydrogen produced was converted from the temperature of the assay to 0°C, before being divided by 22.4 ml/mmol, to obtain the molar amount of hydrogen produced. The molar ratio of hydrogen produced to nutrient consumed was divided by the maximum value (for each nutrient, as shown in Table 2) and the result was expressed as a percentage of the maximum yield.

(18) The efficiency of light is not often given by authors, first because there is more than one definition and second because it is not easy to present a value with certainty. All the values presented in this column were calculated by the authors in the same way, which was also adopted for our calculations, presented in the next column.

(19) The efficiency of light was calculated, using the same expression, for all the references from which we could get the necessary parameters. The expression used was the following (Hall *et al.*, 1995; Markov *et al.*, 1996):

$$\text{efficiency (\%)} = H_2 \text{ production rate} \times H_2 \text{ energy content} / \text{absorbed light energy}$$

Although this definition of light efficiency is the most generally accepted one, each term of the equation can have associated errors: (i) H_2 production rate, as a productivity, is correct for continuous cultures but in batch cultures is a function of time (see item 14 in this list); (ii) H_2 energy content, as combustion enthalpy of H_2, can be expressed as a net calorific value (or lower heating value), but it can also be expressed as a gross calorific value (or higher heating value), when the enthalpy of water vaporization is considered; (iii) the absorbed light energy has not been used but rather the measured light intensity.

Different units can be used for the three factors, in order to make the efficiency a non dimensional number, such as $mol/m^2.s$ for the H_2 production rate, J/mol for the H_2 energy content and W/m^2 for the absorbed light.

The H_2 energy content adopted was the gross combustion enthalpy of H_2 at 0°C and 1 atm, which is 3.05 kcal/l, as shown in Table 3. The absorbed light is assumed to be the same as the measured irradiation energy of the light source, at the level of the culture medium.

The volumetric hydrogen production rate is first converted to 0°C and then multiplied by the culture volume. The obtained volume of H_2 at 0°C (ml) per unit of time (h) is multiplied by the energy content (3.05 cal/ml) and thereafter divided by the irradiation area. The obtained energy produced is expressed by $cal/(h.m^2)$. On the other hand, the light energy consumed is easily converted from W/m^2 to the same unit.

A problem arises when light irradiation is only expressed in lux unit. As there is no conversion to W/m^2, two different proportions were used, from literature when both values were presented, one of them more favorable (100 lux = 1 W/m^2) (Ooshima *et al.*, 1998) and another one less

favorable (55.5 lux = 1 W/m^2) (Nakada *et al.*, 1996). The efficiency calculated with the last proportion is presented, in this column, between brackets. However, in both cases the efficiencies calculated were not trustworthy. In these cases, as well as in others were the culture volume or, mainly, the irradiation area, had to be assumed, the values obtained for efficiencies can not be very accurate and they are pointed out with an asterisk.

Warthmann *et al.* (1993) suggested a slight modification of the equation above referred, that takes into account the combustion energy of the substrates utilized by the anoxygenic phototrophic bacteria, which results in a lower energy yield:

energy yield = (energy of H_2 – energy of the substrate) / light energy

However, in a practical point of view, when organic residues are used as nutrients of the phototrophic bacteria, the energetic content of these compounds is usually worthless.

Most of the times, our calculations could reproduce the ones presented by the authors. In order number 19, the values we obtained for efficiency are even greater than the ones shown by the authors, but the reason is that they used the net combustion enthalpy of H_2 while we have adopted the gross combustion enthalpy. In order number 11, if we had to assume the culture volume, with the available information in the paper, we should obtain values for efficiencies that do not agree with the values presented by the authors. However, we could reproduce these last values, which we show between brackets, and they were obtained using 32.5 ml as culture volume, but this information is missing in that reference. In order number 22 the greater value presented by the authors is just because they did not correct the hydrogen volume from the temperature of the assay (30°C) to 0°C, before using the combustion enthalpy of hydrogen at 0°C.

The reproduction of the few results of efficiencies presented in literature (or the knowledge of the explanation for the differences, when they do not agree with the calculated values) increases the confidence of the calculations provided by ourselves.

TABLE 2
THEORETICAL H_2 PRODUCTION YIELDS FROM DIFFERENT ORGANIC COMPOUNDS

Substrate	Formula	mol H_2 / mol substrate	Reference
Acetate	$C_2H_4O_2$	4	Miyake *et al.* (1984)
Lactate	$C_3H_6O_3$	6	"
Butyrate	$C_4H_8O_2$	10	"
Malate	$C_4H_6O_5$	6	Ooshima *et al.* (1998)
Monosaccharide	$C_6H_{12}O_6$	12	"
Disaccharide	$C_{12}H_{22}O_{11}$	24	"
Benzoate	$C_7H_6O_2$	15	Fiβler *et al.* (1995)
DL-mandelate	$C_8H_8O_3$	17	"
Cinnamate	$C_9H_8O_2$	20	"
Benzoyl formate	$C_8H_6O_3$	16	"
Propionate	$C_3H_6O_2$	7	-

TABLE 3
COMBUSTION ENTHALPY OF H_2 AT 0°C AND 1 ATM

Net calorific value	241.9 kJ/mol (57.87 kcal/mol or 2.58 kcal/l)	Miyake and Kawamura (1987) Markov *et al.* (1996)
Gross calorific value	285.5 kJ/mol (68.3 kcal/mol or 3.05 kcal/l)	El-Shishtawy *et al.* (1998b) Otsuki *et al.* (1998)

The efficiency of a photoprocess where solar photons (or from other source) drive a chemical reaction that stores part of the photon energy as chemical energy of an energy-rich product, such as hydrogen, was defined by Bolton (1996) in a different way, but that equation has not been used. The question of the maximum photosynthetic efficiency has attracted the attention of many researchers (Pirt, 1983). Using different equations for the efficiency calculation Bolton and Hall (1991) state that although the theoretical maximum efficiency of conversion of light to stored chemical energy in green-plant type (oxygen-evolving) photosynthesis in bright sunlight is calculated to be 13.0%, the practical maximum efficiency of photosynthesis under optimum conditions is estimated to be 8-9%.

The main bottleneck of biological hydrogen production seems not to be the hydrogen yield itself but light efficiency. The very few references to light efficiency stress the fact that light intensity, light distribution and light measurement are problematic issues difficult to control, to achieve and to check, respectively.

Studies of light conversion efficiency and H_2 production rate, as a function of light intensity, are not numerous and we feel that a real evolution did not occur in last 12 years. The results presented suggest a compromise between light efficiency and H_2 production rate. Reasonable efficiencies were obtained at low light intensities [7-9% at 50 W/m^2 (Miyake and Kawamura, 1987); 26-35% at 13 W/m^2 (Yamada *et al.*, 1998] but the associated H_2 production rates were too low to be interesting from a practical point of view. An increase of light conversion efficiency and a decrease of H_2 productivity were also obtained by Nakada *et al.*(1998), when light intensity decreased as consequence of penetration through several cell culture layers.

For optimal production of hydrogen by photosynthetic bacteria, to be economically attractive, one has to take into account several parameters from an overall point of view, such as the productivity rate, the light efficiency and the yield from the carbon source. An efficient parameter to evaluate the performance of photobioreactors, in order to maximize hydrogen production is still missing. With this parameter one should also be able to forecast the consequences of scaling-up.

The calculated light efficiencies are, in general, lower than would be desirable. However, some values are quite promising but they were obtained under very peculiar circumstances, namely on a very small scale or with soft irradiation intensities. On the other hand, these values may suggest that the limitation is not biological but are explainable from an engineering point of view. And the challenge is still there!

ACKNOWLEDGMENTS

Jorge Rocha received a postdoctoral fellowship PRAXIS XXI/BPD/ 18840/98 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal
The project was sponsored by the Dutch research program Economy, Ecology and Technology (KIEM 98013)

REFERENCES

Akano, T., Miura, Y., Fukatsu, K., Miyasaka, H., Ikuta, Y., Matsumoto, H., Hamasaki, A., Shioji, N., Mizoguchi, T, Yagi, K. and Maeda, I. (1996). Hydrogen production by photosynthetic microorganisms. *Appl. Biochem. Biotechnol.* **57/58**, 677-688.

Benemann, J.R., Miyamoto, K. and Hallenbeck, P.C. (1980). Bioengineering aspects of biophotolysis. *Enzyme Microb. Technol.* **2**, 103-111.

Benemann, J. (1996). Hydrogen biotechnology: progress and prospects. *Nature Biotechnology* **14**, 1101-1103.

Benemann, J. R. (1998). The technology of biohydrogen, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London,

Bolliger, R., Zürrer, H. and Bachofen, R. (1985). Photoproduction of molecular hydrogen from waste water of a sugar refinery by photosynthetic bacteria, *Appl. Microbiol. Biotechnol.* **23**, 147-151.

Bolton, J.R. and Hall, D.O. (1991). The maximum efficiency of photosynthesis. *Photochem. Photobiol.* **53**, 545-548.

Bolton, J.R. (1996). Solar photoproduction of hydrogen: a review. *Solar Energy* **57**, 37-50.

El-Shishtawy, R.M.A., Kawasaki, S. and Morimoto, M. (1997). Biological H_2 production using a novel light-induced and diffused photoreactor. *Biotechnol. Techniques* **11**, 403-407.

El-Shishtawy, R.M.A., Kitajima, Y., Otsuka, S., Kawasaki, S. and Morimoto, M. (1998a). Study on the behavior of production and uptake of photobiohydrogen by photosynthetic bacterium *Rhodobacter sphaeroides RV*, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 117-122.

El-Shishtawy, R.M.A., Kawasaki, S. and Morimoto, M. (1998b). Cylindrical-type induced and diffused photobioreactors: a novel photoreactor for large-scale H_2 production, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 353-358.

Eroglu, I., Aslan, K., Gündüz, U., Yücel M. and Türker, L. (1998). Continuous hydrogen production by *Rhodobacter sphaeroides* O.U. 001, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 143-149.

Fascetti, E. and Todini, O. (1995). *Rhodobacter sphaeroides* RV cultivation and hydrogen production in a one- and two-stage chemostat. *Appl. Microbiol. Biotechnol.* **44**, 300-305.

Fascetti, E., D'addario, E., Todini, O. and Robertiello, A. (1998). Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. *Int. J. Hydrogen Energy* **23**, 753-760.

Fiβler, J., Schirra, C., Kohring, G.W. and Giffhorn, F. (1994). Hydrogen production from aromatic acids by *Rhodopseudomonas palustris*. *Appl. Microbiol. Biotechnol.* **41**, 395-399.

Fujii, T., Nakazawa, A., Sumi, N., Tani, H., Ando, A. and Yabuki, M. (1983). Utilization of alcohols by *Rhodopseudomonas sp.* No. 7 isolated from n-propanol – enrichment cultures. *Agric. Biol. Chem.* **47**, 2747-2753.

Gest, H. and Kamen, M.D. (1949). Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*. *Science* **109**, 558-559.

Hall, D.O., Markov, S.A., Watanable, Y. and Rao, K.K. (1995). The potential applications of cyanobacterial photosynthesis for clean technologies. *Photosynthesis Research* **46**, 159-167.

Ike, A., Saimura, C., Hirata, K. and Miyamoto, K. (1996). Environmentally friendly production of H_2 incorporating microalgal CO_2 fixation. *J. Mar. Biotechnol.* **4**, 47-51.

Ike, A., Toda, N., Hirata, K. and Miyamoto, K. (1997a). Hydrogen photoproduction from CO_2-fixing microalgal biomass: application of lactic acid fermentation by *Lactobacillus amylovorus*. *J. Ferment. Bioeng.* **84**, 428-433.

Ike, A., Toda, N., Tsuji, N., Hirata, K. and Miyamoto, K. (1997b). Hydrogen photoproduction from CO_2-fixing microalgal biomass: application of halotolerant photosynthetic bacteria. *J. Ferment. Bioeng.* **84**, 606-609.

Kataoka, N., Miya, A. and Kiriyama, K. (1997). Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. *Wat. Sci. Tech.* **36(6-7)**, 41-47.

Khatipov, E., Miyake, M., Miyake, J. and Asada, Y. (1998). Polyhydroxybutyrate accumulation and hydrogen evolution by *Rhodobacter sphaeroides* as a function of nitrogen availability, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 157-161.

Kim, J. S., Ito, K. and Takahashi, H. (1980). The relationship between nitrogenase activity and hydrogen evolution in *Rhodopseudomonas palustris*. *Agric. Biol. Chem.* **44**, 827-833.

Kim, J. S., Ito, K. and Takahashi, H. (1981). Production of molecular hydrogen by *Rhodopseudomonas sp.*. *J. Ferment. Technol.* **59**, 185-190.

Kim, J. S., Ito, K. and Takahashi, H. (1982). Production of molecular hydrogen in outdoor batch cultures of *Rhodopseudomonas sphaeroides*. *Agric. Biol. Chem.* **46**, 937-941.

Kim, J. S., Ito, K., Izaki, K. and Takahashi, H. (1987a). Production of molecular hydrogen by a semi-continuous outdoor culture of *Rhodopseudomonas sphaeroides*. *Agric. Biol. Chem.* **51**, 1173-1174.

Kim, J. S., Ito, K., Izaki, K. and Takahashi, H. (1987b). Production of molecular hydrogen by a continuous culture under laboratory conditions. *Agric. Biol. Chem.* **51**, 2591-2593.

Kobayashi, M. and Kurata, S. I. (1978). The mass culture and cell utilization of photosynthetic bacteria. *Proc. Biochem.* **13**, 27-30.

Krahn, E., Schneider, K. and Muller, A. (1996). Comparative characterization of H_2 production by the conventional Mo nitrogenase and the alternative "iron-only" nitrogenase of *Rhodobacter capsulatus hup*⁻ mutants. *Appl. Microbiol. Biotechnol.* **46**, 285-290.

Maeda, I., Chowdhury, W.Q., Idehara, K., Yagi, K., Mizoguchi, T., Akano, T., Miyasaka, H., Furutani, T., Ikuta, Y., Shioji, N. and Miura, Y. (1998). Improvement of substrate conversion to molecular hydrogen by three-stage cultivation of a photosynthetic bacterium, *Rhodovulum sulfidophilum*. *Appl. Biochem. Biotechnol.* **70-72**, 301-310.

Majizat, A., Mitsunori, Y., Mitsunori, W., Michimasa, N. and Jun'ichiro, M. (1997). Hydrogen gas production from glucose and its microbial kinetics in anaerobic systems. *Wat. Sci. Tech.* **36**, 279-286.

Markov, S.A., Bazin, M.J. and Hall, D.O. (1996). Efficiency of light energy conversion in hydrogen production by cyanobacterium *Anabaena variabilis*. *J. Mar. Biotechnol.* **4**, 57-60.

Markov, S.A. (1998). Bioreactors for hydrogen production, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 383-390.

Matsumoto, M., Yoza, B., Radway, J.C. and Zaborsky, O.R. (1998). Photosynthetic bacteria of Hawaii: potential for hydrogen production, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 163-166.

Mignot, L., Junter, G.A. and Labbe, M. (1989). A new type of immobilized-cell photobioreactor with internal illumination by optical fibres. *Biotechnol. Techniques* **3**, 299-304.

Mitsugi, C., Harumi, A. and Kenzo, F. (1998). We-net: japonese hydrogen program. *Int. J. Hydrogen Energy* **23**, 159-165.

Miyake, J., Tomizuka, N. and Kamibayashi, A. (1982). Prolonged photo-hydrogen production by *Rhodospirillum rubrum*. *J. Ferment. Technol.* **60**, 199-203.

Miyake, J., Mao, X.Y. and Kawamura, S. (1984). Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butyricum. J. Ferment.Technol.* **62**, 531-535.

Miyake, J. and Kawamura, S. (1987). Efficiency of light energy conversion to hydrogen by the photosynthetic bacterium *Rhodobacter sphaeroides. Int. J. Hydrogen Energy* **12**, 147-149.

Miyake, M., Sekine, M., Vasilieva, L.G., Nakada, E., Wakayama, T., Asada, Y. and Miyake, J. (1998). Improvement of bacterial light-dependent hydrogen production by altering the photosynthetic pigment ratio, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 81-86.

Nakada, E., Asada, Y., Arai, T. and Miyake, J. (1995). Light penetration into cell suspensions of photosynthetic bacteria and relation to hydrogen production. *J. Ferment. Bioeng.* **80**, 53-57.

Nakada, E., Nishikata, S., Asada, Y. and Miyake, J. (1996). Hydrogen production by gel-immobilized cells of *Rhodobacter sphaeroides* - distribution of cells, pigments, and hydrogen evolution. *J. Mar. Biotechnol.* **4**, 38-42.

Nakada, E., Nishikata, S., Asada, Y. and Miyake, J. (1998). Light penetration and wavelength effect on photosynthetic bacteria culture for hydrogen production, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 345-352.

Nandi, R. and Sengupta, S., Microbial production of hydrogen: an overview. *Critical Reviews in Microbiology* **24**, 61-84,

Ooshima, H., Takakuwa, S., Katsuda, T., Okuda, M., Shirasawa, T., Azuma, M. and Kato, J. (1998). Production of hydrogen by a hydrogenase-deficient mutant of *Rhodobacter capsulatus. J. Ferment. Bioeng.* **85**, 470-475.

Ogbonna, J.C., Soejima, T. and Tanaka, H. (1998). Development of efficient large-scale photobioreactors: a key factor for practical production of biohydrogen, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 329-343.

Otsuki, T., Uchiyama, S., Fujiki, K. and Fukunaga, S. (1998). Hydrogen production by a floating-type photobioreactor, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 369-374.

Perego, P., Fabiano, B., Ponzano, G.P. and Palazzi, E. (1998). Experimental study of hydrogen kinetics from agroindustrial by-product: optimal conditions for production and fuel cell feeding. *Bioproc. Eng.* **19**, 205-211.

Pirt, S.J. (1983). Maximum photosynthetic efficiency: a problem to be resolved. *Biotechnol. Bioeng.* **25**, 1915-1922.

Pulz, O. and Scheibenbogen, K. (1998). Photobioreactors: design and performance with respect to light energy input. *Advances in Biochemical Engineering/Biotechnology* **59**, 123-152.

Rao, K.K. and Hall, D.O. (1996). Hydrogen production by cyanobacteria: potential, problems and prospects. *J. Mar. Biotechnol.* **4**, 10-15.

Sasaki, K., Tanaka, T., Nishizawa, Y and Hayashi, M. (1990). Production of a herbicide, 5-aminolevulinic acid, by *Rhodobacter sphaeroides* using the effluent of swine waste from an anaerobic digestor. *Appl. Microbiol. Biotechnol.* **32**, 727-731.

Sasaki, K. (1998). Hydrogen and 5-aminolevulinic acid production by photosynthetic bacteria, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 133-142.

Sasikala, C., Ramana, C.V. and Rao, R. (1991). Environmental regulation for optimal biomass yield and photoproduction of hydrogen by *Rhodobacter sphaeroides* O.U. 001. *Int. J. Hydrogen Energy* **16**, 597-601.

Sasikala, C., Ramana, C.V. and Rao, R. (1992). Photoproduction of hydrogen from the waste water of a distillery by *Rhodobacter sphaeroides* O.U. 001. *Int. J. Hydrogen Energy* **17**, 23-27.

Schulz, R. (1996). Hydrogenases and hydrogen production in eukaryotic organisms and cyanobacteria. *J. Mar. Biotechnol.* **4**, 16-22.

Stevens, P., Vertonghen, C., Vos, P. and Ley, J. (1984). The effect of temperature and light intensity on hydrogen gas production by different *Rhodopseudomonas capsulata* strains. *Biotechnol. Lett.* **6**, 277-282.

Taguchi, F., Chang, J.D., Takiguchi, S. and Morimoto, M. (1992). Efficient hydrogen production from starch by a bacterium isolated from termites. *J. Ferment. Bioeng.* **73**, 244-245.

Taguchi, F., Mizukami, N., Yamada, K., Hasegawa, K. and Taki-Saito, T. (1995). Direct conversion of cellulosic materials to hydrogen by *Clostridium sp.* strain no. 2. *Enzyme Microb. Technol.* **17**, 147-150.

Taguchi, F., Yamada, K., Hasegawa, K., Taki-Saito, T. and Hara, K. (1996). Continuous hydrogen production by *Clostridium sp.* strain No.2 from cellulose hydrolysate in an aqueous two-phase system. *J. Ferment. Bioeng.* **82**, 80-83.

Tel-Or, E., Luijk, L.W. and Packer, L. (1977). An inducible hydrogenase in cyanobacteria enhances N_2 fixation, *FEBS Lett.* **78**, 49-52.

The Economist, ed from 1999-03-20, "The fuel-cell car accelerates" (a)

The Economist, ed from 1999-04-24, "Fuel cells hit the road" (b)

Tsygankov, A., Laurinavichene, T., Gogotov, I., Asada, Y. and Miyake, J. (1996). Switching from light limitation to ammonium limitation in chemostat cultures of *Rhodobacter capsulatus* grown in different types of photobioreactor. *J. Mar. Biotechnol.* **4**, 43-46.

Tsygankov, A.A. and Laurinavichene, T.V. (1996). Influence of the degree and mode of light limitation on growth characteristics of the *Rhodobacter capsulatus* continuos cultures. *Biotechnol. Bioeng.* **51**, 605-612.

Tsygankov, A.A., Fedorov, A.S., Laurinavichene, T.V., Gogotov, I.N., Rao, K.K. and Hall, D.O. (1998). Actual and potential rates of hydrogen photoproduction by continuous culture of the purple non-sulphur bacterium *Rhodobacter capsulatus*. *Appl. Microbiol. Biotechnol.* **49**, 102-107.

Türkarslan, S., Yigit, D.O., Aslan, K., Eroglu, I and Gündüz, U. (1998). Photobiological hydrogen production by *Rhodobacter sphaeroides* O.U. 001 by utilization of waste water from milk industry, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 151-156.

Ueno, Y., Kawai, T., Sato, S., Otsuka, S. and Morimoto, M. (1995). Biological production of hydrogen from cellulose by natural anaerobic microflora. *J. Ferment. Bioeng.* **79**, 395-397.

Ueno, Y., Otsuka, S. and Morimoto, M. (1996). Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. *J. Ferment. Bioeng.* **82**, 194-197.

Vrati, S. and Verma, J. (1983). Production of molecular hydrogen and single cell protein by Rhodopseudomonas capsulata from cow dung. *J. Ferment. Technol.* **61**, 157-162.

Vrati, S. (1984). Single cell protein production by photosynthetic bacteria grown on the clarified effuents of biogas plant. *Appl. Microbiol. Biotechnol.* **19**, 199-202.

Wakayama, T., Toriyama, A., Kawasugi, T., Arai, T., Asada, Y. and Miyake, J. (1998). Photohydrogen production using photosynthetic bacterium *Rhodobacter sphaeroides RV*: simulation of the light cycle of natural sunlight using an artificial source, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 375-381.

Warthmann, R., Pfennig, N. and Cypionka, H. (1993). The quantum requirement for H_2 production by anoxygenic phototrophic bacteria. *Appl. Microbiol. Biotechnol.* **39**, 358-362.

Yamada, A., Takano, H., Burgess, J.G. and Matsunaga, T. (1996). Enhanced hydrogen production by a marine photosynthetic bacterium, *Rhodobacter marinus*, immobilized onto light-diffusing optical fibers. *J. Mar. Biotechnol.* **4**, 23-27.

Yamada, A., Hatano, T. and Matsunaga, T. (1998). Conversion efficiencies of light energy to hydrogen by a novel Rhodovulum sp. and its uptake-hydrogenase mutant, in <Biohydrogen> Edited by Oskar R. Zaborsky, Plenum Press, London, 167-171.

Yigit, D.O., Gündüz, U.,Türker, L., Yücel, M. and Eroglu, I., Identification of by-products in hydrogen producing bacteria *Rhodobacter sphaeroides* O.U. 001 grown in the waste water of a sugar refinery. *J. Biotechno.*, in press

Zajic, J.E., Kosaric, N. and Brosseau, J.D. (1978). Microbial production of hydrogen. *Advances in Biochemical Engineering* **9**, 57-109.

Zhu, H., Miyake, J., Anatoly, A. and Asada, T. (1995). Hydrogen production from highly concentrated organic wastewater by photosynthetic bacteria & anaerobic bacteria. *Water Treatment* **10**, 61-68.

Zürrer, H. and Bachofen, R. (1979). Hydrogen production by the photosynthetic bacterium *Rhodospirillum rubrum*. *Appl. Environ. Microbiol.* **37**, 789-793.

Zürrer, H. and Bachofen, R. (1982). Aspects of growth and hydrogen production of the photosynthetic bacterium *Rhodospirillum rubrum* in continuous culture. *Biomass* **2**, 165-174.

HYDROGEN AND POLY- (HYDROXY) ALKANOATE PRODUCTION FROM ORGANIC ACIDS BY PHOTOSYNTHETIC BACTERIA

N. Noparatnaraporn[1], K. Takeno[2] and K. Sasaki[2]

[1]Department of Microbiology, Faculty of Science University,
Bangkok 10900, Thailand
[2]Materials Science and Engineering,
Graduate School of Engineering, Hiroshima Kokusai Gakuin University,
6-20-1, Nakano, Akiku, Hiroshima. 759-0321 Japan

ABSTRACT

Hydrogen production by *Rhodobacter sphaeroides* IL106 and *R. sphaeroides* S from organic acids were investigated considering the bioremediation of sediment mud of oyster farm in Hiroshima Bay. Acidogenic fermentation of sediment suspension (200 g wet mud/L artificial sea water) was first carried out. With the addition of a small amount vitamins (thiamine, nicotinic acid and biotin), acidogenic fermentation was enhanced giving about 2 g/L of acetic acid. In addition, 20 mg/L of phosphate and about 1.0 mg/L of ammonium was released into the culture broth after 7 days.

Hydrogen production from acetic acid as carbon and energy source was investigated using two strains of photosynthetic bacteria (IL106 and S strain) with the synthetic liquor of acidogenic fermentation of sediment mud. When S strain was about 2.0 L of total gas (hydrogen 80%) was evolved after 100 hours culture with acetic acid energy source. However, 0.01 L/L broth of total was evolved by IL106 strain. In this case, large amount of poly-Hydroxyalkanoate (PHA) accumulated in the cells of IL106 (about 70 % of dry weight), but about 40 % of PHA was in the cells of S strain.

PHA and hydrogen production in these strains was closely related and PHA accumulation might be occurred as the consumption of hydrogen (reducing power) in the cells without producing molecular hydrogen extracellularly.

INTRODUCTION

Hydrogen production from waste using photosynthetic bacteria is attractive because energy can be recovered from the wastes derived from renewable resources (Mitsui et al. 1985). Photosynthetic bacteria can produce hydrogen from organic acids (Hillmer and Gest, 1977; Takahashi, 1984; Miyake et al., 1984 and Mao et al., 1986) observed the relatively high hydrogen

production from *Rhodobacter sphaeroides* RV. Recently, several reports about hydrogen production have been reported by photosynthetic bacteria (Sasikala et al., 1991; Turkarslan et al., 1998) using organic wastes.

We have carried out hydrogen production using volatile fatty acid (VFA) considering the utilization organic wastes (Sasaki et al., 1996). Organic wastes are desirable sources for hydrogen production considering environmental use of renewable resources and they are rather easily obtained. Polluted sediment mud by sewage wastewater or food manufacture waste is one of the source for hydrogen production by photosynthetic bacteria, because, this mud is accumulated abundantly in the bottom river, lake and pond and purification (bioremediation) of this mud by photosynthetic bacteria results the hydrogen production (renewable energy production) simultaneously.

On the other hand, photosynthetic bcteria can produce poly-hydroxyalkanoate (PHA) intracellularly under nitrogen source deficient condition (Brandl et al., 1991; Suzuki et al., 1996). PHA(s) are recognized as the biodegradable plastic and some of them were already on market of environmental friendly plastic. PHA accumulation in the cells is explained as the energy saving process when excess amount of energy by photosynthesis is provided. However, the relationship between hydrogen production and PHA accumulation are not elucidated.

In this paper, hydrogen production and PHA accumulation from the sediment mud using photosynthetic bacteria were carried out considering the bioremediation of sediment mud and hydrogen energy recovery. In addition, the relationship between hydrogen production and PHA accumulation were discussed .

MATERIALS AND METHODS

Rhodobacter sphaeroides S (Sasaki et al., 1991) and *R. sphaeroides* IL106 (Takeno et al., 1999) were used.

A Glutamate-malate medium (Sasaki et al., 1987) was used for preculture. For hydrogen and PHA production, carbon source was changed sometimes to acetic acid or the mixture of acetic and proponic acid in place of DL-malic acid (Modified medium).

Preculture was carried out as described previously (Sasaki et al., 1987). 300 ml conical flask (100 ml medium) was used for preculture under static light conditions with 5 klux (17.5 W/m^2) illumination (tungsten bulbs) on the surface of the vessel at 30°C for 2-3 days.

Hydrogen and PHA production were carried out as described previously (Sasaki et al., 1996; Sasaki, 1998) in a 1.5 L bottle (1 L liquid). Glutamate-malate and Modified medium was filled in the Roux bottle and cultured under anaerobic light condition. Culture broth was gently mixed with magnetic stirrer (about 100 rpm) to maintain homogenous culture condition. Hydrogen produced was in gas holder (Sasaki et al., 1996). Illumination was provided at 5-30 klux (17.5-105 W/m^2) on the surface of the vessel with two tungsten bulb. Temperature was controlled 30±0.2°C, and pH was controlled at 7.0±0.1 by adding 4N-HCL and 4N NaOH solutions.

Anaerobic digestion of sediment mud was carried out in 5L conical flask (liquid 2 L). Sediment mud (200-400 g wet basis) from the bottom of oyster farm in Hiroshima Bay was suspended in artificial sea water (Takeno et al., 1999). Flask was fermented for 7 days at 30°C under static condition by adding 10% seed culture broth (fresh sediment from the sea bottom). The flask was mixed every one day shaking by hand to maintain homogenous broth condition. If necessary, 1 mL vitamin solution (1 g, thimaine; 10 mg, biotin; 1 g nicotinic acid in 1 L of 20% ethanol solution) was added.

Cell mass, hydrogen, residual acetic aid propionic acids and nitrogenase activity in the cell were measured as described previously (Sasaki, 1996). PHA content of the cells was measured

by the method by Barandl et al. (1991).

RESULTS AND DISCUSSION

1. Hydrogen Production From Acetic acid

To utilize organic wastes production, acetic and propionic acid should be utilized by photosynthetic bacteria because organic wastes are usually converted to volatile fatty acids (VFA) such as acetic, propionic and butyric acids during anaerobic digestion. Especially, acetic and propionic acid are major VFA in the digestion liquor. Therefore, acetic and propionic acids were used for hydrogen production as a carbon and energy source with Modified medium. As shown in Fig. 1, hydrogen production with acetic acid (a) and a mixture of acetic and propionic acids (b) was investigated. Hydrogen was produced in both cases, although the maximum production rate (33 mL/g cells/h) was low compared to L-malic acid (80.3 mL/g cell/h)(Sasaki, et al., 1996). Nitrogenase was actually induced intracellularly in both culture condition (a, b). Hydrogen gas was about 80% of total gas in this bacterial hydrogen production (other gas, CO_2). However, it was confirmed that hydrogen can be produced by acetic and propionic acid. There are some possibilities to utilize VFAs from anaerobic digestion liquor from organic wastes.

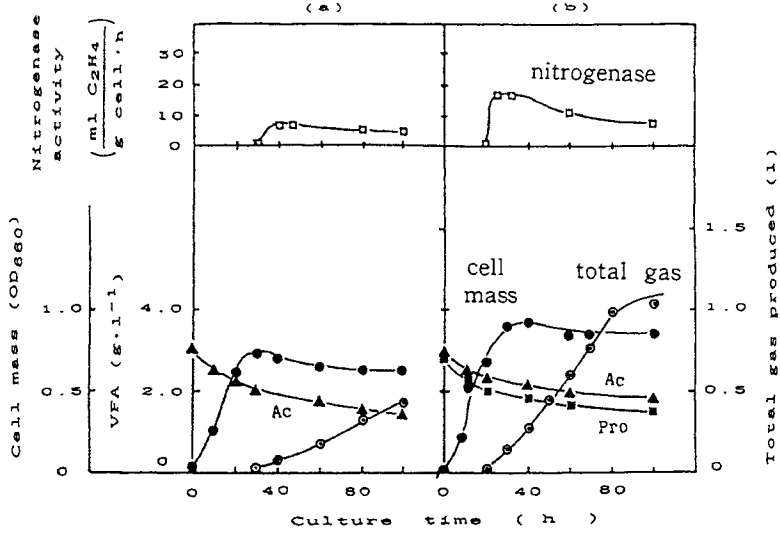


Figure 1: Photohydrogen production by *Rhodobacter sphaeroides* S from acetic and propionic acid (VFA) (30°C, 10 klux, ph 7.0±0.1): (a) acetic acid 3 g/L +glutamate-Na 0.17 g/L; ●, cell mass; ◎, total gas evolved; ▲, residual acetic acid; ■, residual propionic acid; □, nitrogenase activity.

2. Anaerobic Digestion of Sediment Mud

Sediment mud from oyster farm consists of about 95% sea sand and 5% of organic matters. In the matters, COD, high molecule organic fatty substances, total phosphorous and total nitrogenous compounds present with relatively high amount (Takeno et al., 1999). Anaerobic digestion was first carried out to release such organic matters into the liquor from the mud and decompose them to the low molecule organic matters. As shown in Fig. 2, acetic acid and phosphate were not released into the fermentation liquor without addition of vitamin solutions suggesting that anaerobic fermentation was not proceeded. On the other hand, acetic acid and phosphate were significantly released into the liquor after 7 days fermentation with addition of vitamins. It was suggested that vitamins addition enhanced acidogenic fermentation. Only small amount of ammonium release was the result of consumption by the acidogenic fermentation bacterial growth. Other VAF except acetic acid was not produced during this fermentation (Takeno et al., 1999). Therefore, by vitamins addition, acidogenic fermentation, bacteria such as homo-acetic acid fermentation bacteria might be activated and digestion of organic matters from the mud might be proceeded.

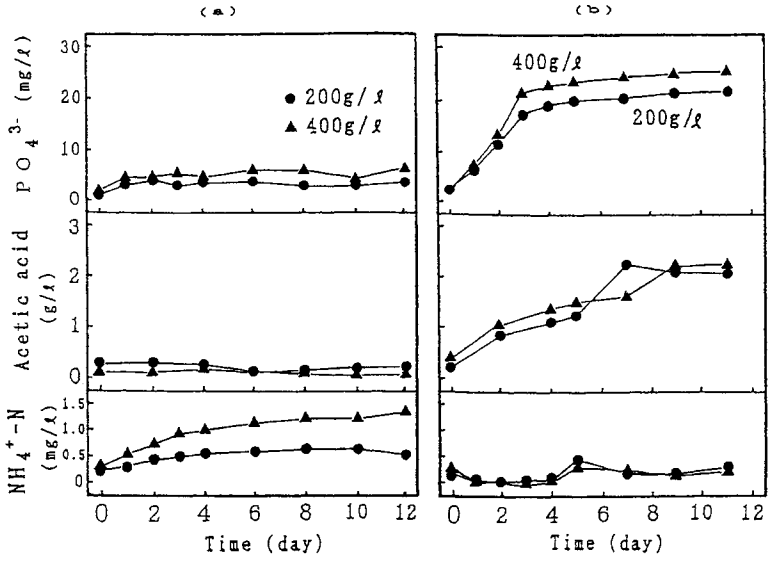


Figure 2: Anaerobic acidogenic fermentation of sediment mud suspension without vitamin addition (a) and with vitamin addition (b). The vitamin concentration added to the suspension was 1 mg/L of thiamine, 1 mg/L of nicotinic acid and 10 μg/L of biotin. ●, 200 g sediment mud/L artificial sea water; ▲, 400 g sediment mud/L artificial sea water.

3. Growth and Utilization of Acetic acid by Rhodobacter sphaeroides IL106

Growth and utilization of acetic acid by *Rhodobacter sphaeroides* IL106 was investigated when ammonium was added as a nitrogen source. As shown in Fig. 3, growth (OD_{660} and dry cell) was attained up to about 3 g/L after 2 days culture. And acetic acid was consumed by these cells together with the drastic consumption of phosphate. Ammonium and nitrate consumed. It was confirmed that this bacteria consume acetic acid in anaerobic digestion liquor sediment mud and produced cells under 3 % NaCl concentration condition and with some other substances such as trace amount of heavy metals.

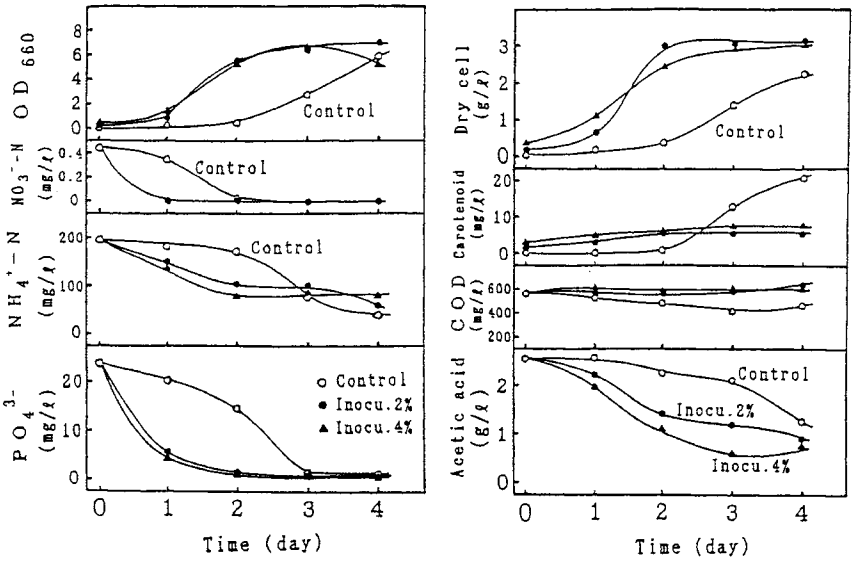


Figure 3: Profiles of cell growth (OD_{660} and dry cell), acetic acid, phosphate, ammonium, nitrate COD and intracellular carotinoid by *R. sphaeroides* IL106 on anaerobic acidogenic fermentation liquor of sediment mud. ○, control (no inoculation of IL106); ●, 2% (v/v) inoculation of IL106; ▲, 4% (v/v) inoculation of IL106. 200 mg/L of ammonium (NH_4^+-N) as added as $(NH_4)_2SO_4$.

4. Hydrogen Production with Strains Photosynthetic Bacteria

Hydrogen production was out from synthetic anaerobic digestion liquor of sediment mud (Modified medium) with low ammonium concentration two strain of *R. sphaeroides*. These two strains have relatively high growth activity among *Rhodobacter* sp. In addition, S strain can produce hydrogen from acetic acid as observed in Fig. 1. As shown in Fig. 4, gas production was started after growth was ceased at 30 hours culture in S strain and final gas produced was about 400 mL/L culture broth. Gas production rate was about 10 ml/g cell/h. However, in IL106 strain, gas production was quit low level. Thus, hydrogen production was quite differ depend on the strain of *Rhodobacter* sp. tested.

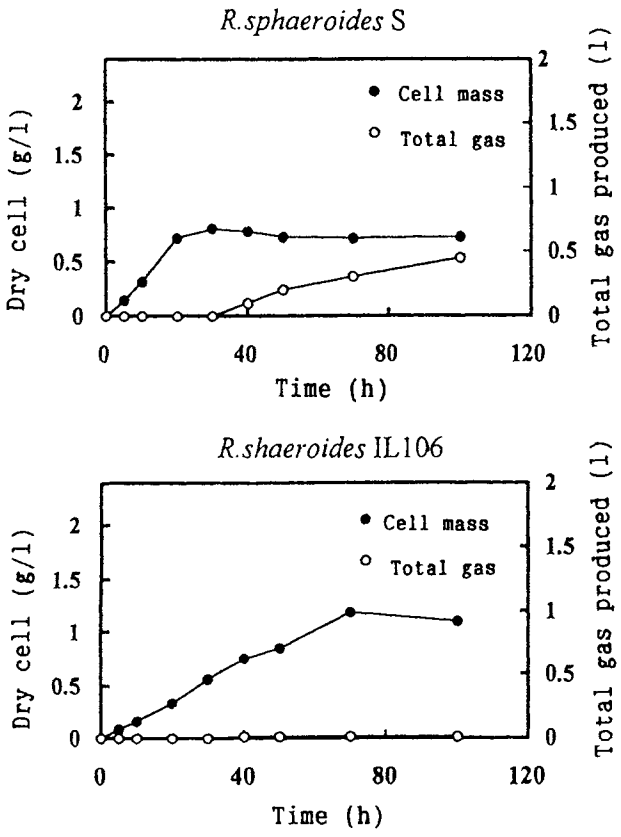


Figure 4: Hydrogen production and cell growth of *R. sphaeroides* S and *R. sphaeroides* IL106. ●, cell mass; ○, total gas (about 80% is hydrogen).

5. Relationship Between Hydrogen Production and PHA Accumulation

Quantitative relationship among hydrogen production, cell mass production and intracellular PHA accumulation were summarized in Table I with the experimental results using L-malic acid as the carbon and energy source after 4 days cultivation. Hydrogen production was relatively high level when L-malic acid was used as the energy source but intracellular PHA accumulation was relatively low in both strains. When acetic acid was used, hydrogen production was observed in S strain but quit low level of production in IL106 strain. However, PHA accumulation in IL106 strain was relatively high compared with that of S strain. This accumulation was comparable with that of *R. sphaeroides* under strict nitrogen deficient condition (Brandl et al., 1991). Major PHA in *R. sphaeroides* is recognized as PHB (poly-hydroxybutyrate) (Brandl, 1991; Suzuki et al., 1995). From these results, it that reducing power

produced in the cells of S strain by cyclic phosphorelation (photosynthesis) was easily consumed for hydrogen production but this power tended to for PHA accumulation IL106 strain. This phenomenon might be observed by the results of the competition between nitrogenase and PHA synthetase activities under each condition. However, it is still obscure why such system balance is formed. Ammonium concentration important role to share the reducing power to hydrogen and PHA synthesis. Because, biomass synthesis including protein synthesis was actually proceeded under ammonium rich condition and hydrogen production and PHA synthesis was completely. It is well known that ammonium presence repress the nitrogenase induction in the cell of photosynthetic bacteria (Hillmer and Gest, 1997) Khatipov et al. (1998) reported that hydrogen production and PHA accumulation were controlled by the nitrogen source. This problem should be elucidated in the further study.

TABLE 1
RELATIONSHIP BETWEEN HYDROGEN PRODUCTION AND PHA ACCUMULATION IN *R. SPHAEROIDES* S AND *R. SPHAEROIDES* IL106 WITH DIFFERENT CARBON AND ENERGY SOURCES

	R. sphaeroides S			*R. sphaeroides* IL106		
Carbon source	Total H_2 (ml)	Cell mass (g/l)	PHA (%)	Total H_2 (ml)	Cell mass (g/l)	PHA (%)
Malic acid	1970	0.95	10.6	235	0.84	27.3
Acetic acid	490	0.78	38.7	13	1.10	66.5

4 days culture

Hydrogen production and PHA accumulation in the cells of *Rhodobacter sphaeroides* was closely correlate and the balance of enzyme activities might results hydrogen production or PHA accumulation.

In addition, the possibility of hydrogen production acetic acid was observed. Although the production rate still low level, this observation might be significant considering the hydrogen production from the organic waste such as sediment mud of sea and lake.

REFERENCES

1. Brandl,H. Gross, R.A. Lentz, R.W., Lloyd, R., and Fuller, R.C. (1991). The accumulation of poly(3-hydroxyalkanoates) in *Rhodobacter sphaeroides*. *Arch. Microbiol.* **155**, 357-340.
2. Hillmer, P., and Gest, H. (1977). H_2 metabolism in the photosynthetic bacterium Rhodopseudomonas capsulata, production and utilization of H_2 by resting cells. *J. Bacteriol.*, **129**, 732-739.
3. Khatipov, E., Miyake, M., Miyake, J., and Asada, Y. (1998). Polyhydroxybutyrate accumulation and hydrogen evolution by *Rhodobacter sphaeroides* as a function of nitrogen avaibility, in BioHydrogen, ZaborSky, O.R. (ed.) Plenum Press, York and London, pp157-161.
4. Mao, X. Y., Miyake, J. and Kawamura, S. (1986). Screening photosynthetic bacteria for hydrogen production from organic acids. *J. Ferment. Technol.* **64**, 245-249.

5. Mitsui, A., Matunaga, H., Ikemoto, H., and Renuka, B.R. (1985). Organic and inorganic waste treatment and simultaneous photoproduction of hydrogen by immobilized photosynthetic bacteria, in Develoment in Industrial Microbiology. *Society of Industrial Microbiology* pp. 209-222.
6. Miyake, J., Mao, X. Y., Kawamura, S. (1984). Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butyricum*. *J. Ferment. Technol.* **62**, 531-535.
7. Sasaki, K., Ikeda, S., Nishizawa, Y., and Hayashi, M. (1987). Production of 5-aminolevulinic acid by photosynthetic bacteria. *J. Ferment. Technol.* **65**, 511-515.
8. Sasaki, K., Noparatnaraporn, N., and Nagai, S. (1991). Use of photosynthetic bacteria on the production of SCP and chemicals from sgroindustrial waset, in Bioconversion of Waste Materials to Industrial Products, Martin, A.M.(ed.), ElSevier Science Publishers, New York, London, pp. 223-265.
9. Sasaki, K., Takeno, K., and Emoto, Y. (1996). Utilization of organic acids and volatile fatty acids and hydrogen production by photosynthetic bacteria. *J. Soc. Water. Environ.* **19**, 63-70, (in Japanese).
10. Sasaki, K. (1998). Hydrogen and 5-aminolevulinic acid production by photosynthetic bacteria, in BioHydrogen, Zaborsky, O.R. (ed.) Plenum Press, New York, London, pp.133-142.
11. Suzuki, T., Tsygankov, A.A., Miyake, J., Tokiwa, Y., and Asada, Y. (1995). Accumulation of poly-(hydroxybutyrate) by a non-sulfur photosynthetic bacterium, *Rhodobacter sphaeroides* RV at different pH. *Biolechnol. Lett.* **17**, 395-400.
12. Takahashi, T. (1984). Hydrogen production nitrogenase of photosynthetic bacteria, in Kougousei Saikin (Photosynthetic Bacteria), Kitamura, H. et al.(ed.), Gakkai Shyppan Center, Tokyo, pp. 196-204.
13. Takeno, K., Sasaki, K., Watanabe, M., Kaneyasu, T. and Naomichi, N. (1999). Removal of phosphorous from sediment mud in oyster farm using a photosynthetic bacterium, *Rhodobcter sphaeroides* IL106. *J. Biosci. Bigen.* in press.
14. Turkarslan, S., Yigit, D., O., Aslan, K., Eroglu, I., and Gunduz. U. (1998). Photobuilogical hydrogen production by *Rhodobcter sphaeroides* O.U.001 by utilization of waste water from milk industry, in BioHydrogen, Zaborsky, O. Z. (eds), Plenum Press, New York, London, pp 151-156.

HYDROGEN FROM BIOMASS

T. Wakayama and J. Miyake,

National Institute for Advanced Interdisciplinary Research,
AIST/ MITI,
1-1-4 Higashi, Tsukuba, Ibaraki, 305-8562 Japan

ABSTRACT

Hydrogen, which does not liberate carbon dioxide during combustion, is considered to be an important alternative energy resource of the future. Hydrogen can be produced by the unused energy source as biomass. Biomass is produced by the actions of air, water and soils using sunlight as energy source, it is an renewable source of clean energy. However, only a small portion of biomass is presently used, and it is the unused source that should be used effectively.

Biological hydrogen production could be a potentially environmentally acceptable energy production method. Particularly, photo-hydrogen production by photosynthetic bacteria has the advantage that with sunlight as the energy source, hydrogen and biomass can be produced simultaneously. This feature is affective for the promotion of environmentally acceptable energy production technology aimed at producing hydrogen from organic wastewater.

KEY WORDS:

Photosynthetic bacteria, biomass, photo-hydrogen production, sunlight, organic waste water,

1. INTRODUCTION

Biomass is produced by the actions of air, water and soils using sunlight as energy source, it is an renewable source of clean energy. However, only a small portion of biomass is presently used, and it is the unused source that should be used effectively (TABLE 1). Approximately two billion tons of biomass is present on the earth, and 0.2 billion tons are increased every year. Biomass produced per year corresponds to approximately 10 times of yearly world energy consumption. Biomass can also be used as a substrate for energy production, particularly for hydrogen production.

TABLE 1
THE BIOMASS ENERGY UTILIZATION IN THE WORLD

	Population (million)	Total demand of energy (EJ)	Consumption Biomass: Traditional technology	Consumption Biomass: New technology
North America	276	95.6	1.6	0.8
Western Europe	454	57.9	0.8	0.4
East Europe	386	68.8	1.3	0.4
Japan, Australia	144	21.1	0.2	0.3
Total of a developed nation	1,263	243.4	5.9	1.9
South America	448	17.5	5.3	1.9
Middle and Near East, North Africa	271	12.3	0.9	0.0
Sahara Africa	501	12.2	5.9	0.2
Pacific, Southeast Asia	1,163	45.8	14.6	0.7
South Asia	1,146	20.9	8.6	0.3
Total of a developing nation	4,029	108.3	35.3	3.1
Total of the world	5,292	352.3	39.2	5.0

New Renewable Energy Resources, 1994 (World Energy Council)

By use of microorganisms, hydrogen can effectively be obtained from wood and marine biomass according to purposes. Diverse microorganisms are able to produce hydrogen, widely distributing from photosynthetic microorganisms that depend on light energy for acquiring necessary energy for growth to non-photosynthetic microorganisms that depend on organic and inorganic compounds (TABLE 2). Microorganisms capable of producing hydrogen are classified into anaerobic bacteria, fermentation bacteria, aerobic bacteria, photosynthetic bacteria, and algae. These microorganisms can be used alone or in mixture of multiple microorganisms depending on the biomass to be used.

TABLE 2
CLASSIFICATION OF HYDROGEN EVOLUTION BACTERIA

Available Energy Form	Enzyme of H_2 Evolution	A class of Bacteria		A Genus of Bacteria	Electron Donor
Photosynthesis	Hydrogenase	Green Algae		*Chlamydomonas*	Water
		Blue-Green Algae	Heterocyst	*Chlorella*	↑
				Anabaena	↑
			Non-Heterocyst	*Oscillatoria*	↑
	Nitrogenase	Photosynthetic Bacteria	Non-sulfur Bacteria	*Rhodopseudomonas*	Organic Matters (Organic Acids)
				Rhodobacter	↑
				Rhodospirillum	↑
			Sulfur Bacteria	*Chromatium*	Sulfates
				Thiocapsa	↑
Non-Photosynthesis	Hydrogenase	Obligate Anaerobes		*Clostridium*	Organic Matters (Sugers)
				Methanobacterium	↑
		Facultative Anaerobes		*Escherichia*	↑
	Nitrogenase	Nitrogen Fixing Bacteria	Facultative Aerobes	*Azotobacter*	↑
				Clostridium	↑
			Facultative Anaerobes	*Klebsiella*	↑

2. PHOTO-BIOLOGICAL HYDROGEN PRODUCTION BY PHOTOSYNTHETIC MICROORGANISMS (BACTERIA)

The advantage of the use of photosynthetic microorganisms is capability of using light energy such as sunlight as an energy source(Figure 1). Photosynthetic microorganisms are roughly divided into algae (including cyanobacteria) that are able to use water as electron donor and photosynthetic bacteria that depend on organic compounds. Both microorganisms are capable of photobiological hydrogen production, however, algae and cyanobacteria are not suitable for hydrogen production, because these microorganisms does not directly degrade organic compounds such as biomass. Accordingly, in this chapter, photo-biological hydrogen production by photosynthetic bacteria is described.

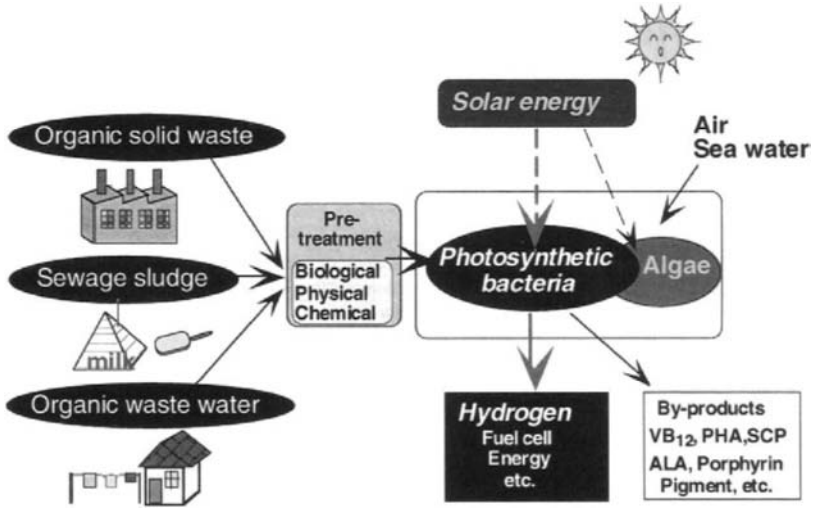


Figure 1: Conceptual illustration for photo-hydrogen production by photosynthetic organisms.

As an advantage of photo-biological hydrogen production by photosynthetic bacteria, waste water disposal and energy production can be simultaneously performed using organic waste water as a substrate(TABLE 3). Photosynthetic bacteria are able to grow by utilizing organic acids, carbohydrates, sulfuric compounds such as hydrogen sulfide. Photosynthetic bacteria are able to rapidly assimilate volatile fatty acids represented by acetate and propionate even at a high concentration. Thus, photosynthetic bacteria are practically used in waster water disposal of Tofu factory and animal barn. Alcohol is also a promising substrate for hydrogen production because of a high hydrogen to carbon ratio.

TABLE 3
PHOTO-HYDROGEN PRODUCTION FROM BIOMASS

Substrates	Strains	Production rate
Organic acids		
Malate	*Rba. capsulatus*	130–168 $mm^3 \cdot h^{-1} \cdot mg\ dcw.^{-1}$
Malate	*Rba. sphaeroides*	138–262 $mm^3 \cdot h^{-1} \cdot mg\ dcw.^{-1}$
Lactate	*Rba. sphaeroides* RV	62 $ml \cdot h^{-1} \cdot g\ dcw^{-1}$
Lactate	*Rsp.* Gunes	0.6 $dm^3\ H_2 \cdot dm^3\ culture^{-1} \cdot h^{-1}$
Mixed VFA	*Rba. sphaeroides* NR3	1.7 $dm^3\ H_2 \cdot dm^3\ culture^{-1} \cdot d^{-1}$
Mixed VFA	*Rba. sphaeroides* RV	2.0 $dm^3\ H_2 \cdot dm^3\ culture^{-1} \cdot d^{-1}$
Aromatic acids	*Rps. palustris* DSM 131	310 $\mu mol\ H_2 \cdot h^{-1} \cdot g\ dcw.^{-1}$
Sugar		
Raw corn	*Rps. gelatinosa* T-20	74 $dm^3 \cdot kg\ corn\ starch^{-1}$

Raw potato	*Rps. gelatinosa* T-20	1.3 $ml \cdot h^{-1}$
Raw cassava	*Rps. gelatinosa* T-20	0.5 $ml \cdot h^{-1}$
Glucose	*Rsp. rubrum* KS-301	91 $ml \cdot h^{-1}$
Sulfur compounds		
Hydrogen sulfate	*Chromatium* sp. PBS 1071	6 $mol \cdot h^{-1} \cdot mg\ dcw.^{-1}$
Food waste		
Yogurt waste	*Rps. rubrum* S-I	12-20 ml $H_2 \cdot dm^3\ culture^{-1} \cdot 10\ d^{-1}$
Whey waste	*Rps. rubrum* S-I	8-20 ml $H_2 \cdot dm^3\ culture^{-1} \cdot 10\ d^{-1}$
Sugar refinery	*Rps. palustris*	35-50 µl $H_2 \cdot h^{-1} \cdot mg\ dry\ cell^{-1}$
Sugar cane	*Rps. capsulata* DSM 1710	14 µl $H_2 \cdot mg \cdot Chl^{-1} \cdot h^{-1}$
Tofu waste	*Rba. sphaeroides* RV	12.9 ml $H_2 \cdot ml\ culture^{-1}$
Agricultural waste		
Orange process waste	*Rps.* sp. Miami PBE2271	90 $mm^3 \cdot g\ dcw.^{-1}$
Still waste	*Rba. sphaeroides* O.U.001	0.5 $m^3 \cdot 144\ h^{-1}$
Starch waste	*Rps.* sp. BHU 1-4	88 µl $H_2 \cdot h^{-1} \cdot mg\ dcw.^{-1}$
Glucose waste	*Rps.* sp. D	0.5 $dm^3\ H_2 \cdot dm^3\ culture^{-1} \cdot d^{-1}$
Cow dung	*Rps. rubrum* S-I	6.3 $mm^3\ H_2 \cdot h^{-1} \cdot mg\ dry\ cell^{-1}$
Rice	*Rps. rubrum* S-I	35 $mm^3\ H_2 \cdot h^{-1} \cdot mg\ dry\ cell^{-1}$
Organic waste water		
Paper mill	*Rsp. molischianim*	70–139 µl $H_2 \cdot h^{-1} \cdot mg\ dry\ cell^{-1}$
Heated sludge	*Rba. sphaeroides* RV	0.7 $dm^3\ H_2 \cdot dm^3\ culture^{-1} \cdot d^{-1}$
Lactic fermentated vegetable	*Rba. sphaeroides* RV	62 ml $H_2 \cdot g\ dcw.^{-1}$

2.1. Photo-biological Hydrogen Production from Organic Acids

Photosynthetic bacteria can rapidly assimilate volatile fatty acids and grow. Because it assimilates organic compounds, hydrogen production from various fatty acids has been investigated. As for volatile fatty acids, lactate obtained after lactate fermentation of carbohydrates and acetate contained in processed solution after methane fermentation can be used as a substrate.

A high hydrogen production by various photosynthetic bacterial strains using malate and lactate as single substrates has been reported, such as 130–168 $mm^3 \cdot h^{-1} \cdot mg\ dcw.^{-1}$ produced by *Rps. capsulata* (*Rba. capsulatus*) and 138–262 $mm^3 \cdot h^{-1} \cdot mg\ dcw.^{-1}$ produced by *Rba. sphaeroides*. As for lactate, a production velocity of 62 $ml \cdot h^{-1} \cdot g\ dcw.^{-1}$ was obtained using *Rba. sphaeroides* RV strain, eliminating 38% of the substrate lactate, and the conversion rate from substrate to hydrogen reached 42%. It has been reported that using immobilized bacteria of *Rsp.* genus, photobiological hydrogen was produced from lactate and the velocity of 0.6 $dm^3\ H_2 \cdot dm^3\ culture^{-1} \cdot d^{-1}$ was obtained.

In organic waste water contains various organic compounds, particularly volatile fatty acids. Therefore, study of photo-biological hydrogen production from substrate mixture consisting of two or three of acetate, butyrate, and propionate various ratios has been proceeded by *Rba. Sphaeroides* NR3 and RV strains. Hydrogen production and organic acid consumption velocities were markedly improved at a certain ratio of organic acid mixture.

The efficient hydrogen production from organic acid mixture is useful information for utilization of actual waste water.

Photo-biological hydrogen production from aromatic compounds, which are unlikely to serve as substrates, has been attempted. Using various immobilizing agents, *Rps. palustris* DSM 131 strain was immobilized and photo-biological hydrogen production from aromatic fatty acids was investigated. A velocity of 310 μmol $H_2 \cdot h^{-1} \cdot g$ dcw.$^{-1}$ was obtained under 10 klux. Bacterial cells immobilized by alginate gel showed a greater hydrogen production velocity than free bacteria.

2.2. Photo-biological Hydrogen Production from Carbohydrates

Not only monosaccharide such as glucose, polysaccharides such as starch are degraded to glucose and used as substrates for hydrogen production. It is possible to produce hydrogen from soluble starch, which is generally difficult to use as a substrate, as well as directly from raw starch such as corn, potato, and cassava.

A strain closely related to *Rps. gelatinosa* (*Rubrivivax gelatinosus*) was isolated from tropical soil. The substrate range applicable to hydrogen production is gradually widening with discovery of new species and genera. Using *Rps. gelatinosa* T-20 strain, hydrogen production from various starch has been investigated. The hydrogen production was decreased in the order of raw corn, raw potato, and raw cassava. This order was the same as that of hydrolytic activity of T-20 strain Amylase for each starch. T-20 strain produced a maximal level of 74 dm^3 hydrogen per kg raw corn starch.

Using *Rhodosprillum rubrum* KS 301 strain, continuous photo-biological hydrogen production was tried using a 1.5 l Nossle loop type photo-bioreactor. With glucose substrate, a hydrogen production velocity of 91 $ml \cdot h^{-1}$ was obtained under 12 klux irradiation.

2.3. Photo-biological Hydrogen Production from Sulfuric Compounds

Since photosynthetic sulfur bacteria are able to grow using sulfuric compounds as electron donors, and hydrogen production using sulfuric compounds are being investigated. Not only environment-integrated Photo-hydrogen production but also active elimination of environmental pollutant is expected. Hydrogen sulfide is a causative substance of offensive odor in waste water and sewage disposal plants. A minute amount is also contained in exhaust gas from methane fermentation and a large amount is recovered from oil purification processing.

Using purple and green photosynthetic sulfur bacteria; *Chromatium*, *Thiocapsa*, and *Thiocystis*, hydrogen production has been investigated using sulfuric compounds such as Na_2S as a substrate. Utilizing this ability, technology of processing sulfuric compounds and hydrogen sulfide is being investigated. It has been reported that free sulfur was produced by eliminating hydrogen sulfide using *Ectothiorhodospira*. Not only sulfur recovery from hydrogen sulfide, but also hydrogen production was studied and hydrogen production was possible. Immobilized marine purple photosynthetic sulfur bacteria *Chromatium* sp. Miami PBS 1071 strain produced hydrogen at a velocity of 6 $mol \cdot h^{-1} \cdot mg$ dcw.$^{-1}$. Similarly, it was reported that hydrogen was obtained by outdoor culture of marine *Chromatium* at a hydrogen sulfide concentration from 2.5 $mol \cdot m^{-3}$ to 20 $mol \cdot m^{-3}$.

2.4. Photo-biological Hydrogen Production from Food Waste

Agricultural/livestock waste and organic waste water are obtained in large amounts at relatively many sites with low cost, and are also useful materials for hydrogen production. Waste water from food factory particularly contains large amounts of organic components and the components are stable with a small variation, and thus are usable as substrates of photo-biological hydrogen production. Substrates that photosynthetic bacteria do not directly use can be used by combining with methane or lactiate fermentation. It is also advantageous that using waste water as substrate, hydrogen production and swage disposal can be performed simultaneously.

Hydrogen production for several weeks by *Rps. rubrum* S-1 strain from yogurt waste water and whey discharged from daily product factory has been reported. Using *Rps. palustris* immobilized by agar, hydrogen production from waste water from sugar refinery has been studied. It tried photo-biological hydrogen production from sugar waste water using *Rps. capsulata* DSM 1710 strain, and a velocity of approximately 14 $\mu l\ H_2 \cdot mg \cdot BChl^{-1} \cdot h^{-1}$ was maintained even if the concentration of waster water was changed.

Photo-biological hydrogen production from waste water of Tofu factory was also tried. Photo-biological hydrogen production from agricultural/livestock waste is also being investigated. It reported that a marine isolate belonging to genus *Rps.* produces hydrogen using pectin and cellulose, which are contained in agricultural waste in large quantities, as electron donors, in addition to use of various organic acids and carbohydrates. Using marine photosynthetic bacteria *Rps.* sp. Miami PB2271 strain immobilized on polyethylene film, 90 $mm^3 \cdot mg\ dcw.^{-1}$ of hydrogen was produced from waste water of orange processing, and TOC in the waste water was decreased from 430 $g \cdot m^{-3}$ to 270 $g \cdot m^{-3}$. Using *Rba. sphaeroides* O.U.001 strain (ATCC 49419; DSM 58649), 0.5 m^3 of hydrogen was produced during 144 hours from waste water of distillation tank.

Hydrogen production from supernatant of acid-fermented vegetables and fruit scraps by *Rba. sphaeroides* RV strain has been investigated. Medium containing various essential metals was added to the supernatant and the mixture was subjected to photo-biological hydrogen production. It was confirmed that Mo affects the growth and hydrogen production. Using *Rps.* sp. BHU 1-4 strain, photobiological hydrogen production from starch and sugar waste waters was investigated at 45°C. Immobilized bacteria produced hydrogen at a velocity of 88 $\mu l\ H_2 \cdot h^{-1} \cdot mg\ dcw.^{-1}$. Using *Rps.* sp. D strain immobilized by agar, photo-biological hydrogen production from glucose and lactate was investigated. As for glucose substrate, 70% of COD was eliminated.

2.5. Photo-biological Hydrogen Production from Organic Waste Water

Studies of photo-biological hydrogen production from organic waste water and organic compounds, which were generally considered difficult to use as substrates, have been reported. Since organic waste water and sludge contain large amounts of organic compounds and are obtained in large quantity at relatively many sites with low cost, these are promising unused source.

With *Rsp. molischianim* immobilized by agar, hydrogen production from papermill water waste was continued for 35 days, and 30% of COD was treated. Photo-biological hydrogen production from heat-eliminated liquid of sludge was investigated using *Rba. sphaeroides* RV strain. Stable continuous culture for 90 hours was possible even if it was outdoor culture. *Rps. palustris* DSM 131 strain was immobilized by various immobilizing agents and photo-biological hydrogen production from aromatic fatty acids was studied. The conversion rates from consumed fatty acids; benzoate and sinamate, were 88% and 86%, respectively. From

the major components of insecticides such as 2,4-D and monochlopotos, 170 μl·vessel^{-1} of hydrogen was produced under 2.4 klux.

For hydrogen production by photosynthetic bacteria using sunlight and organic waste water, immobilization of bacterial cells to various supports is being investigated to physically stabilize and improve the hydrogen-producing activity. As immobilizing supports by including immobilizing method, agar, which is natural macromolecular gel, calcium alginate, and carrageenan are frequently used.

They attempted photo-biological hydrogen production from organic waste water using immobilized *Rha. sphaeroides* O.U.001 strain. By immobilizing bacteria, the bacteria was made more tolerable in a high concentration of waste water than free bacteria and the hydrogen-producing ability was increased 10-fold or more.

2.6. Photo-biological Hydrogen Production Using Sunlight

As the greatest advantage of photo-biological hydrogen production by photosynthetic microorganisms, sunlight can be used as energy source. However, only a limited number of study on outdoor hydrogen production has been performed, despite that utilization of sunlight is essential for practical photo-biological hydrogen production.

Factors interfering the use of sunlight for photo-biological hydrogen production include those dependent on sunlight property such as great variation of sunlight by weather and season and large daily change. There are other interfering factors; photo-biological hydrogen production is saturated under the light intensity at meridian transit, the light is absorbed and scattered by photosynthetic bacteria and does not permeate deep in the photo-bioreactor, and photosynthetic bacteria-dependent factors such as dependency of photo-hydrogen production on temperature.

Practical places for photo-biological hydrogen production using sunlight are so-called sunbelt zone, where strong sunlight intensity and long daylight hours are available. Photosynthetic bacteria absorb near infrared spectrum lights and the reactor temperature increases. Therefore, it is desirable to use heat-resistant bacteria.

As for photosynthetic bacteria, it has been reported that a maximal hydrogen production velocity of approximately 4 dm^3·h^{-1} was obtained using heat-resistant strain of *Rba. sphaeroides* isolated in Thailand in batch culture in a 33 dm^3 reactor. Using a 6 dm^3 reactor, continuous hydrogen production was performed by semi-continuous batch culture for 45 days. It was reported that photo-biological hydrogen production from degraded cassava starch was performed in Bangkok, Thailand, and 737 ml·l^{-1} of hydrogen was produced from Arabic gum substrate. They reported a maximal production velocity of 1.6 dm^{3}H$_2$·m^{-2}·h^{-1} by immobilized *Rps. palustris* 42 OL strain. It also obtained production of 138 mm^3·h^{-1}·mg dcw.$^{-1}$ hydrogen at 40°C by *Rba. sphaeroides* B5 strain isolated from the tropical zone. Screening of photosynthetic bacteria with high hydrogen-producing ability is proceeding, and a novel phenotype *Rba. marinus* NKPB0021 strain was reported, which was isolated from sea and has hydrogen-producing ability. Highly hydrogen-producing *Rba. sphaeroides* RV strain was characterized and 190μl·h^{-1}·mg dcw.$^{-1}$ of hydrogen was produced at 35°C under 5 klux. They screened for photosynthetic bacteria with a high hydrogen-producing ability at a high temperature and isolated M0006 strain, which produced 11 ml·h^{-1}·70 ml^{-1} of hydrogen.

Since a bacterial strain with hydrogen-producing ability at 45°C or higher has not yet been obtained, investigation and development of bacterial strains capable of producing hydrogen at a high temperature is desired to make photo-biological hydrogen production more efficient.

Sunlight at meridian transit is excess for hydrogen production by photosynthetic bacteria,

and the conversion rate from light to hydrogen is markedly decreased. To overcome this problem, diffuse irradiation of excess sunlight is being investigated.
With *Rba. sphaeroides* RV strain, spatial diffusion of excess light was investigated using a reactor equipped with shades. By setting shades on the light-receiving surface of the reactor, the conversion rate was improved by 1.4-fold. Temporal diffusion of sunlight is also being investigated.

3. BIOLOGICAL HYDROGEN PRODUCTION BY NON-PHOTOSYNTHETIC MICROORGANISMS

It is known that anaerobic bacteria such as *E. coli* produce hydrogen in anaerobic carbon metabolism. Currently used microorganisms in hydrogen production study other than photosynthetic microorganisms are mainly anaerobic bacteria; genera *Clostridium* and *Klebsiella*. Hydrogen production is catalyzed by Hydrogenase in genera *Clostridium* and *Citorobacter* and by Nitrogenase in genera *Klebsiell*a and *Azotobacter*. These microorganisms assimilate organic compounds such as glucose and the hydrogen production velocity is high. However, because they depend on organic compounds for all energy required for growth and reducing power, unlike photosynthetic microorganisms, they can not completely degrade substrates and additionally produce organic acids, the final product of anaerobic metabolism.

3.1. Biological Hydrogen Production by Anaerobic Bacteria

In genus *Clostridium* that uses Hydrogenase, hydrogen-producing reaction is an equilibrium reaction and the energy consumption is small. When glucose is substrate, it is theoretically possible to produce 4 mols of hydrogen from 1 mol of glucose, however about 1-2 mols are experimentally obtained.

Hydrogen production by Nitrogenase requires ATP, however ATP synthesis by anaerobic fermentation is not high in genus *Klebsiella*, resulting in a low hydrogen production per unit substrate. Theoretical hydrogen yield is 0.5 mol per 1 mol glucose. Non-photosynthetic microorganisms are also immobilized in many cases. *Clostridium butyricum* is immobilized to various supports and used for hydrogen production from glucose. As immobilizing supports, a mixture of agar + acetylcellulose is superior. The hydrogen production efficiency was improved using agar + acetylcellulose compared to those using polyacrylamide and agar alone. Addition of peptone and riboflavin facilitated hydrogen production.

As for anaerobic bacteria, hydrogen production by *Clostridium butyricum* from alcohol fermentation water waste has been investigated and use of produced hydrogen for fuel battery has been attempted and a maximal electricity of 13 W was obtained.

Hydrogen production from cellulose by thermophillic *Clostridium butyricum* is being investigated. Hydrogen production from plant leaves by *Enterobacter* and from rice straws and garbage by rumen bacteria are being investigated.

4. PHOTO-BIOLOGICAL HYDROGEN PRODUCTION BY MIXED CULTURE WITH PHOTOSYNTHETIC BACTERIA

Since biomass is unused source relatively readily obtained in a large quantity, it is very valuable as unused energy source. Anaerobic bacteria easily degrade biomass such as macromolecular polysaccharides and produce hydrogen at a high velocity. However, anaerobic bacteria cannot completely degrade organic compounds and organic acids accumulate. Accordingly, the hydrogen production efficiency is low. In contrast, photosynthetic bacteria utilize organic acids more than organic compounds. Furthermore, photosynthetic bacteria are able to completely degrade substrates to hydrogen by use of light energy, resulting in a high hydrogen production efficiency. Accordingly, hydrogen production by mixed culture system was considered, in which a combination of the two bacterial species is used, making use of the characteristics of each species. Anaerobic bacteria degrade organic compounds to organic acids and hydrogen, and the produced organic acids are converted to hydrogen by photosynthetic bacteria. This method improves hydrogen production efficiency and widen the range of organic materials useful for hydrogen source.

4.1. Photo-biological Hydrogen Production by Mixed Culture with Anaerobic Bbacteria

In 1984, Miyake et al. investigated hydrogen production from glucose using *Clostridium* and *Rba. sphaeroides* RV strain immobilized by agar. *Rba. sphaeroides* RV strain does not produce hydrogen from glucose, and *Clostridium* produces only a minute amount, about 1 mol. However, in mixed culture system of these bacterial strains, seven-fold higher hydrogen production was possible. They reported photo-biological hydrogen production from starch by mixed culture of *Clostridium butyricum* and *Rba.* sp. M-19 strain. It is likely that both bacteria synergistically affect the hydrogen production in mixed culture systems. A combination of *Chlorobium* and *Desulfuromonas* is being investigated.

Researchers investigated hydrogen production from glucose, cellulose, and sawdust by agar-immobilized mixed culture system of *Klebsiella pneumoniae* and *Rsp. rubrum.* Hydrogen production from cellulose by mixed culture system of *Cellulomonas* sp. strain ATCC21399 and *Rba. capsulatus* B100 strain has been also tried. In this combination, *Cellulomonas* does not have hydrogen-producing ability. Another researchers also tried photo-biological hydrogen production from cheese whey using *Rsp. rubrum* S-II and *E. coli* wild strain. They reported hydrogen production by mixed culture system of *Chlamydomonas reinharditii* C-238, which is algae, and *Rsp. rubrum* NCIB 8255. Photo-biological hydrogen production from starch by mixed culture of *Rba. sphaeroides* and *Pseudomonas*, and *Rhodobium marium* and *Vibrio fluvialis* or *Proteus vulgalis* was also reported.

In recent years, global warming phenomena due to greenhouse gases such as carbon dioxide have received attention as global environmental problems. To decrease the amount of carbon dioxide emission, it is necessary to decrease consumption of fossil fuels and increase the use of new regenerative energies represented by natural energy. A typical new energy source is sunlight.

To collect widely dispersed energy such as sunlight, rapidly progressing biotechnology and microbial conversion technique have been attracting attention. Photosynthetic microorganisms, which acquire energy by photosynthesis and grow, possess variety of specific

functions such as carbon dioxide fixation, nitrogen fixation, oxygen production and hydrogen production, and are particularly expected for use of such functions.

From such a background, construction of environmental purification system and environment-integrated energy production system using useful functions of photosynthetic microorganisms such as photosynthetic bacteria and microalgal fungi has been attempted. Among these attempts, photobiological hydrogen production using photosynthetic bacteria can be applied to construction of a system that purifies the environment and produces clean energy simultaneously because photosynthetic bacteria are able to use solar energy as the energy source as well as use unused resources as the substrate such as organic waste water.

REFERENCES

1. Hall, D. O. (1999). Will Biomass Environmentally Friendly Fuel of the Future?. *Biomass and Bioenergy* **15**, 357-367.
2. WEC. (1994). New Renewable Energy Resources. World Energy Council.
3. Kitamura, H. et al., Ed. (1984). Kougousei Saikin (means Photosynthetic Bacteria, in Japanese), Gakkai Syuppan Center Biseibutsu, Tokyo, Japan.
4. Benemann, J.R. (1994). Feasibility analysis of photo-biological hydrogen production, Hydrogen Energy Progress. *Int. J. Hydrogen Energy*. **2**, 931-940.
5. Weaver, P. F. et al. (1980). Photo-biological Production of Hydrogen. *Solar Energy* **24**, 3-45.
6. Miyake, J. (1998). The Science of Biohydrogen: An Energetic View. p. 7-18. In Zaborsky, O. R., Benemann, J. R., Matsunaga, T., Miyake, J., Pietro, A. S. (eds.), Biohydrogen, Plenum publishing corp. New York.
7. Asada, Y. et al. (1999). Photobiological Hydrogen Production. *J. Biosci. Bioeng* **88**, 1-6.
8. Miyake, J. et al. (1999). Simulation of the Daily Illumination Pattern of Sunlight for Bacterial Photo-hydrogen Production. *J. Biosci. Bioeng.* **88**, 659-663.
9. Wakayama, T. et al. (2000). Effect of Light/dark Cycle on Bacterial Hydrogen Production by *Rhodobacter sphaeroides* RV -From hour to second range-. *App. Biochem. Biotechnol.* **84-86**, 431-440.
10. Miyake, J. et al. (1984). Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic Bacterium and *Clostridium butyricum*. *J. Ferment. Technol.* **62**, 531-535.

This Page Intentionally Left Blank

HYDROGEN PHOTOPRODUCTION FROM STARCH IN ALGAL BIOMASS

A. Ike, H. Kawaguchi, K. Hirata, and K. Miyamoto

Environmental Bioengineering Laboratory,
Graduate School of Pharmaceutical Sciences,
Osaka, University, 1-6 Yamadaoka Suita, Osaka 565-0871, Japan

ABSTRACT

For the photoproduction of H_2 from a CO_2-fixing algal biomass, a two-step process consisting of lactic acid fermentation as pretreatment preceding the H_2 production, and single-step process in which a halotolerant bacterial community directly converted starch in the algal biomass to H_2, were proposed. The bacterial community designated BC1 contains three halophilic or halotolerant bacterial species, *Vibrio fluvialis*, *Rhodobium marinum* and *Proteus vulgaris*. Among them, *V. fluvialis* and *R. marinum* play roles in the degradation of starch into acetic acid and ethanol, and the production of H_2 from the degraded products, respectively. A study using the starch-rich microalgae, *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta*, demonstrated the H_2 production efficiencies of the above two processes.

INTRODUCTION

Among the many global environmental problems facing society today, "the green house effect" is widely recognized as being one of the most serious. To address this problem, CO_2 fixation by microalgae is considered to be an environmentally friendly, energy-saving measure. On the other hand, microalgal systems produce large amounts of waste biomass which could easily reverted to CO_2. Therefore, it is necessary to establish processes by which algal biomass can be recycled for resource production. Algal biomass could be supplied from the oxidation pond in waste-water treatment plants, or from eutrophicated lakes. It can be converted to various energy media, such as methane (1) and oil (2). We have proposed a system for converting algal biomass into H_2, biologically. Among various microorganisms which produce H_2, photosynthetic bacteria have the advantage that they can carry out H_2 production from organic substrates using solar energy. Therefore, a cost effective energy production process could be achieved using photosynthetic bacteria.

In this article, we propose two processes to convert algal biomass into H_2 using photosynthetic bacteria (Fig. 1). Starch, the major constituent of algal biomasses, is rarely degraded by photosynthetic bacteria alone to the low-molecular-weight substrates convertible

to H_2. Some thermochemical, enzymatic, or biological degradation of the polymers is required as a pretreatment process for the biomasses. Furthermore, pretreatment processes are necessary for the liquefaction of algal cells enveloped within hard cell wall. We propose a process involving a two-step conversion, *i.e.*, starch fermentation to lactic acid and them H_2 production from the lactic acid. Using a starch-degrading lactic acid bacterium, *Lactobacillus amylovorus*, starch in algal cells is degraded with high efficiency into lactic acid (3, 4). Lactic acid is a suitable substrate for H_2 production by photosynthetic bacteria.

In terms of cost-saving, however, a single-step biological cultivation process is more desirable than a two-step process. There have been only a few reports concerning single-step H_2 production from starch (5, 6). In our previous work, we obtained a halophilic bacterial community which could directly utilize starch for H_2 production in a single-step culture process (7, 8). In this work, the constituent bacterial species in the community were isolated and their roles in H_2 production from starch were characterized. Furthermore, algal biomass were fed into coculture of starch-degrading and photosynthetic bacterial strains isolated from the community.

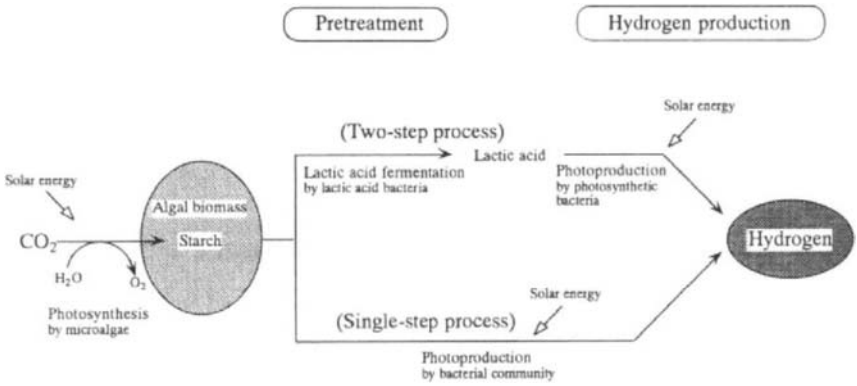


Figure 1: Pathways of H_2 production from an algal biomass.

MATERIALS AND METHODS

Algal Biomass Used for H_2 Production

A freshwater green alga *Chlamydomonas reinhardtii* (IAM C-238) and a marine green alga *Dunaliella tertiolecta* (ATCC 30929) were grown photoautotrophically in modified Bristol medium (9) and modified f/2 medium (10), respectively. Algae were cultivated at 30°C for 10 d in a cylindrical glass bottle (working volume, 2 *l*; diameter, 10 cm) in the presence of 1% CO_2 gas supplied continuously, under fluorescent lamp illumination (10 W/m^2). Algal cells were harvested by centrifugation (13,000 X g, 10 min) and diluted in the medium used for the H_2 production tests without the substrate, to give a final starch concentration of 4.05 g/*l*.

H_2 Production by Photosynthetic Bacteria and Bacterial Communities

The photosynthetic bacteria used for H_2 production were *Rhodobacter sphaeroides* RV, *Rhodobacter capsulata* (ATCC 11166), *Rhodospirillum rubrum* (ATCC 11170), *Rhodovulum sulfidophilus* (ATCC 35886), and *Rhodobium marinum* (ATCC 35675). The medium components contained in the basal medium (3) in addition to 10 mM sodium glutamate and 0.15% $NaHCO_3$ were added to the lactic acid fermentate, raw algal biomass or artificial media. After degassing, the fermentate (60 ml), the raw algal biomass (60 ml) or the medium (65 ml) was placed in a glass tube (volume, 70 ml; diameter: 2.5 cm) with a gas outlet and a sampling nozzle. An actively growing culture of photosynthetic bacteria or bacterial communities were harvested by centrifugation (6300 X g, 10 min) and resuspended in each test medium to obtain a final OD_{660} of 0.6. The glass tubes were incubated at 30°C under tungsten lamp illumination (330 W/m^2) until H_2 evolution ceased (approx. 100 h). The evolved gas trapped in a 100 ml glass cylinder above a water bath.

Lactic Acid Fermentation of Algal Biomass

For lactic acid fermentation, a lactic acid bacterium *Lactobacillus amylovorusr* (ATCC 33620) capable of hydrolysing starch (11) was employed. Two point five ml of an actively growing *L. amylovorus* cell suspension culture (OD_{600} = ca. 9) was harvested by centrifugation (17,000 X g, 10 min) and added to 25 ml of the concentrated raw algal biomass described above. The inoculated samples were incubated at 37°C for 2-4 d in 50 ml plastic bottles with screw caps. To prevent cessation of fermentation caused by a dropping pH accompanying the lactic acid production, 500 mg of $CaCO_3$ was added before the fermentation, or 10 N NaOH was every 6 h throughout the lactic acid fermentation (to adjust pH to 6.0), as a neutralizer.

Bacterial Community Consisting of Anaerobic Bacteria and Photosynthetic Bacteria

A bacterial community was obtained from activated sludge from a night soil (human feces) treatment plant which employs a seawater dilution system to prevent rise in reactor temperature due to the high BOD content of night soil. After one month of enrichment under light illumination, red, orange or pinkish colonies were isolated from the sludge, and one type of colony was found to exhibit the highest H_2 production in the presence of 3% NaCl. This bacterial community was designated as BC1 and used for further experiments.

Characterization and Identification of Bacterial Strains Isolated From BC1.

The bacterial strains constituting BC1 were isolated by spreading the culture onto LB agar plates (8). A photosynthetic bacterial strain was identified by sequence analysis of its 16S rRNA gene. The numerical profiles of the other isolated bacteria were tested using API 20E strips (Bio Merieux S. A, France).

Assay Methods

The composition of algal biomasses and their lactic acid fermentate, and the amount of H_2 gas evolved were estimated using methods described previously (3). The assay of ethanol was also performed using methods described previously (8).

RESULTS AND DISCUSSIONS

Two-step H_2 Production from Algal Biomass

Most photosynthetic bacteria cannot use starch, the major constituent of algal biomass, as an electron donor of H_2 production. Therefore, we attempted to pretreat the algal biomass, to degrade algal starch by lactic acid fermentation (Table 1). In lactic acid fermentation, starch was converted into lactic acid, an ideal substrate for H_2 production by a photosynthetic bacterium. Table 1 shows that the starch accumulated in the *C. reinhardtii* and *D. tertiolecta* biomass was reduced by fermentation and converted into lactic acid. In addition, the lactic acid bacterium was found to play a role in degradation of the algal cell wall of *C. reinhardtii*, as well as the degradation of starch in the cells. It was significant that the algal cell was broken and liquified without any thermochemical pretreatment, which had usually been used in the past but had high energy cost. Furthermore, the fermentation needed no more additional nutrients than those that came from the algal cells.

TABLE 1
COMPOSITION CHANGES OF *C. REINHARDTII* AND *D. TERIOLECTA* BIOMASS BY LACTIC ACID FERMENTATION

Strain	*C. reinhardtii*		*D. tertiolecta*	
	Biomass [a] (g/l)	Fermentate (g/l)	Biomass [b] (g/l)	Fermentate (g/l)
Total sugar [c]	51.7	10.8	20.3	3.8
Starch [d]	41.8	1.0	17.2	0.3
Total lipid [e]	11.3	7.3	21.6	17.7
Triglyceride	8.1	5.7	7.5	7.2
Glycerol	0.1	0.1	6.3	6.5
Protein	13.4	10.5	34.4	26.8
Lactic acid	<0.1	38.9	0.7	16.9
Volatile fatty acids [f]	0.6	0.6	<0.1	0.8
Ammonia	<0.1	0.2	<0.1	0.3

Condensed algal biomasses (100 times as dense as original algal culture) were freeze-thawed (-30 °C) and inoculated with *L. amylovorus*. Inoculated samples of *C. reinhardtii* and *D. tertiolecta* biomass were incubated at 37 °C for 4 d and 2 d, respectively.

[a] The dry weight of algal biomass was 76.2 g/l

[b] The dry weight of algal biomass was 72.2 g/l

[c] Total sugar contains starch.

[d] Starch was detected as glucose.

[e] Total lipid contains triglyceride.

[f] Volatile fatty acids are the sum of acetic, propionic, *n*-butyric, *iso*-butyric, *n*-valeric, *iso*-valeric acids.

Several strains of photosynthetic bacteria were applied for H_2 production from the lactic acid fermentate of *C. reinhardtii* biomass (Fig. 2). *R. marinum* (ATCC 35675) produced the largest amount of H_2 (124 mmol) from 1*l* of the culture over a period of 100 h. The molar yield of H_2 by *R. marinum* from the starch accumulated in an algal biomass was 7.9 mol H_2/mol starch-glucose.

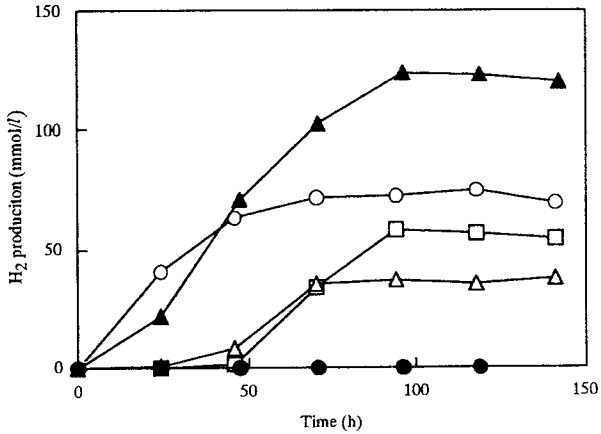


Figure 2: H_2 production from the lactic acid fermentate of the *C. reinhardtii* biomass by various photosynthetic bacteria. The fermentate of *C. reinhardtii* biomass was diluted to give a lactic acid concentration of 30 mmol/l, inoculated with one of the five strain of photosynthetic bacteria (○, *Rhodobacter sphaeroides*; △, *Rhodobacter capsulata*; □, *Rhodospirillum rubrum*; ●, *Rhodovulum sulfidophilus*; ▲, *Rhodobium marinum*), and incubated under illumination of 330 W/m^2 at 30°C.

Bacterial Strains Constituting BC1

BC1 was obtained from night soil treatment sludge for single-step H_2 production from an algal biomass. BC1 produced H_2 from starch in the presence of 0-5% NaCl, and the highest H_2 production was obtained with 3% NaCl, suggesting the halophilic growth characteristic of BC1. This NaCl concentration (3%) was used in all subsequent experiments. It was suggested that BC1 is suitable for H_2 production in high-salt environments such as in a system using seawater. To the best of our knowledge, a bacterial community capable of producing H_2 from starch under high-salt conditions (i.e., 3% NaCl) has not previously been reported. Ten strains were isolated from BC1. Among them, only strain A-501 was photosynthetic, and the others (designated as strains T-51, T-522, and T-53 to T-59) showed no phototrophic growih. All the strains were Gram-negative and facultatively anaerobic and could grow in the presence of 3% NaCl. The strain A-501 was identified as *R. marinum* Strains T-522 and T-59 identified as *Vibrio fluvialis*. All the other strains were found to be *Proteus vulgaris*.

The Role of the Isolated Strains in H_2 Production From Starch

The photosynthetic bacterium *R. marinum* A-501, which was considered to be the main (or only) H_2 producer in BC1, could utilize various organic substrates for the photoproduction of H_2 (Table 2). However, among the substrates tested, starch, cellobiose and acetic acid, which are utilized by BC1 as substrates for H_2 production, could not be used for H_2 production by a pure culture of strain A-501. The results indicate that the conversions of starch, cellobiose and acetic acid into H_2 require the contribution of other bacteria in BC1. Among the isolated strains, only *V. fluvialis* T-522 and T-59 possessed starch-degrading activity. This result suggests that *V. fluvialis* T-522 and T-59 contribute to the degradation of starch to supply some substrate(s) in a form that can be readily utilized by *R. marinum* A-501 for H_2 production.

TABLE 2
H_2 PRODUCTION BY *R. MARINUM* A-501 AND BC1 WITH VARIOUS SUBSTRATES

Substrate	H_2 production by *R. marinum* A-501 (mmol/*l*)	H_2 production by BC1 (mmol/*l*)
Starch	0.0	39.1
Glucose	21.6	19.9
Maltose	13.4	17.6
Cellobiose	0.0	21.7
Sucrose	12.3	18.3
Acetic acid	0.2	56.1
Lactic acid	37.3	82.9
Malic acid	23.4	26.4
Glycerol	8.3	15.9

Substrate concentrations were adjusted stoichiometrically to yield 300 mmol H_2 per liter of culture by photosynthetic bacteria. Actively growing cultures of BC1 or strain A-501 were inoculated into each medium to obtain a final OD_{660} of 0.6. The samples were incubated at 30℃ under tungsten lamp illumination (330 W/m^2) until H_2 evolution ceased (approx. 100 h).

BC1 produced approx. 40 mmol H_2 from 1*l* of the starch-containing H_2 production medium. Almost the same amount of H_2 was evolved by the 2-membered cocultures consisting of strains A-501 and T-522, and of strains A-501 and T-59. The other 2-membered cocultures, consisting of *R. marinum* A-501 and *P. vulgaris* T-51 or T-53 to T-58, failed to evolve H_2. Thus, it was confirmed that H_2 production from starch was a result of cooperation between *R. marinum* and *V. fluvialis*.

To identify the substrates obtained from starch and to confirm that the substrates were available for H_2 production in BC1, soluble starch (4.05 g/*l*) was fed to a pure culture of *V. fluvialis* T-522 (Fig. 3A). The fermentate, sampled after approx. 120 h, contained a large amount of acetic acid (approx. 400 mg/*l*) as the major metabolite, and approx. 100 mg/*l* ethanol as the by-product. These results suggest that acetic acid and ethanol might be the major substrates for H_2 production by strain A-501 in BC1. Subsequently, the fermentate was fed to a pure culture of strain A-501 (Fig. 3B). As shown in Fig. 3B, the concentrations

of acetic acid and ethanol decreased, and the maximum H_2 production reached a level comparable to that by BC1. The amount of evolved H_2 was nearly equal the value which be stoichiometrically calculated from the consumption of acetic acid and ethanol (calculation not shown). This indicates that acetic acid and ethanol in the fermentate were utilizes by strain A-501 as the preferred substrates for H_2 production. However, contrary to the case of the fermentate, H_2 evolution by strain A-501 in pure culture was not observed when using synthetic media containing acetic acid and/or ethanol by strain A-501 in pure culture (Table 2, data not shown for ethanol). These findings strongly suggest that strain T-522 might supply some as yet unknown factors that induce H_2 production by strain A-501 from acetic acid and ethanol.

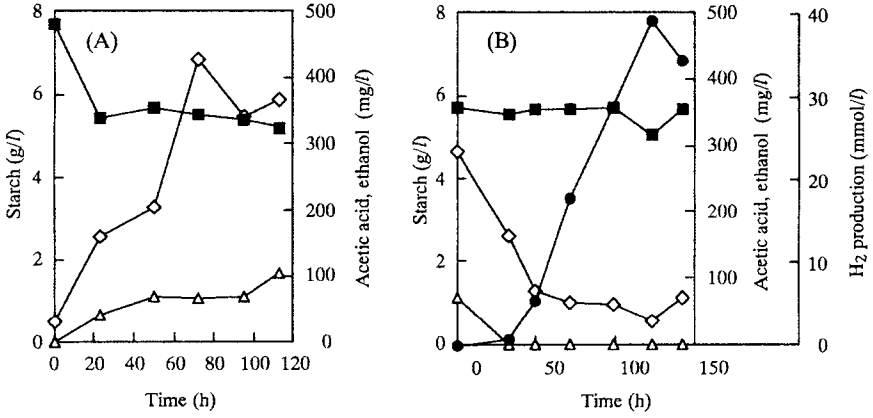


Figure 3: Fermentation of starch by V. fluvialis T-522 (A) and H_2 production by *R. marinum* A-501 from the fermentate (B). Synthetic medium containing 4.05 g/l soluble starch was inoculated with *V. fluvialis* T-522 and incubated at 30°C. The fermentate produced by *V. fluvialis* T-522 was inoculated with *R. marinum* A-501 and incubated under illumination of 330 W/m^2 at 30°C. The evolved H_2 (●), starch concentration (■), acetic acid concentration (◇), and ethanol concentration (△) were analyzed.

Single-step H_2 Production From Algal Biomass

We applied the coculture of *R. marinum* A-501 and *V. fluvialis* T-522 (designated as BC2) to H_2 production from raw algal biomasses of *C. reinhardtii* or *D. tertiolecta* (Fig. 4). The amount of H_2 evolved reached 140 mmol and 60 mmol per liter in the cultures of *C. reinhardtii* and *D. tertiolecta* biomasses, respectively, 40 mmol of H_2 was evolved from pure soluble starch. BC2 produced 6.2 mol H_2 from 1 mol starch-glucose in a *C. reinhardtii* biomass. Thus, the bacterial community BC1, which consisted of *R. marinum*, *V. fluvialis* and *P. vulgaris*, and which could produce H_2 from starch in a single-step process under high-salt conditions, was developed. A cost-effective H_2 production process could be achieved using seawater for the preparation of the culture medium.

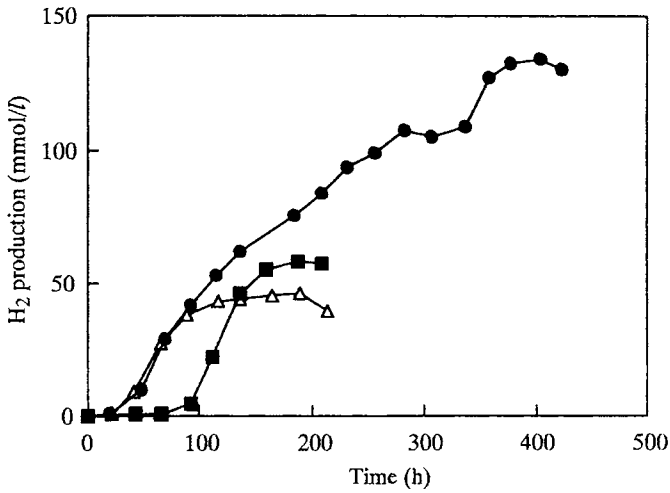


Figure 4: H_2 production from an algal biomass by BC2. *C. reinhardtii* (●) and *D. tertiolecta* (■) biomasses were prepared to give a final starch concentration of 4.05 g/l. Algal biomass and pure soluble starch (△) medium were inculated with BC2 containing *R. marinum* A-501 and *V. fluvialis* T-522 at the ratio of 1:2 (on dry weight basis), and incubated under illumination of 330 W/m^2 at 30°C.

In this paper, we proposed two-step and single-step processes for H_2 photoproduction from algal biomass. The two-step and single-step processes had the highest H_2 yields of 7.9 and 6.2 mol H_2/mol starch-glucose, respectively. We cannot compare the efficiency of these processes until we obtain the pilot scale data. However, one reason for the lower efficiency of the single-step process than the two-step process appears to be that acetic acid, the main substrate for *R. marinum* in the single-step process, is not effective electron donor for H_2 production compared with lactic acid in the two-step process. Therefore, it is necessary to investigate the possibility of using a bacterial community consisting of lactic acid bacteria and photosynthetic bacteria. However, the period needed for the single-step process was 2.5 times longer than for the two-step process (data not shown). The period for the single-step process could be shortened by increasing the concentration of algal biomass fed to the bacterial community.

We have shown that a bacterial community consisting of an anaerobic bacterium and a photosynthetic bacterium could produce hydrogen from starch-rich biomasses. To use this bacterial community in continuous H_2 production, we must develop a method to keep the bacterial community stable. It is important to regulate populations of bacterial strains and to maintain a high H_2 yield during hydrogen production.

REFERENCES

1. Regoni-Stern, S., Rismond, R., Szpyrkowicz, L., Zilio-Grandi, F,, Vigato, P. A. (1990). Anaerobic digestion of nitrophilic algal biomass from the Venice lagoon. *Biomass.* **23**, 179-199.
2. Kishimoto, M., Okakura, T., Nagashima, H., Yokoyama, S., and Yamaberi, K. (1994). CO_2 fixation and oil production using micro-algae. *J. Ferment. Bioeng.* **78**, 479-482.
3. Ike, A., Toda, N., Hirata, K, and Miyamoto, K. (1997). Hydrogen photoproduction from CO_2 fixing microalgal biomass: application of lactic acid fermentation by *Lactobacillus amylovorus*. *J. Ferment. Bioeng.* **84**, 428-433.
4. Ike, A., Toda, N., Tsuji, N., Hirata, K, and Miyamoto, K. (1997). Hydrogen photoproduction from CO_2-fixing biomass: application of halotolerant photosynthetic bacteria. *J. Ferment. Bioeng.* **84**, 606-609.
5. Yokoi, H., Mori, S., Hirose, J., Hayashi, S., and Takasaki, Y. (1998). H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp.. *Biotechnol. Lett.* **20**, 895-899.
6. Buranakari, L., Cheng-ying, F., Ito, K, Izaki, K, and Takahashi, Y. (1985). Production of molecular hydrogen by photosynthetic bacteria with raw starch. *Agric. Biol, Chem.* **49**, 3339-3341.
7. Ike, A., Toda, N., Murakawa, T., Hirata, K., and Miyamoto, K. (1998): Hydrogen Photoproduction from Starch in CO_2-Fixing Microalgal Biomass by a Halotolerant Bacterial Community. In BioHydrogen. (Ed.) Zaborsky O. R. Plenum Press. New York and London. pp.311-318.
8. Ike, A., Murakawa, T., Kawaguchi, H., Hirata, K., Miyamoto, K., (1999): Photoproduction of Hydrogen from Raw Starch Using a Halophilic Bacterial Community. *J. Biosci. Bioeng.* **88**, 72-77 (1999).
9. Miura, Y., Yagi, K., Shoga, M., and Miyamoto, K., (1982): Hydrogen production by a green alga Chlamydomonas reinhardt~'i, in an alternating light/dark cycle. *Biotechnol. Bioeng.* **24**, 1555-1563.
10. Guillard, L. and Ryther, J. H., (1962): Studies of marine planktonic diatoms. *Can. J. Microbiol.* **8**, 229-239.
11. Nakamura, L. K., (1981): Lactobacillas amylovorus, a new starch-hydrolyzing species from cattle waste-corn fermentations. *Int. J. Syst. Bacteriol.* **31**, 56-63.

This Page Intentionally Left Blank

PHOTOBIOLOGICAL HYDROGEN PRODUCTION AND NITROGENASE ACTIVITY IN SOME HETEROCYSTOUS CYANOBACTERIA

H. Masukawa, K. Nakamura, M. Mochimaru and H. Sakurai

Department of Biology, School of Education, Waseda University
Nishiwaseda 1, Shinjuku, Tokyo 169-8050, Japan

INTRODUCTION

Nitrogen-fixing heterocystous cyanobacteria are potential candidates for the development of photobiological hydrogen production systems. They produce H_2 under aerobic conditions using water as an electron donor. Hydrogen metabolism in these cyanobacteria involves at least three enzymes: nitrogenase, uptake hydrogenase, and bidirectional hydrogenase. Some researchers favor hydrogenase over nitrogenase as the hydrogen evolving system because of its high energy efficiency, but it was reported that continued production of H_2 in air was mediated by nitrogenase in the heterocysts.

To develop the hydrogen producing systems by cyanobacteria based on nitrogenase activity, it is important to find cyanobacterial strains which have a high activity of H_2 production. We reported the activities of nitrogenase and H_2 evolution in three cyanobacterial strains and some factors which affected these activities [1]. In the present work, we continue our efforts to find suitable strains and study the activities of nitrogenase and H_2 production of 12 nitrogen-fixing filamentous strains.

MATERIALS AND METHODS

2.1. Strains and Culture Conditions

Strains studied were as follows: *Anabaena* sp. PCC7120, *Anabaena cylindrica* IAM M-1, *Anabaena variabilis* IAM M-58, *Anabaenopsis circularis* IAM M-4, *Nostoc muscorum* IAM M-14, *Nostoc linckia* IAM M-30, *Nostoc commune* IAM M-13, *Nostoc carneum* IAM M-35, *Cylindrospermum muscicola* IAM M-32, *Anabaena cylindrica* UTEX B 629, *Anabaena flos-aquae* UTEX 1444, and *Anabaena flos-aquae* UTEX LB 2558. *Anabaena* sp. PCC7120 was kindly donated by Dr. Ohmori of the University of Tokyo. UTEX and IAM strains were obtained from the Culture Collection of Algae at the University of Texas and the Institute of Applied Microbiology, University of Tokyo, respectively.

All strains were grown in BG11 medium and washed with $BG11_0$ (N-free medium) medium. For induction of nitrogenase, washed cells were transferred to $BG11_0$ and the day

will be referred to as 0 day in the Figures and the Table. The cultures were grown in $BG11_0$ at 26-30 °C with continuous illumination by white fluorescent light of 20 μE $m^{-2}s^{-1}$ PAR on a shaker.

2.2. Assay of H_2 Production and Nitrogenase Activity

A portion of the cultures was concentrated to 5-10 μg Chl *a*/ml by centrifugation on the days indicated in the Figures and the Table. One ml of each of the concentrated cultures was transferred to 7.5-ml glass vials, sealed with rubber stoppers, and incubated for 1-2 hrs under illumination of 60 μE m^{-2} s^{-1} PAR after exchange of the gas phases to Ar for the assay of H_2 production and to 12% (v/v) C_2H_2 in air for the assay of nitrogenase activity, respectively. The H_2 and C_2H_4 were monitored by gas chromatography (Shimazu GC-8A) as described in [2].

RESULTS AND DISCUSSION

Differentiation to heterocysts from vegetative cells in many cyanobacteria usually takes several days [3], and our cultures were grown for 3-6 days after transfer to $BG11_0$ medium. The observed highest values of H_2 production and nitrogenase activity of each strain are summarized in Table 1. Of the strains studied, *Anabaena flos-aquae* UTEX LB 2558 had the highest nitrogenase activity, followed by *Anabaena cylindrica* IAM M-1 and *Anabaena variabilis* IAM M-58. *A. variabilis* IAM M-58 was most active in H_2 production, and the amount of H_2 produced was markedly higher than that of the other species studied. The incubation time required to attain the highest values differed among the species.

TABLE 1

MAXIMUM RATES OF C_2H_2 REDUCTION AND H_2 PRODUCTION ACTIVITIES OF STRAINS UNDER N_2-FIXING CONDITIONS

	Product (nmol μg chl a^{-1} h^{-1})	
Strain	C_2H_4	H_2
Anabaena flos-aquae UTEX LB 2558	23	3.2
Anabaena variabilis IAM M-58	19	4.2
Anabaena cylindrica IAM M-1	19	2.1
Anabaena sp. PCC7120	14	2.6
Nostoc linckia IAM M-30	13	0.17
Nostoc muscorum IAM M-14	12	0.60
Anabaenopsis circularis IAM M-4	10	0.31
Anabaena flos-aquae UTEX 1444	9.2	1.7
Anabaena cylindrica UTEX B 629	8.1	0.91
Nostoc commune IAM M-13	1.5	0.25
Cylindrospermum muscicola IAM M-32	ND	ND
Nostoc carneum IAM M-35	ND	ND

ND, not detectable

Figure 1 shows the time courses of nitrogenase activity and H_2 production of 6 cyanobacterial strains after transferring the cells to $BG11_0$ medium. The change in H_2 production activity of most strains except for *A. variabilis* IAM M-58 were mirrored in nitrogenase activity of the respective species. All nitrogenase activities were notably, or slightly, decreased shortly after the highest activities were attained. The decrease of nitrogenase activity may be due to accumulation of NH_3 or combined nitrogen.

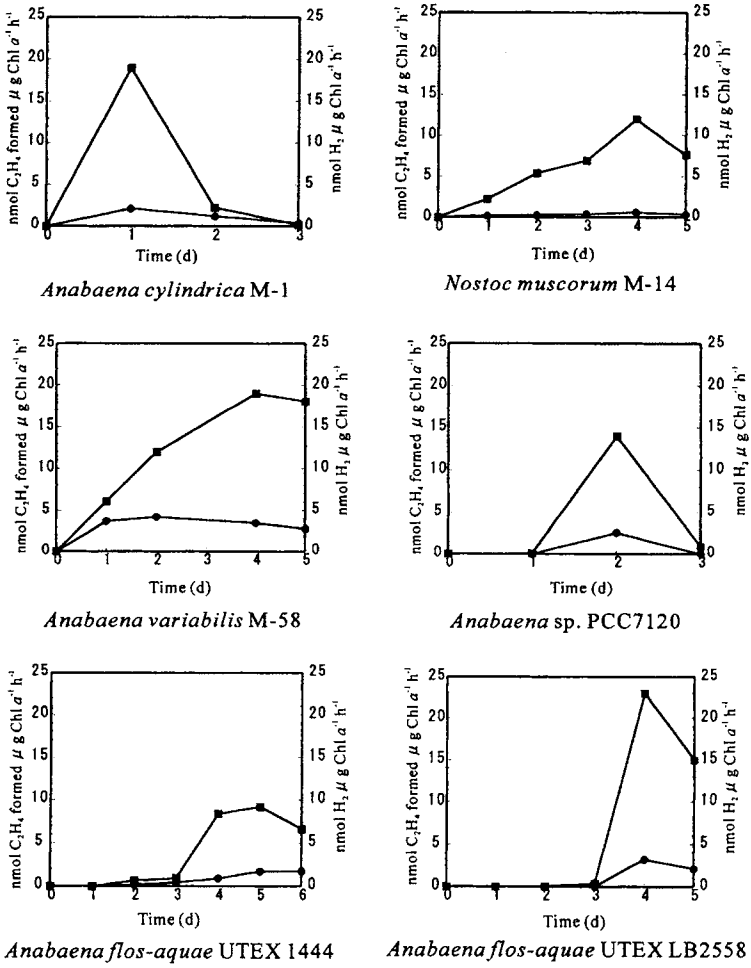


Figure 1: Time course of in vivo nitrogenase (■), and H_2 producing (●) activities of some cyanobacteria. Culture were transferred to $BG11_0$(N-free) medium zero time.

On the other hand, *A. variabilis* IAM M-58 showed a markedly higher H_2 production activity from the first day, prior to when the nitrogenase activity had reached its maximum. When nitrogenase activity was assayed in 12% (v/v) C_2H_2 in Ar, it was 25-40% higher than that in air, whereas in *Anabaenopsis circularis* IAM M-4 only about 10% higher activity was observed in Ar (data not shown).

In summary, *A. variabilis* IAM M-58 seems to possess certain advantages which make it one of the potential candidates for the development of photobiological H_2 producing systems because of its higher nitrogenase activity and ability to maintain continual H_2 evolution.

ACKNOWLEDGMENTS

This work was supported by grants from RITE to H. Sakurai.

REFERENCES

1. Masukawa, H., Mochimaru, M. and Sakurai, H. (1998). in Photosynthesis: Mechanisms and Effects (Garab. G., ed.) pp.4143-4146, Kluwer Academic Publishers, Dordrecht.
2. Almon, H. and Böger, P. (1988). in Methods in Enzymology, vol. 167 (Packer, L. and Glazer, A. N. eds.) pp. 459-467, Academic Press, San Diego.
3. Golden, J. W., Robinson, S. J. and Haselkorn, R. (1985). *Nature* **314**, 419-423.

STRATEGIES FOR IMPROVING OXYGEN TOLERANCE OF ALGAL HYDROGEN PRODUCTION

M. Seibert, T.Y. Flynn, and M. L. Ghirardi

National Renewable Energy Laboratory,
Golden, CO 80401 U.S.A.

ABSTRACT

The direct photoevolution of H_2 from water by green algae is a transient phenomenon, due to the rapid inactivation of the reversible hydrogenase (the enzyme catalyzing the reduction of protons to H_2) by O_2 a by-product of photosynthesis. Moreover, the expression of the algal H_2 production activity requires prolonged dark, anaerobic treatment of the cells in order to induce the enzyme. We are addressing the O_2-sensitivity problem of algal hydrogenase by means of both classical genetics and molecular biology approaches. Results of studies on the former topic will be presented. The ultimate goal of our research is to generate a *Chlamydomonas reinhardtii* mutant that is sufficiently tolerant to O_2 to produce H_2 under aerobic conditions. The availability of such a mutant will permit the development of a commercial photobiological H_2 production system that is cost effective, renewable, scalable and non-polluting.

The classical mutagenesis/selection approach that we have developed to obtain such a desirable mutant takes advantage of the reversible activity of the algal hydrogenase. We have designed two selective pressures that require mutagenized algal cells to survive by either metabolizing (photoreductive selective pressure) or evolving (H_2-evolution selective pressure) H_2 in the presence of O_2 concentrations that inactivate the wild-type (WT) enzyme. Given the generally low specificity of the two selective pressures, the surviving organisms were subsequently subjected to a positive screen using a chemochromic sensor that detects H_2 evolved by the algae. Clones that were found to exhibit high H_2-evolution activity in the presence of O_2 were characterized in more detail using biochemical assays. The strategy currently employed consists of re-mutagenizing, re-selecting and re-screening first generation mutants under higher selective stringency in order to accumulate single-point mutations, and thus, to further increase the O_2 tolerance of the organism.

Recent results include the isolation of a clone, 104G5 with 14 times higher tolerance to O_2 than the WT (obtained by the application of one round of photoreductive selective pressure); and the isolation of first and second generation mutants with, respectively, 3-4 and 10 times higher tolerance to O_2 than the WT (by application of two rounds of the H_2-evolution selective pressure).

INTRODUCTION

Green algae such as *Chlamydomonas reinhardtii* can photoproduce H_2 from water in a reaction catalyzed by the reversible hydrogenase enzyme (Gaffron and Rubin 1942). In the light, electrons released by the oxidation of water molecules are transferred through photosystems II and I to the low redox potential carrier ferredoxin. Normally, reduced ferredoxin supplies electrons to the CO_2 fixation pathway. However, following an anaerobic treatment in the dark, algal cells induce the reversible hydrogenase (Ghirardi et al 1997b), an enzyme that can combine photosynthetically-generated electrons and protons to generate H_2 gas. The hydrogenase pathway competes with CO_2 fixation for electrons from reduced ferredoxin. This competition is short lived, though, due to the prompt deactivation of hydrogenase by O_2 that is concomitantly released by photosynthetic water oxidation (Schulz 1996). The O_2-sensitivity trait of the hydrogenase has precluded the use of green algae in a direct biophotolysis H_2-production system (Benemann 1996).

The occurrence of reversible hydrogenase enzymes is not restricted to algae. They are present in many anaerobic microorganisms, in photosynthetic bacteria, and in cyanobacteria (Adams 1990, Wu and Mandrand 1993, Albracht 1994). The physiological roles and biochemical characteristics of these hydrogenases are variable. However, in all instances the enzyme is ultimately inactivated by O_2. However, mutant organisms containing hydrogenases that are able to operate at higher O_2 concentrations have been described (Gogotov 1986, McTavish et al. 1995, Weaver et al. 1999), suggesting that the enzyme is amenable to manipulations that may affect its O_2 tolerance. These observations led us to investigate several approaches to generate and isolate *C. reinhardtii* mutants that can produce H_2 in the presence of O_2.

We originally proposed to use random mutagenesis, followed by employing selective pressures under gradually increasing O_2 concentrations, to isolate the desired phenotype. Two selective pressures were designed (Ghirardi et al. 1996, 1997, 1997b), based on the reversible activity of the algal hydrogenase, e.g. H_2-production and H_2-uptake. Under H_2-production selective pressure, algal cells are required to survive a short treatment with metronidazole (MZ), a drug that competes with the hydrogenase for electrons from photosynthetically-reduced ferredoxin. Reduced MZ generates a radical that in the presence of O_2 produces superoxide radicals and H_2O_2, both of which are toxic to the algae. The selective pressure is applied in the presence of O_2 levels that are known to deactivate the WT hydrogenases. In organisms that have a hydrogenase that is active following exposure to O_2, some of the electrons from reduced ferredoxin can be used for H_2 production instead of MZ reduction and decreased toxicity can be observed (Ghirardi et al. 1996, 1997, 1997b). Similarly, the photoreductive pressure requires the algal cells to survive in an atmosphere of CO_2, H_2, and controlled concentrations of O_2 in the presence of the herbicides 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and atrazine. These herbicides block photosynthetic O_2 evolution and electron flow on the reducing side of photosystem II and prevent electrons from water from reaching the hydrogenase enzyme. The surviving organisms grow by fixing CO_2 with electrons obtained from the oxidation of H_2, catalyzed by an O_2-tolerant hydrogenase, and ATP generated by cyclic electron transport around photosystem I (Ghirardi et al. 1997).

However, it quickly became apparent that the selective pressures were not specific enough and yielded a mixed population of survivors. A chemochromic sensor was then developed in collaboration with D. Benson at NREL to allow us to quickly screen the survivors of the selective pressures for H_2-producing clones. The screening was based on the ability of a multilayer, thin film device containing WO_3 and Pd to change color (from transparent to blue) upon exposure to nanomoles of H_2. The usefulness of the film in

detecting H_2 evolved by algal colonies on agar plates was demonstrated previously (Ghirardi et al. 1998; Seibert et al. 1998). We will discuss a procedure that was developed to detect H_2 production by O_2-tolerant algal mutants using the chemochromic sensor, and present evidence that the combination of mutagenesis, selection and screening steps successfully results in the isolation of clones with increased tolerance to O_2.

The use of random mutagenesis to generate *C. reinhardtii* mutants, followed by selection for the desired phenotype has been successfully used in biochemical research for many years. This technique is employed when the gene that encodes a particular protein has not been cloned, precluding the use of site-directed mutagenesis or other molecular biology techniques. The algal hydrogenase protein has been isolated to purity by Happe and Naber (1993), who also sequenced 24 amino acid residues from the N-terminal portion of the enzyme. However, the DNA sequence of the gene encoding the hydrogenase enzyme in *C. reinhardtii* has not been determined at this point because the use of RT-PCR (reverse transcriptase polymerase chain reaction) approaches by Happe and our own group have not as yet been successful.

MATERIALS AND METHODS

Cell Growth

Wild type (WT) *C. reinhardtii* (137c+) was a gift from Prof. S. Dutcher, University of Colorado, Boulder. Algal cells were grown photoautotrophically in basal salts (BS), a modification of Sueoka's high salt medium (Harris 1989) that includes citrate to prevent salt precipitation. This formulation contains the following salts: 10 mM NH_4Cl, 1 mM $MgSO_4$, 7.5 mM KH_2PO_4, 7.5 mM K_2HPO_4, 1.5 mM Na_3-citrate, 0.5 mM $CaCl_2$, 20 mM $FeCl_3$, and 1/2 x Hutner's trace elements. This medium can be solidified with 1.5% w/v agar and amended with 0.5 g/l yeast extract (Difoo) for plates, and may be supplemented with 10 mM sodium acetate depending on the experiment. Liquid cultures were grown under continuous cool white fluorescent lamp illumination (70 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) at 25°C and agitated on a shaker. Cells were harvested by centrifugation at 2000 x g for 10 min and resuspended in liquid BS medium.

Mutagenesis

Mid-log phase cultures were harvested and resuspended in liquid BS to yield a 10 ml suspension of $7x10^6$ cells/ml. Ethylmethane sulfonate (EMS) was added to a final concentration of 5 ml/ml (46 mM), and the cells were incubated with gentle agitation for various periods of time. At the end of the incubation period the cells were washed and resuspended in 50 ml of the same medium lacking EMS. For the 5-bromouracil (5BU) mutagenesis, 550 ml of liquid BS medium were inoculated with 20 ml of mid log phase culture to give an initial cell density of 4.9×10^4 cells/ml. The culture was grown overnight under fluorescent illumination (70 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) and then sparged with 2% CO_2 (50 ml/min). A filter-sterilized stock solution of 5-bromouracil (dissolved in BS) was then added to the culture to a final concentration of 1 mM. The culture was incubated under the same conditions for another 48 h, at which point the cells were harvested, washed, and resuspended in 50 ml of BS medium. Liquid cultures from either the EMS or 5BU mutagenesis were grown in the light as above for at least 7 days before being submitted to the selective pressures.

Photoreductive Selection (PR) Procedure

Liquid cultures of mutagenized algal cells (250 ml, 2.8 x 10^5 cells/ml) in BS were treated with 15 μM each of DCMU and atrazine, and the flasks were placed in anaerobic jars. The gas phase contained 16.5% H_2, 2% CO_2, 5-20% O_2, balanced with Ar. The cultures were grown for a couple of weeks with stirring and illuminated with fluorescent light (70 $\mu E \cdot m^{-2} \cdot s^{-1}$). At the end of the selection period, the cells were washed with BS medium and revived in liquid BS medium plus 10 mM sodium acetate.

H_2-Production Selection (MZ) Procedure

A suspension of anaerobically-induced algal cells was mixed with an anaerobic MZ-Na azide solution to final concentrations of 40 mM MZ and 1 mM sodium azide at 2.8 x 10^6 cells/ml. While maintaining darkness, O_2 was added to 5% in the gas phase, and the mixtures were shaken vigorously for 4 min. Immediately following the O_2 treatment, the cultures were exposed for 6 min to light (320 $\mu E \cdot m^{-2} \cdot s^{-1}$) filtered through a solution of 1% $CuSO_4$ with mixing. At the end of the selection period, the cells were washed with BS medium and either resuspended in the same medium or plated for cell counting.

Chemochromic Screening

Individual colonies surviving mutagenesis and selection were transferred to square petri dishes that can easily accommodate an 8 x 8 colony matrix and a square chemochromic sensor. Following a 7-14 day growth period, the agar plates were made anaerobic overnight to induce the algal hydrogenase and then preexposed to 21% O_2 for different periods of time in the dark to deactivate the WT phenotype. The plates were immediately transferred to an anaerobic glove box, the sensor applied, and the colonies were illuminated for 3 minutes to photoevolve H_2 (see Fig. 1). At the end of the illumination period, the sensors were analyzed for the location of blue dots, corresponding to the algal colonies that still evolved H_2 following the O_2 pretreatment (see Results and Discussion). The identified clones were then transferred from the original plate to liquid BS + 10 mM acetate and cultivated for further characterization.

H_2-Evolution Assay

Mid-log phase algal cultures were harvested and resuspended in 20 ml phosphate buffer (Ghirardi et al. 1997b), supplemented with 15 mM glucose and 0.5% v/v ethanol, and they were then made anaerobic with Ar bubbling. Concurrently, 2 ml of an enzymatic O_2-scrubbing system (Packer and Cullingford 1978) that consisted of 1mg/ml glucose oxidase and 27,720 units/ml catalase (Sigma # C 3155) was dispensed into dialysis tubing (6-8 kD MW cutoff) and made anaerobic as above. The dialysis bags were added to the cell suspensions and the vials were sealed, covered with aluminum foil, and incubated at room temperature for 4 h. Following this induction treatment, the cell suspensions were kept at 4°C overnight. The assay reaction consisted of exposing the cells to various levels of O_2 for two minutes, reestablishing anaerobiosis, and adding reduced methyl viologen to serve as the electron donor to the hydrogenase. The reaction mixtures were incubated in the dark for 15 minutes at 30°C in a shaking water bath, and the reaction was stopped by adding trichloroacetic acid. The presence of H_2 was detected by gas chromatography.

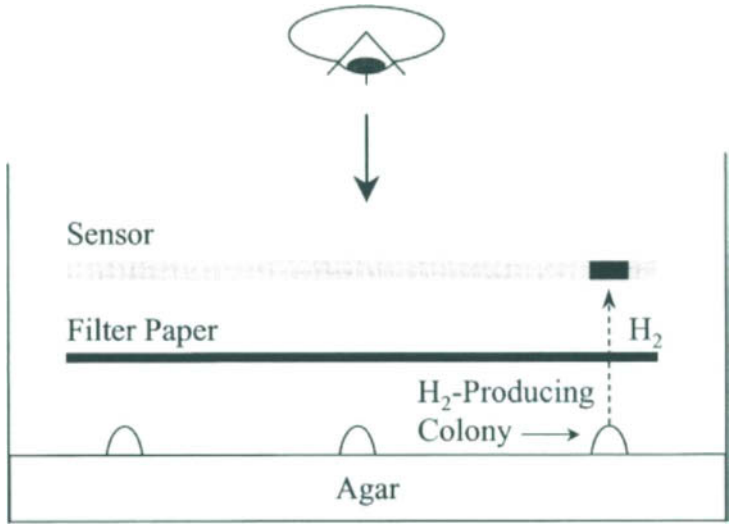


Figure 1: Chemochromic sensor system for identifying H_2-producing algal colonies plated put on a petri dish. After the cells were induced, they were illuminated from below to initiate H_2-production activity. Specific areas on the sensor film in closest contact to H_2-producing colonies turne from transparent to a blue color when H_2 was detected.

RESULTS AND DISCUSSION

Random mutagenesis of WT *C. reinhardtii* cells was done with either ethylmethane sulfonate (EMS) or 5-bromouracil (5BU), in order to generate different types of mutants. EMS alkylates adenine and guanine and gives rise to tautomeric shifts, allowing adenine to pair with cytosine and guanine to pair with thymine (Klug and Cummings 1983). As a result, A-T $\leftrightarrow$ G-C transition mutations are created. Bromouracil is an analog of thymine that, instead of pairing with adenine, pairs with guanine, causing A-T $\leftrightarrow$ G-C transition mutations (Klug and Cummings 1983). The frequency of mutants among survivors increases with mutagen dose, but so does the damage to the genetic background (Bos 1987). Therefore, killing rates of less than 60% were chosen to minimize damage to the remainder of the genome, at the expense of high mutation frequency. The problem of decreased mutant frequencies among the survivors is normally solved by employing effective selection procedures.

Populations of algal cells treated with either of the two mutagens for different periods of time were then submitted to either the PR or the MZ selective pressure, in the presence of controlled amounts of O_2. Figure 2 summarizes the fate of each mutagenized population. Cells mutagenized with EMS were initially submitted to the PR selective pressure in the presence of 5% O_2. The survivors from this selective pressure (populations PR8, PR9, and PR10) were grown for 7-14 days, plated, and chemochromically screened (Fig. 3) following deactivation with atmospheric levels of O_2 for 2 min. Clones that produced the best signal during the screening procedure were characterized in a more detailed manner using a

polarographic method for determination of O_2 tolerance. None of the selected clones had a significant increase in O_2 tolerance, although the 24gl mutant and others showed up to 4 times higher rates of H_2 evolution compared to the WT control (Ghirardi et al. 1998). The lack of improvement in O_2-tolerance in this first round of mutants forced us to re-assess the PR selection protocol. We determined that the initial O_2 concentration of 5% O_2 (set in anaerobic treatment jars) was significantly decreased by cellular respiration of the cultures during the application of the selective pressure. This problem was solved in subsequent experiments by replacing the gas mixture daily until the culture became chlorotic, indicating that the majority of the cells were dead.

The PR8, PR9 and PR10 populations, which contained mutants with higher rates of H_2 evolution than that of WT (see above), were subsequently submitted to the MZ selective pressure following deactivation by 5% O_2. The surviving populations (less than 3% of the initial cell density), MZ12, MZ13 and MZ14, were resuspended in liquid medium and plated to yield single colonies. Two hundred and forty clones from each of the three populations were screened as above, and selected clones were further assayed to determine their O_2 tolerance relative to WT cells.

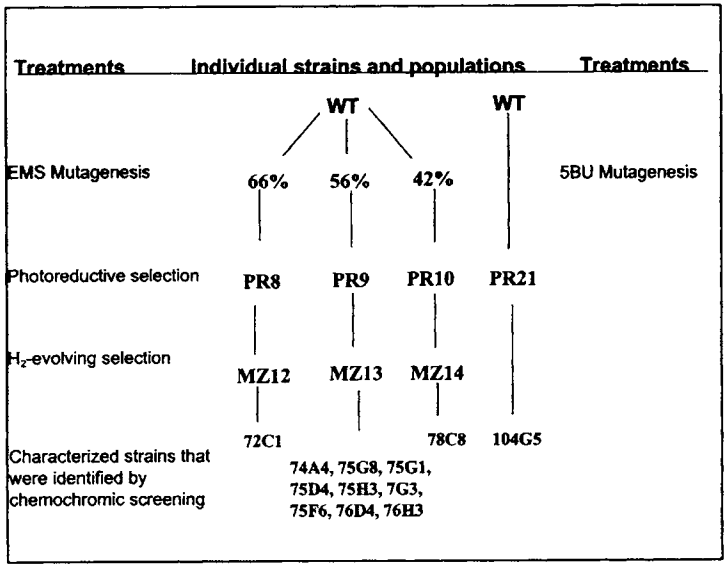


Figure 2: Treatment history of the various strains.

In addition, one population of WT cells was mutagenized with 5-bromouracil (see Fig. 2, right), which was done by adding the mutagen to actively dividing cells for a total incubation time of 2 days. This long exposure may have allowed more than a single mutation to occur. The resulting population was submitted to the PR selection in the presence of 20% O_2. Gasses were exchanged in the anaerobic jar on a daily basis. The survivors from this PR selection were screened (see Fig. 3), and the best clones were also assayed for O_2 tolerance.

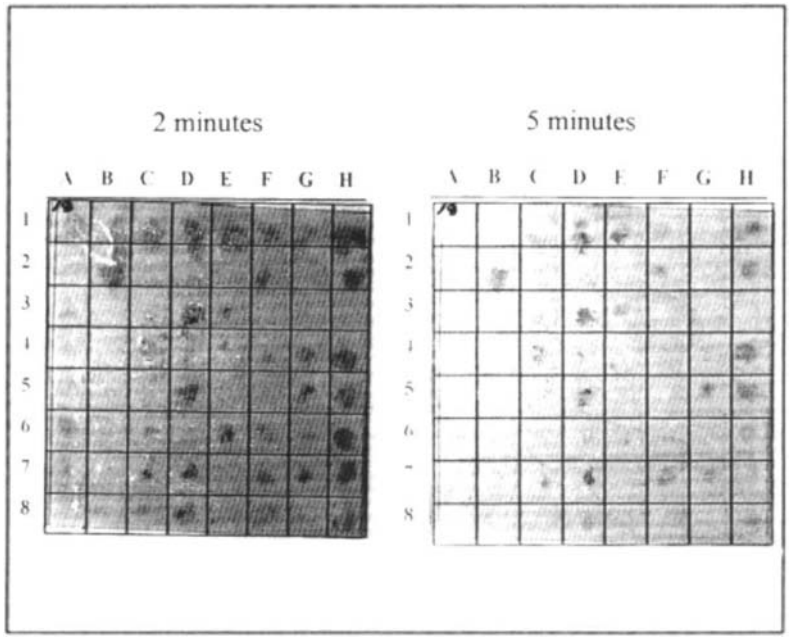


Figure 3: Chemochromatic sensors films that had been exposed to 64 mutant colonies of algae located in a grid array. The colonies were selected by photoreductive pressure for H_2-production ability in the presence of O_2. The spots represent those colonies that still produce H_2 after being preexposed to O_2 for either 2 or 5 min. The colonies that produced the most intense spots were chosen for further testing.

In the past, measurement of the O_2 tolerance of the clones had been done by determining an O_2 I_{50} for H_2 evolution, that is, the concentration of O_2 added to the gas phase that inhibited the rate of algal H_2 evolution by half of the value measured in the absence of added O_2 (Ghirardi et al. 1996, 1997, 1997b, 1998, 1999; Seibert et al. 1998). Rates of H_2 evolution were measured polarographically with a Clark-type electrode, poised for the detection of H_2. Anaerobically induced algal cells, resuspended in the presence of an O_2-scrubbing system (to insure maintenance of anaerobiosis) were treated with O_2 in the dark for 2 min and illuminated. The rate of H_2 produced during the first 5 min of illumination was used to calculate an initial rate of H_2 evolution by the algal cells. There was one major problem with this procedure. The presence of the O_2-scrubbing enzymatic system in the cell suspension was very effective in keeping the culture anaerobic by quickly consuming the O_2 added to the gas phase during the dark deactivation phase of the enzyme assay, resulting in variable concentration of O_2 throughout that period. Thus, the O_2 I_{50}s previously obtained, may have been overestimated. In order to eliminate these two problems, a new assay procedure to estimate O_2 tolerance of selected clones was adopted and optimized. The new procedure involved physically separating the algal cell suspension from the O_2-scrubbing enzymatic system during the dark anaerobic induction period, and then transferring only the cells to a separate vial where O_2 deactivation and H_2-evolution activity measurements were done (see

Materials and Methods section). To deactivate WT hydrogenases, a controlled amount of O_2 was added to the anaerobic cell suspension in the sealed vial and incubated under vigorous mixing for 2 min. Anaerobic conditions were then rapidly re-established, and methyl viologen (reduced by addition of sodium dithionite) was added to serve as the electron donor to the hydrogenase. The mixture was then incubated in the dark for 15 min at 30°C, and the reaction was stopped by the addition of trichloroacetic acid. The amount of H_2 produced was detected by gas chromatography.

Table 1 summarizes the characteristics of the indicated selected clones from each experiment. The survivor derived from the MZ13 selection showed the best response to the chemochromic screening (possibly because better mutants had been generated by the EMS mutagenesis step), and thus contributed the most clones to the assay. The parameters used to initially characterize the O_2 tolerance of the mutants included the maximum rate of H_2 evolution measured without any exposure to O_2 (V_0) and the amount of H_2-evolution activity remaining after an exposure to 2% O_2 for 2 min (% of V_0). The % of V_0 parameter was used to roughly compare the relative O_2 tolerance of the mutant clones to the WT strain. Four strains were more fully characterized by titrating the H_2-evolution activity following deactivation of the enzyme with increasing levels of O_2 for 2 min. The O_2 I_{50} was then estimated by fitting the data to a single exponential decay function. Figure 4 shows O_2 titration curves for, respectively, WT, 76D4 and 104G5 clones. The estimated O_2 I_{50}s are shown in Table l.

TABLE 1
CHARACTERISTIC OF SELECTED STRAINS

Parental population	Strain	V_0 (μmoles H_2/(mg Chl x h)	% of V_0	O_2 I_{50}
WT	-	39 ± 10 (n = 6)	0.26	0.22
MZ12	72C1	81	14	-
MZ13	76D4	78	18	0.82
	76H3	72	35	0.96
	74A4	64	15	-
	75G8	50	26	-
	75G1	82	17	-
	75D4	88	18	-
	75H3	67	27	-
	75G3	73	29	-
MZ14	78C8	64	9	-
PR21	104G5	82	59	2.8
76D4	141F2 (after a 2nd MZ treatment)	88	42	2

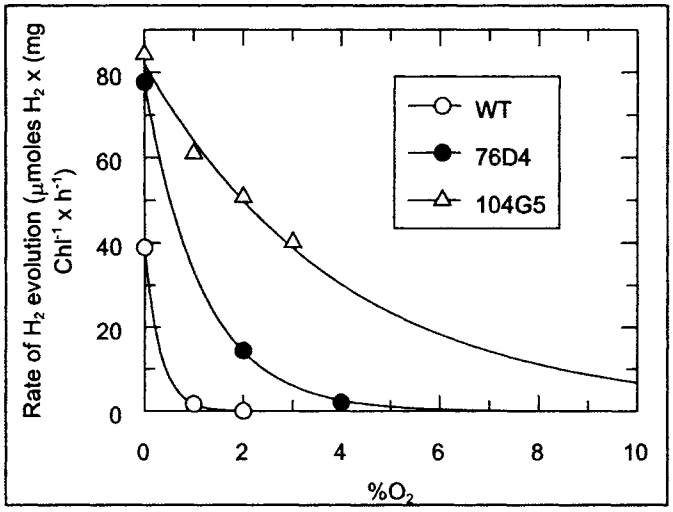


Figure 4: O_2 titration of the rates of H_2 evolution by selected colonies. The mutants were exposed to one round of H_2-production (76D4) or one round of photoreductive (104G5) selective pressure.

Further inspection of Table 1 reveals that all of the mutants identified by the screening assay were improved with respect to V_0 and O_2-tolerance, compared to their parental WT strain. The V_0S were increased in all of the mutants, with a 2.3-fold increase observed in clone 75D4. The O_2 I_{50}s were increased by 3.7-4.4-fold in clones 76D4 and 76H3. The least improved mutant, 78C8 had only a 9% increase in O_2-tolerance compared to the WT strain, which may represent the minimum phenotype for surviving the conditions used in this H_2-evolving selection experiment. The 76D4 clone was remutagenized with EMS (61% survival), reselected using a second MZ procedure with a selective pressure of 40% O_2 in the dark for 5 min, and finally rescreened, following deactivation with 100% O_2 for 5 min (similar to Fig. 3, right). Table 1 shows that one of the resulting clones, 141F2 had over a 2-fold increase in I_{50} compared to its parent (76D4) and almost a 10-fold improvement compared to the grandparent WT strain. The 104G5 clone from the PR21 population is our best isolate to date and has an O_2 I_{50} about 14 times higher than the WT strain. It is important to point out that this strain was isolated after a single photoreductive selection, and verifies that the modification of the PR procedure has been successful.

Given the range of increased O_2-tolerance measured with the different clones in Table 1, one could argue that there is more than one genotype that gives rise to the O_2-tolerant phenotype. The following three obvious possibilities exist: (a) different amino acid substitutions at a single critical residue, (b) random substitutions distributed throughout the O_2-sensitive domain, or (c) mutations on genes other than the hydrogenase, causing a decrease in intracellular O_2 concentration, such as through increased rates of respiration or decrease cell membrane permeability to atmospheric O_2. It is noteworthy mentioning that two rounds of mutagenesis, MZ selection, and screening yielded organisms with an increasingly higher O_2-tolerance, supporting either of the last two possibilities discussed above.

In conclusion, we have developed two types of selective pressures and a screening

procedure to generate mutants of *C. reinhardtii* that exhibit H_2-production capacity with improved yield and over an order of magnitude increase in O_2 tolerance. Additional rounds of mutagenesis, selection and screening are expected to result in further improvements in the near future. These mutants will be useful at some point in developing applied systems for generating large amounts of H_2 fuel from water and sunlight to satisfy society's needs for non-poluting, renewable energy in the 21st century.

ACKNOWLEDGMENTS

We thank the U.S. Department of Energy Hydrogen Program for supporting this research and appreciate the help of D. Benson and S.-H. Lee who provided the sensors.

REFERENCES

Adams, M.W.W. (1990). The Structure and Mechanism of Iron-Hydrogenases. *Biochim. Biophys. Acta* **1020**, 115-145.

Albracht, S.P.J. (1994). Nickel Hydrogenases: in Search of the Active Site. *Biochim. Biophys. Acta* **1188**, 167-204.

Benemann, J. (1996). Hydrogen Biotechnology: Progress and Prospects. *Nature Biotechnol.* **14**, 1101-1103.

Bos, C.J. (1987). Induction and isolation of mutants in fungi at low mutagen doses. *Curr. Genet.* **12**, 471-474.

Gaffron, H. and J. Rubin. (1942). Fermentative and Photochemical Production of Hydrogen in Algae. *J. Gen. Physiol.* **26**, 219-240.

Ghirardi, M.L., S. Markov and M. Seibert. (1996). Development of an Efficient Algal H_2-Producing System. In *Proceedings of the 1996 U.S. DOE Hydrogen Program Review*, 285-302, NREL/CP-430-21968.

Ghirardi, M.L., T. Flynn, S. Markov and M. Seibert. (1997). Development of an Efficient Algal H_2-Producing System. In *Proceedings ofthe 1997 US DOE Hydrogen Program Review*, 11-24, NREL/CP-430-23722.

Ghirardi, M.L., R.K. Togasaki and M. Seibert. (1997b). Oxygen Sensitivity of Algal H_2-Production. *Appl. Biochem. Biophys.* **63-65**, 141-145.

Ghirardi, M.L., T. Flynn, M. Forestier and M. Seibert. (1998). Development of an Efficient Algal H_2-Producing System. In *Proceedalgs of the 1998 U.S DOE Hydrogen Program Review,* 43-60m NREL/CP-570-25315.

Ghirardi, M.L., T. Flynn, M. Forestier, A. Iyer, A. Melis, P. Danielson and M. Seibert. (1999). Generation of *C. reinhardtii* Mutants that Photoproduce H_2 from H_2O in the Presence of O_2. In *Photosynthesis' Mechanisms and Effects* (G. Garab, ed.), Kluwer Academic Publishers, The Netherlands, vol. III, 1959-1962.

Gogotov, I.N. (1986). Hydrogenases of Phototrophic Microorganisms. *Biochimie* **68**, 181-187.

Happe, T. and J.D. Naber. (1993). Isolation, Characterization and N-terminal Amino Acid Sequence of Hydrogenase from the Green Alga *Chlamydomonas reinhardtii. Eur. J. Biochem.* **214**, 475-481.

Harris, E.H. 1989. *The Chlamydomonas Sourcebook*, Academic Press, New York.
Klug, W.S. and M.R. Cummings. (1983). *Concepts in Genetics*. Charles E. Merrill Publishing Company, Columbus, Ohio.

McTavish, H., L.A. SayavedraSoto and D.J. Arp. (1995). Substitutions of *Azotobacter vinelandii* Hydrogenase Small-Subunit Cysteines by Serines Can Create Insensitivity to Inhibition by O_2 and Preferentially Damages H_2 Oxidation over H_2 Evolutiont. *J. Bacteriol.* **177**, 3960-3964.

Packer, L. and W. Cullingiord. (1978). Stoichiometry of H_2 Production by an *in vitro* Chloroplast, Ferredoxin, Hydrogenase Reconstituted System. *Z Naturforsch.* **33c**, 113-115.

Schulz, R. (1996). Hydrogenases and Hydrogen Production in Eukaryotic Organisms and Cyanobacteria. *J. Mar. Biotechnol.* **4**, 16-22.

Seibert, M. T. Hynn, D. Benson, E. Tracy and M. Ghirardi. (1998). Development of Selection and Screening Procedures for Rapid Identification of H_2-Producing Algal Mutants with Increased O_2 Tolerance. In *Biohydrogen* (O. Zaborsky et al., eds.), Plenum Press, New York, p. 227-234.

Weaver, P. (1999). Biological Hydrogen Production. In Proceedings of the U.S. DOE Hydrogen Program Review, in press.

Wu, L.-F. and M.A. Mandrand. (1993). Microbial Hydrogenases: Primary Structure, Classification, Signatures and Phylogeny. *FEMS Microbiol. Rev.* **104**, 243-270.

This Page Intentionally Left Blank

II. Hydrogen World

This Page Intentionally Left Blank

EMERGING HYDROGEN ENERGY SYSTEMS AND BIOLOGY

T. Ohta

Yokohama National University, Prof Em.
E-Mail: tokiota@ka2.so-net.ne.jp

ABSTRACT

The emerging energy-converters need the hydrogen-fuel, which has no alternatives because of the increasing environmental demands. The proton exchange membrane fuel cell (PEMC). The hydrogen fueled turbine, and the metalhydride battery are typical. In order to cope with the increasing demand of hydrogen, multifarious hydrogen production systems must be examined. Especially, the water-splitting technologies by use of the renewable energies and the biological hydrogen production methods must emphasized. The mechano-catalyic water-splitting which has discovered early in 1998 is also stressed. A type of tunnel reaction is proposed to explain this new water-splitting. However, both the renewable energy systems, the biological systems and the mechano-catalyic systems are not necessarily simple and deterministic, that is, they belong to rather complexity. Complexity for the those systems is considered, and a scientific complex body of knowledge is proposed including the complexity field.

KEY WORDS:

hydrogen-fueled turbine, solid polymer electrolysis, proton exchange membrane fuel cell, metalhydride-reaction, metalhydride-battery, depletion of energy resources, recycle demands, environmental demands, photoelectrochemical water-splitting, biological and biochemical water-splitting, mechano-catalytic water-splitting, frictional electricity, tunnel reaction, complex systems, scientific complex

1. EMERGING HYDROGEN ENERGY TECHNOLOGIES.

We have no alternative of hydrogen fuel for the high efficient and no polluting energy converters. The fundamental reasons are as follows. First, the energy conversion is based upon the chemical reaction,

$$H_2 + (1/2)\, O_2 = H_2O \qquad \text{(Eq. 1)}$$

The reaction produces only water, which can be recycled if the renewable energies are applied, so that hydrogen fuel can be produced freely from the fossil fuels. Today, more than trillion cubic meter of hydrogen gas are a provided to the industries, and they are produced from the chemical processes:

(i) Methane resource: $CH_4 + O_2 = CO_2 + 2H_2$

(ii) NAPHTHA resource (mixture of hydrocarbons), for example, from benzene $C_6H_6 = 3H_2 + 6CO_2$

the both of which emit large amount of carbon dioxides.

Next, the sensible energy generated from 1 g of H_2 by Eq. (1) is 141.86 kL/g at the highest value, which is as high as 2.7 times compared to that of gasoline.

Thirdly, the reaction velocity of Eq. (1) has one of the fastest values among all chemical reactions. The reaction velocity represents generally the chemical wattage. The high value of the chemical wattage for H_2 has been applied to the rocket-fuel.

Fourthly, the chemical reaction of hydrogen is done with the proton generated from the molecules. The proton is one of the elementary particles, and makes chemical reactions like as physical reactions, which are nearly reversible. This precious reaction has been utilized to, for example, metalhydride-battery.

We shall introduce the three typical emerging technologies herein below.

1.1. Hydrogen-fueled Turbine

The energy efficiency of any heat engine is limited to the Carnot's efficiency:

$$\eta_c = I - T_2/T_1 \qquad \text{(Eq. 2)}$$

where T_1 and T_2 represent the inlet and the outlet temperatures, respectively. The typical steam turbine of the conventional nuclear plants has T_1 = 350°C and T_2 = 140°C, so that η_c =0.34. More than 65% oh the nuclear energy are wasted to heat up the sea water. It has been the important aim to raise up the energy efficiency of the thermal plants. How to raise up the inlet temperature is the key of this task. Japan has challenged to develop the hydrogen-fueled gas turbine [1]. The inlet temperature of the gas turbine has been improved markedly since 20 years ago, that is to say, it was 500°C, 750°C and 1,000°C at 1970s, 1980s, and 1990s, respectively. If hydrogen fuel is applied with oxygen as the oxidizing agent, the inlet temperature will become 17,000°C. The temperature limit is due to the mechanical and thermal resistances of the turbine blade even in the case, where the cooling system is simultaneously used. The Japanese project has verified, by the turbine with power capacity of 500 MW, that the inlet enthalpy with 1,700°C and 5O ata gives more 60 % efficiency. The best power system is to apply the hydrogen-fueled turbine as the topping regenerative cycle. There exists no alternative fuel of hydrogen if we consider both the efficiency and the no-polluting emission.

1.2. Solid Polymer Electrolysis (SPE) and Proton-exchange Membrane Fuel Cell (PEMFC)

The most efficient water-electrolysis with a compact size, today, is due to the SPE. Figure 1 shows a diagram for the principle of the SPE. The external battery takes off the electrons from the water contacted with the anode (A) to give the reaction;

$$H_2O - e^- = 2H^+ + (1/2)O_2 \quad \text{(Eq. 3)}$$

The protons pass through the solid polymer electrolyte (P. e.g., Nafion) and attains to combine with the electrons which are provided from the battery to evolve hydrogen at the cathode C;

$$2H^+ + 2e^- = H_2 \quad \text{(Eq. 4)}$$

Equation (3) and (4) mean that the supply of the energetic e^- is needed to split water. This is the basic principle of water-electrolysis. The PEMFC is just the reverse operation of the SPE. Hydrogen fuel is decomposed into $2e^-$ and $2H^+$ by the catalytic cathode. The protons pass through the solid polymer (electrolyte) and arrive at the anode (A) to react with the electrons and the supplied oxygen. Then, water is produced. The electrons come to A via the external resistance. This fuel cell generates, ideally, about 1V-direct current power. A stack of the cells is constructed to give the output power with, for example, 25 kW, which is set together to drive the vehicles.

The Daimlar Benz [2] has made the hydrogen bus named NEBUS whose output power is 250 kW. The PEMC costs about US$ 250 per W, which the same level as that of nuclear plants. We may expect a rapid spread of PEMFC, which is applied not only to the vehicles but also to the on-site generating systems including the domestic uses, in the near future. However, a special notice is stressed about the strong environmental pollution which may be caused by the wasted matters of used PEM, of which pH-value is very small (high acidity).

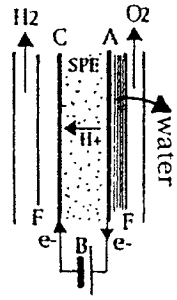


Figure 1: Structure of solid polymer electrolyte electrolyzer (SPE).

1.3. Metalhydride Systems [3]

The reaction equation of metalhydride (MHx) with hydrogen is written as

$$\{2 / (y - x)\}MH_x + H_2 = \{2 / (y - x)\}MH_y + Q \quad \text{(Eq. 5)}$$

where Q (= 10 – 20 kJ / mol.H_2 in most cases). This reaction is almostly reversible by virtue of the week coupling between the hydrogens and metal atoms. The applications of MH_x – H_2 cycles have been studied widely and the following fives are regarded as practical;

(i) Hydrogen storage,
(ii) Heat storage,
(iii) Heat pump,
(iv) Robotic actuater,
(v) Secondary battery.

The actuation principles for the devices listed in the above is so clear that the explanation is not necessary. The metals or the alloys, which are appropriate to the listed devices, are classified into three categories;

(i) Titanium-iron (Ti-Fe) systems
(ii) Mischmetal-nickel (Mm-Ni) systems
(iii) Magnesium-nickel (Mg-Ni) systems

More than 100 types of the alloys have been studied.

One of the most representative applications of the reversible metal-metalhydride cycle has been realized as the secondary battery. Figures 2a add 2b show the discharging and charging processes of the MH battery.

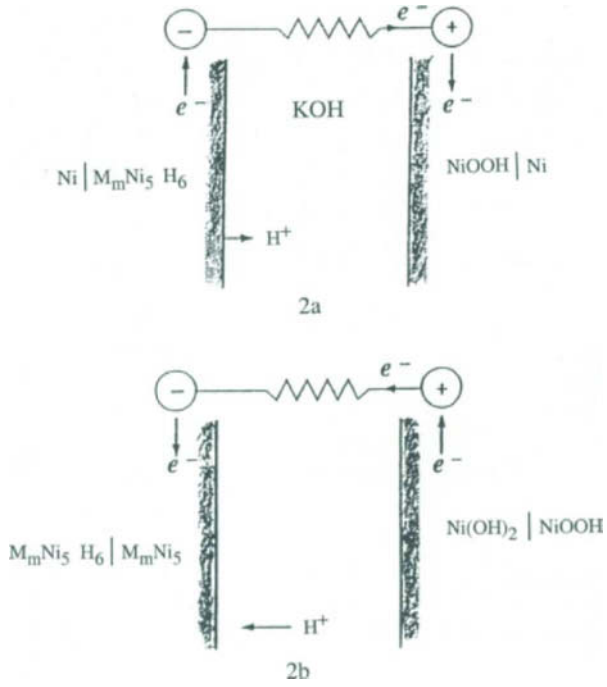


Figure 2: Discharging (2 a) and charging (2 b) processes of MH battery.

(a) Discharging process

A proton H^+ is emitted to the KOH electrolyte from the cathode (Ni / $MmNiH_x$) and the electron e^- goes to the anode via the external resistance. At the anode (NiOOH / Ni), the attained electrons are caught by NIOOH by the reaction:

$$e^- + H^+ + NiOOH = Ni(OH)_2$$

(b) Charging process

An electron is removed from the Ni $(OH)_2$ at the anode, and the reverse reaction occurs to yield NiOOH and H^+, which goes to the cathode to react to produce the metal hydride. The merits of this type of battery are that the cathode reaction is simple, and the metalhydride can store large amount of hydrogen. It is possible to replace the liquid electrolyte by the solid polymer electrolyte.

2. HYDROGEN PRODUCTION BY RENEWABLE ENERGIES

As mentioned previously, production of hydrogen - the ultimate fuel - from the fossil fuels will be very limited. Accordingly, every technology which applies renewable energy must be studied.

2.1. Solar Cell Combined with Water-electrolysis

The global improvement of the solar cell production in recent years has been remarkable. The total amount of the solar cell production in 1988 was 151.7 MW, which is 20.6 % increase compare to the previous year. The country's share was 53.7 % (USA), 49.2 % (Japan), 30.1 % (EU), and 18.7 % (others).

Almost of all the solar cells are made from silicon (Si), which is so abundant that more than 28 % of the earth's crust are composed of Si. The absorption spectrum of Si for solar light is fit, and Si is not noxious to the living things.

The cell efficiency of a single crystalline Si solar cell reaches 18- 0 % in the mass production line. The poly crystalline and cast Si solar cell shows 15-18% on average. The cell efficiency of amorphous Si solar cells (a-Si) is 8-9 %. Silicon solar cell generates electric power of direct current with about 1 V, a few combinations of which are suitable to apply to water-electrolysis. Therefore, if Si solar cell is combined with SPE, the system efficiency will be 10 % in practical use. This value is the highest among the systems which produce hydrogen with use of renewable energies as will be described here in-below.

2.2. Photoelectrochemical Water-splitting

A water-splitting device has been invented [4], where photo-semiconductor and platinum are used as the cathode and the anode, respectively, instead of setting both the solar cell and the electrolyzer, separately. This method is called "photoelectrochemical (PEC) water-splitting" or "photo semiconductor electrode method". The key phenomenon of PEC water-splitting is the steep rise (fall) of the potential at the interface between the n-(p-) semiconductor and the liquid electrolyte (e.g., KOH). If photons irradiate onto the interface, both the electrons (e^-) and positive holes (h^+) are excited to their conductive energy bands where they can move freely, so that e^- and h^+ are separated by the interface potential difference. The h^+ react with water by the equation:

$$H_2O + 2\,h^+ = 2H^+ + (1/2)O_2 \qquad \text{(Eq. 6)}$$

and the e^- goes to the counter electrode via external load, and reacts with H^+ by the equation:

$$2H^+ + 2e^- = H_2$$

where H^+ attains through the electrolyte.

Figure 3 shows the diagram for the principle of the PEC water-splitting. Notations A, C, M and R represent the cathode, the anode, and the external load, respectively. The equipment set is simpler than the solar cell-SPE system, however the selection of the photo-semiconductor is very limited. The good materials such as Si cannot be available, because Si is corrosive to the electrolyte. The photo-semiconductors such as TiO_2, $SrTiO_3$, WO_3, Fe_2O_3, and MoS_2 are known, however the energy conversion efficiency of solar ray is less than 4 %. Unless more excellent material is invented, this device falls behind the solar cell-SPE systems. The key - point of this technology is the choice of the electrode material.

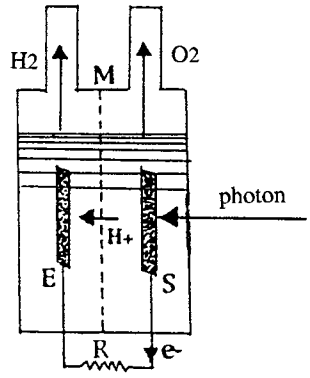


Figure 3: Principle of the photo-electrochemical water-splitting.

2.3. Photo-catalytic Water-splitting By Using Dye

Paying attention to the fact that dye is sensitive to solar ray, one may construct a solar energy conversion system with use of a synthesized dye. The excited state of the charge transfer the a complex of rubidium-bipyridine, $[Ru\ (bpy)]_3^{2+}$, has a long life time (about 685 ns) and the complex has strong oxidizing and reducing actions. In addition, its photo-absorption band, whose maximum wavelength is 450 nm, can cope with solar spectrum. Therefore, this complex has been investigated [5] as a hopeful photo-catalysis for solar energy conversion.

Figure 4 shows a diagram of the principle for the photo-catalytic water-splitting, where the photo-catalysis is assumed to be $Ru[bpy]_3^{2+}$. The $2Ru^{3+}$ reduce water to evolve O_2 and $2H^+$. Solar photons induce the reaction:

$$2Ru^{2+} + 2H^+ + \text{photons} = H_2$$

where the protons come from the reducing reaction.

Although it seems fascinating, this method is under experimental stage.

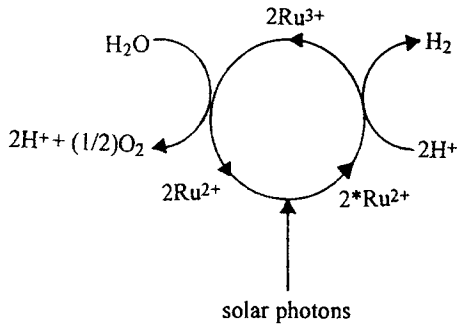


Figure 4: Principle of the photo-catalytic water-splitting.

2.4. Biological and Biochemical Water-splitting

We will be readily aware of applying the chlorophyllous materials in place of the photo-semiconductors or photo-sensitive complexes. This idea leads us to the subjects as the above title. According to A. Mitsui we will introduce briefly the water-splitting methods using the living thing's bodies, herein below. This title can be classified into three types.

(i) Living system such as algae and photosynthetic bacteria evolve hydrogen gas as shown in Fig. 5

We can construct a large breeding pond structure with the transparent roof, from which the evolved hydrogen is gathered, purified, and compressed to use. We must pay attention that Fig. 5 shows the principle of water-splitting not only for the biological systems, but also for every physico-chemical system {ref. Eqs. (3), (4), (6)}. One should be aware that this system is not so imaginary but can be realized with the same efficiency to the solar cell combined with water-electrolyzer systems, if an efficient living system is applied.

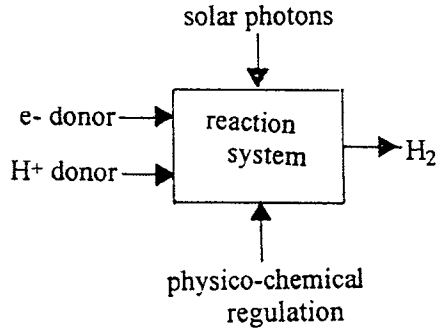


Figure 5: Hydrogen evolution system-model by the living things.

(ii) Cell free systems

The pulverised selected biomass which contains the stabilized electron- and the proton-donors generates hydrogen when solar beam irradiates onto it. This H_2-production mechanism is very similar to photosemiconductor- and photo-catalysis- methods.

(iii) Fermentation systems

Hydrogen evolution from glucose in dark fermentation by anerobic bacteria has been studied [7]. This is a traditional technology of chemical engineering systems, and can be done on a large scale. As a recycling utilization method for the wastes from the food industries, and the agriculture, this systems may be applied as one of the clean energy systems.

Lastly, we must pay attention that the biological systems have two main problems. One of them is the need of the fertilizers, and another is the needs of protection systems from the harmful living things.

3. MECHANO-CATALYTIC WATER-SPLITTING

3.1. Outline of the Experiment

A the beginning of 1998, the research group of Tokyo Institute of Technology headed by Prof. K Domen has found simultaneous O_2 and H_2 evolution by just stirring metal-oxide powder such as Cu_2O, NiO, Co_3O_4, Fe_3O_4, etc. (p-semiconductors) in distilled water [8]. The group has shown also that water is splitted if the stirring rod is the semiconductor foil instead of mixing the powder. We must pay attention to that the stirring rod is wrapped by the semiconductor foil instead of mixing the powder. We must pay attention to that the stirring rod (plastic) must be kept contacting with the bottom surface of the glass vessel, in the both cases. The energy efficiency was about 4%. Figures 6 and 7 show the experimental arrangement and the time of gas evolution, respectively. The stirring speed ranges from 0 to 1500 rpm. The reaction cell has a flat bottom (G) which is made of Pyrex glass, and the stirring rod (R) is covered by Teflon. The density of the semiconductor powder (S) is typically 0.1 g per 200 cc of water.

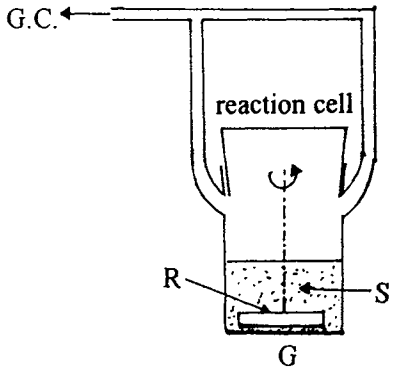


Figure 6: Experimental arrangement of mechano-catalytic water-splitting.

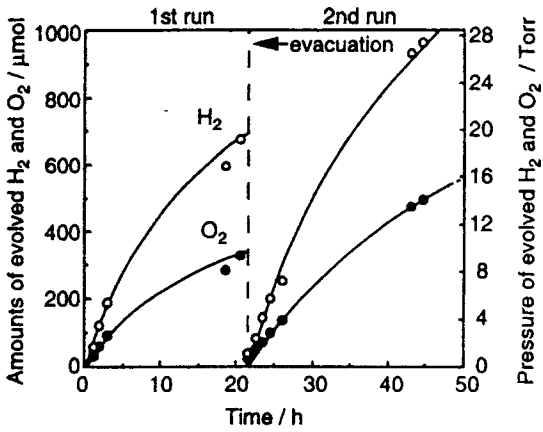


Figure 7: Time course of gas evolution.

3.2. A preliminary Theory

The mechano-catalyic water-splitting outlined in the above is very complicated phenomenon, so that it is not easy to establish the clear theory.

The author [9] has published a preliminary theory, which is introduced herein below.

(i) The body of glass is made of SiO_2, which has the ionic bonding structure. Glass surface is vulnerble to external force, and even a touch of a finger yields many pin-striped fine cracks, of which depth is order of 1 μm. Usually, the surface is hardebed by coating tin-chloride ($SnCl_2$), and the body is doped by some ionic materials. Even the strengthened glass such as Pyrex, the frictional rubbing between R and G generates numerous micro cracks, which cut the bonding to yield the dangling bonds and the electrons transfer from R to G to the bonding sites, and are trapped there. This is the generation mechanism of the frictional electricity.

(ii) Examples of the existence of the strong electrostatic fields in water the electric fishes, the thunderbolts, and so on. The underlying conditions are:
(a) Water must be non-conductive, (b) Electric generation must be continually undertaken, (c) Electric field is weakened by the dielectric shielding ($1/\varepsilon$, where ε is the dielectric constant), so that the field is not always strong enough to be detected at a separated point, (d) The micro cracks are widened and deepened by the water pressure, (e) The interaction between the trapped electrons and the water molecule is negligible.

(iii) The pin-striped micro cracks will cross each other, so that there exist many enlarged open spaces at the crossed places. Some percentage of the semiconductor particles may be suspended at the spaces, then these particles will be subject to a very strong field ($E_0 = q / \delta^2$, where q and δ represent the electric charge around the space and the average distance between the particle and electrons, respectively.

The value of E_0 is estimated to be the order of 10^7 V / m.

(iv) The work function of the positive hole is transformed in the presence of E_0, and the thickness of potential barrier is given by

$$X_m = \{e/ (16\pi\varepsilon E_0)\}^{1/2} \quad \text{(Eq. 7)}$$

This equation shows that the tunnel effect is possible. In addition, the emitted carrier has the energy of the order of $e E_0\lambda$, where λ is the mean free path. This energy is a little less than 1 eV, and the positive hole with this energy can react with water to yield H^+, and evolve oxygen, after Eq. 6. Two protons react with the two trapped electrons at the micro cracks to evolve hydrogen.

(v) The semiconductor particles (S) suspended at the open spaces will contact periodically to the bottom of R, which is rotating about the vertical axis. A this moment of contact, the positive holes are supplied to S from R, so that the intermittent emission of the positive holes is realized, which occurs the said tunnel reaction. Figure 8 shows the system diagram of the proposed model for the mechano-catalytic water-splitting. In the case of foil-wrapped experiment, one side of foil is kept contacting to R, and the other side is periodically subjected to the strong electric fields due to the fixed spaces.

(vi) The suspended particles are always subjected to the turbulence, so that some of them will remain at the spacings, while others will leave. Mechano-catalytic water-splitting phenomenon belongs eminently to the systems. We enumerate the related statistical and probabilistic matters herein after.
(a) Formations and the distribution of crossed open spaces.
(b) The probabilistic dynamics of trapping and the leaving of the particles at the spacings.
(c) The probabilistic dynamics of touching the particle onto the bottom of R.
However, if the turbulence plays another essential role in this phenomenon, the theory will be re-examined anew.

4. DETERMINISTIC SYSTEMS, ADAPTIVE COMPLEXITY, AND SCIENTIFIC COMPLEX

Hydrogen energy systems are indispensable to the ultimate energy systems and are deterministic only if hydrogen is supplied from the fossil fuels. However, we shall face to the strong limits of the environments and the depletant fossil fuel resources. Therefore, we must apply the renewable energy sources to produce hydrogen from water, and the renewable energies are not always deterministic (they are statistical and probabilistic).

Thus, hydrogen energy systems based upon the renewable energies must be re-examined from the complexity science, which will give a clear and deterministic story to a complicated phenomena. Such a study as mentioned in the above has been approached [10]. The mechano-catalytic water-splitting is a typical and important example in this field, that is to say, it is composed of many different scientific branches; rubbing mechanics, ionic structure of matter, frictional electricityin water, semiconductor, tunnel effect, particle dynamics in water, turbulence, chemical reaction, and so on. It can be said that the mechano-catalytic water-splitting is a scientific complex including the complexity.

We are looking forward to an improvement of this emerging field including also the biological areas.

REFERENCES

1. H. Sugishita et al. (1999). A Study of Hydrogen Combustion Turbine System, Proc. New Energy Systems and Conversions, Ed. by T. Ohta, M. Ishida and K. Matsuura, Osaka), p.77.
2. A. Brinner et al. (1998). DLR's membrane Fuel Cell Test Facility for Stationary and Mobile XApplication, Proc. 12Th WHEC, Bolcich and T. N. Veziroglu, Buenos Aires, p. 1597.
3. T. Ohta. (1994). ENERGY TECHNOLOGY, Sources, Systems and Frontier Conversions, , Pergamon Press.
4. A. Fujishaima and K. Honda. (1972). *Nature* **239**, 37.
5. M. Graetzel et al. (1982). *Pure Appl. Chem.* **54**, 2369.
6. A. Mitsui. (1979). Biological and Biochemical Hydrogen Production, Chap. 8 in Solar-Hydrogen Energy Systems, Ed. By T. Ohta, Pergamon Press.
7. National Institute of Bioscience & Human-Technology, et al. (1999). Hydro Production as an Environmentally Friendlly Technology, Abstracts of Bio-Hydrogen'99 Symposium (1999, Tsukuba), p.6-3.
8. S. Ikeda et al. (1998): *Chem. Commun.* 2185.
9. T. Ohta. (1999). Energy Technologies as approached from Adaptive Complexity. Proc. New Energy Systems and Conversions, Ed. by T. Ohta, M.Ishida, and K. Matsuura, Osaka, p.481.
10. T. Ohta. Energy Systems and Adaptive Complexity (Pergamon Press), To be published.

This Page Intentionally Left Blank

BIOHYDROGEN: AN OPPORTUNITY FOR INTERNATIONAL COOPERATION

A. San Pietro[1] and J. R. Benemann[2]

[1] Department of Biology, Jordan Hall 142, Indiana University, 1001 E Third St., Bloomington, IN 47405-3700

[2] Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA 94720

INTRODUCTION

The dramatic and sudden escalation in crude oil prices in the 1970s served to focus worldwide attention on the urgent need for alternative energy resources and technologies. Solar energy has duly been recognized as our largest sustainable and ultimate non-fossil, non-nuclear energy resource. It can be converted into chemical or electrical energy but unfortunately it is a diffuse and intermittent energy source. Thus, the land area necessary for fuel or power generation on a large scale may well be prohibitive unless the efficiency of solar energy conversion technologies is high. Here we discuss photobiological hydrogen, one such technology, as well as the recent R&D directions in the United States in this field and the need and opportunities for international collaborative R&D efforts.

As a consequence of the first oil crisis, the leadership in Japan and the United States realized that a collaborative effort in alternative energy resources would be beneficial to our two countries. In 1975, Prime Minister Fukuda of Japan met with President Carter in Washington, DC, and they signed a Memorandum of Agreement for a collaborative research and development program in Photosynthesis and Photoconversion. The agencies involved were the National Science Foundation (NSF) and the Department of Energy (DOE) in the United States and the Science and Technology Agency (STA) and Ministry of Education (Mombusho) in Japan. Further, Prime Minister Fukuda and President Carter presided over the dedication of the Solar Energy Research Institute (SERI) in Golden, CO, the national laboratory dedicated to solar energy research and known presently as the National Renewable Energy Laboratory (NREL). The dedication was the highlight of the celebration of the nationally proclaimed "SUN DAY". One of us (ASP) was very fortunate to attend the dedication and to be involved later in the collaborative program as a member of the Steering Committee on behalf of the NSF. The other (JRB) benefited from the scientific exchange supported by the program through mutual visits with Prof. Kazuhisa Miyamoto of Osaka University (Miyamoto et al, 1987).

During the intervening years crude oil prices have declined, recently approaching those of pre-oil crisis times. Complacency replaced desire and resolve and, as expected, U. S. federal support for biosolar research became sporadic and intermittent. The decline in crude oil prices did not, however, eradicate the long-term need for alternative resources and technologies. That need is just as urgent presently as a quarter century ago but for somewhat different reasons. And not simply because the lower-cost fossil fuels may be depleted in the foreseeable future, but also, and perhaps more critically, because of the potential for global climate change from fossil CO_2 emissions. Both of these problems remain as very serious concerns to the scientific and governmental communities in many countries. Thus, it is important to address and resolve as soon as possible the basic technical and economic feasibility of alternative energy technologies, such as biohydrogen production, to allow for better allocation of scarce resources between and within applied R&D programs.

What then is the appropriate direction for our future efforts to develop biohydrogen as an energy source? What then should be the goals and objectives of such an endeavor? Is an allocation of resources to a few approaches, or even a single concept, justifiable by present knowledge? Or should research efforts be spread over a multitude of approaches? Is it timely to initiate now a major integrated engineering development effort or is the field still in the basic research stage? Should we emphasize single purpose processes or integrate biohydrogen production into other processes, such as waste management? Each country must provide its own national answers to such issues and for solar energy technologies in general. The answers will reflect the relative emphasis placed by each country on providing for energy and environmental security, on self-sufficiency vs the ability to compete in the world markets and on basic vs applied science. Although the priorities and direction of the R&D efforts may well differ country by country, it is inevitable that large areas of coincidence and common interest will exist and can provide a basis for International Collaboration.

Here we discuss the current US DOE Hydrogen Program priorities and directions and attempt to identify areas of common interest which could form the basis for an International Collaborative effort.

THE US DOE HYDROGEN PROGRAM

The Hydrogen Program was initiated in mid 1970s within the then recently created US Department of Energy to minimize our dependence on oil imports, primarily from the Middle East Exporting Community. The need to remove this dependency and to develop a national and sustainable energy economy was the driving force of the early Hydrogen Program. At that time, hydrogen was touted as the "Fuel of the Future" and we became aware of the "Hydrogen Economy Concept".

Initially, hydrogen production concepts were based on the use of what was then assumed would be inexpensive nuclear-derived electricity. As the real cost of nuclear energy became clear, it became apparent that hydrogen from nuclear power would not be economically competitive. The focus of the program shifted then to hydrogen production from renewable resources such as solar and wind. The increasing focus over the past two decades on air and water pollution, as well as global warming, have identified even greater benefits of hydrogen.

The U. S. Congress recognized the importance of hydrogen energy R&D as early as

1980 although it was not until a decade later that the "Spark M. Matsunaga Hydrogen Research, Development and Demonstration Act of 1990 (P. L. 101-566)" was signed into law by President George Bush. This legislation, often referred to as the "Matsunaga Act", also created the Hydrogen Technology Advisory Panel (HTAP) which is advisory to the Secretary of Energy. Further, the Matsunaga Act shifted the focus of the Hydrogen Program to emphasize production from renewable resources with fossil fuels serving an interim transitional role. The "Energy Policy Act of 1992 (P. L. 102-486)" also emphasized the importance of hydrogen R&D and the "Hydrogen Future Act of 1996 (P. L. 104-271)" provided for increased long term R&D in hydrogen energy. The present day DOE Hydrogen Program is attempting to balance both the long- and short-term objectives of hydrogen R&D, spanning the range from long term and basic R&D to the development of a hydrogen infrastructure in the transportation sector. The following statement is the twenty year vision statement for the Hydrogen Program (US DOE, 1998); namely,

"In the next twenty years, concerns about global climate change and energy security will create the platform for penetration of hydrogen into several niche markets. Ultimately, hydrogen and electricity will come from sustainable renewable energy resources, but fossil fuels will be a significant transitional resource during this period. The growth of fuel cell technology will provide a base for the establishment of the hydrogen option into both transportation and electricity supply markets."

Realization of this vision will require a major, increasing, long-term and stable federal R&D investment in hydrogen production, transmission, storage and utilization given the major remaining scientific, technological and economic obstacles to commercial deployment of hydrogen energy systems. One major ultimate goal of the Hydrogen Program is the production of hydrogen from sunlight and water. To achieve this goal, the Hydrogen Program will support long-term photoelectrochemical and photobiological research and development with a target cost of hydrogen of $10-$15/MMBtu. This is a major technical and economic challenge which may limit the processes that can be considered to only those with the potential of achieving the highest solar conversion efficiencies at the lowest possible cost.

PHOTOBIOLOGICAL HYDROGEN PRODUCTION (BIOHYDROGEN)

The three processes currently under study by the US DOE Hydrogen Program for solar driven bioproduction of hydrogen from water (Table I, Entries 1, 2 and 3A) are all based on variants of the process of oxygenic photosynthesis and often referred to as biophotolysis (Lien and San Pietro, 1975; Benemann, 1996, 1998). Dark fermentation processes per se are not currently being considered in the US DOE Hydrogen Program except for the conversion of CO (from gasification processes) to H_2, a dark reaction catalyzed by photosynthetic bacteria (Weaver et al, 1998).

TABLE I

PHOTOBIOLOGICAL HYDROGEN PRODUCTION

1. Single stage: Direct Photoproduction of Hydrogen by Microalgae: Simultaneous production of hydrogen and oxygen without intermediate CO_2 fixation.
Requirements:
- Oxygen tolerant hydrogenase or nitrogenas
- Efficient hydrogen and oxygen separation system

2. Two stage: Indirect Photoproduction of Hydrogen; Green Microalgae or Non-heterocystous N-fixing Cyanobacteria; Temporal Separation: Alternating production of biomass and hydrogen in a single reactor. Cells are grown in the light and under conditions where they evolve oxygen and store cell biomass high in carbohydrates. They are then made, or allowed, to go anaerobic to induce the hydrogenase and evolve hydrogen in the dark initially and then under illumination.
Requirements:
- Control of PSII to inhibit oxygen evolution under anaerobic conditions
- Induction of H_2 production system under anaerobic conditions in light and dark

3. Two stage: Indirect Photoproduction of Hydrogen; Physical (or Spatial) Separation

A. Green Microalgae or Non-hetereocystous Cyanobacteria: Alternating production of biomass and hydrogen in two separate reactors. Cell are grown in the light under conditions where they evolve oxygen and store carbohydrates. They are then concentrated and moved to a second reactor where they go anaerobic to induce the hydrogenase and evolve hydrogen in the dark and thereafter in the light. Either the same or another organism (usually photosynthetic bacteria) may be used to evolve hydrogen from biomass produced in the oxygenic phase.
Requirements:
- Hydraulic control to harvest the organisms for transfer between reactors
- Control of PSII to inhibit oxygen evolution under anaerobic conditions

B. Heterocystous Cyanobacteria: Production of a reduced substrate in the vegetative cells which is transported to the heterocyst where it is oxidized and hydrogen is released using the nitrogenase or hydrogenase enzymes as the catalyst.
Requirements:
- Development of low maintenance heterocysts
- Separation of H_2 and O_2 produced simultaneously
- Replacement of the inefficient nitrogenase-based H_2 production with a hydro-genase-based system

4. Single and Two stage systems using Photosynthetic Bacteria: Quantitative conversion, in the light, of organic substrates, including microalgal derived fermentation products and organic wastes, into hydrogen and carbon dioxide.
Requirements:
- Suitable substrates/wastes and/or substrate/waste pre-treatment methods
- Replacement of inefficient nitrogenase with hydrogenase

In principle, the simultaneous production of hydrogen and oxygen from water (Table I, Entry 1), also called direct biophotolysis, would be the process of preference. It occurs in a single reactor and would not require the intermediate reduction, and subsequent evolution of CO_2 in the hydrogen evolution stage, as required in the two indirect biophotolysis processes (Table I, Entries 2 and 3; see below). It does, however, require an oxygen-tolerant hydrogenase enzyme and reaction, as well as a method of separating hydrogen and oxygen. As discussed earlier in these Proceedings by Michael Seibert (Seibert et al, pp) there is an active research program underway at NREL to develop an oxygen-tolerant hydrogenase in microalgae using the classical approach of mutagenesis and mutant analysis (McBride et al, 1977). A second major problem is that a potentially explosive mixture of H_2 and O_2 would need to be handled and would require separation, a technically feasible but economically costly step.

The alternative approaches of indirect photoproduction of hydrogen (Table I, Entries 2 and 3A) obviate the need for an oxygen-tolerant hydrogenase and are receiving increased attention in the US DOE Hydrogen Program. In the first stage, microalgae are grown on CO_2 to produce cell biomass high in storage carbohydrates (starch, glycogen, etc) concomitant with the release of molecular oxygen (see Benemann, 1997). The cell culture is then made or allowed to go anaerobic and the reversible hydrogenase becomes activated and/or induced. Hydrogen gas is evolved, some in the dark but mostly in the light, in a PSI mediated or assisted reaction, until the storage carbohydrates are depleted. At this point the cycle would be repeated by initiation of a new phase of aerobic CO_2 fixation. The two stages can be separated temporally (Table I, Entry 2), with the culture remaining in the same reactor, or physically (Table I, Entry 3A) with the two stages being carried out in different reactors. The choice between processes could be based on the scale; for large-scale systems, a two reactor process may be preferred, where the aerobic stage is carried out in large low-cost open ponds and the anaerobic stage in much smaller enclosed photobioreactors. Small systems might be single or dual reactor processes in which the entire process occurs within closed photobioreactors.

Both the direct and indirect biophotolysis processes could also be carried out with nitrogen-fixing cyanobacteria (Table 1, Entries 1-3). Indeed, much, perhaps even most, of the work in photobiological hydrogen production has used nitrogen-fixing cyanobacteria and photosynthetic bacteria in hydrogen evolution studies. However, the nitrogenase enzyme is even more oxygen sensitive than the reversible hydrogenase and thus even more unlikely in a direct biophotolysis process. And, most important, even for indirect biophotolysis processes, the great inefficiency of this enzyme (4 ATP per H_2 produced) limits the potentially achievable efficiency by such systems to only about half of what could, at least in theory, be possible with hydrogenase-based systems. Although hetereocystous cyanobacteria can produce O_2 and H_2 simultaneously, their application is questionable due to the high energy demand of the heterocyst for respiration, maintenance and biosynthesis. While biophotolysis based on nitrogenase-mediated H_2 production can be readily demonstrated with heterocystous or non-heterocystous cyanobacteria, the inherently low efficiency of such systems is the basis for their removal from the US DOE Hydrogen Program.

BIOPHOTOLYSIS ECONOMICS AND PHOTOSYNTHETIC EFFICIENCIES

A preliminary assessment of a conceptualized two stage indirect biophotolysis system was reported by John Benemann (1997) to the International Energy Agency (IEA) which in

summary stated:

"A two stage indirect biophotolysis system was conceptualized and general design parameters extrapolated. The process comprises open ponds for the CO_2 fixation stage, an algal concentration step, a dark adaptation and fermentation stage, and a closed tubular photobioreactor in which hydrogen production would take place. A preliminary cost analysis for a 200 ha system, including 140 ha of open ponds and 14 ha of photoreactors was carried out. The cost analysis was based on prior studies for algal mass cultures for fuels production and a conceptual analysis of a hypothetical photochemical process, as well as the assumption that the photo-bioreactors would cost about $\$100/m^2$. Assuming a very favorable location, with 21 MJ/m^2 total insolation, and a solar conversion efficiency of 10% based on CO_2 fixation in the large algal ponds, an average cost of \$10/GJ H_2 is projected. Of this almost half is due to the photobioreactors, one fourth to the open pond system, and the remainder to the H_2 handling and general support systems".

In a follow-up report, Tredici et al (1998) carried out a similar analysis for an indirect single stage process in which the entire process would take place in closed tubular photoreactors. Based on an actual analysis for a defined photobioreactor design (not just an assumption as in the earlier effort), a cost of only $\$50/m^2$ for the photobioreactors was concluded. And in this case it included overhead and other indirect capital costs. Overall costs from such single stage, or at least entirely closed, systems were projected at about \$15/GJ, a value within the economic target range of the US DOE Hydrogen Program. However, both studies are based on many favorable assumptions. Each study assumed a 10% solar energy conversion efficiency by the microalgal cultures. Without such an efficiency, the cost of H_2 would rapidly increase as conversion efficiency declines. Achieving a 10% conversion efficiency for CO_2 under sunlight conditions will be a major challenge in developing any such process. Thus, solar energy conversion efficiencies are a new focus for support by the US DOE Hydrogen Program.

It is generally accepted that the photosynthetic machinery can convert the light energy of only about 200 photons/sec into useful chemical energy. This rate is apparently dictated by the seemingly irreducible turnover time of electron transfer between the two photosystems; namely, about 5 msec. At full sunlight, the photon flux reaches about 2000 photons/sec or some 10 times the rate that absorbed photons can be usefully converted into chemical energy. That is, 90% of the absorbed photons are lost as heat or fluorescence. Thus, we get the normally noted value of 10% for the maximum solar energy conversion efficiency at high light intensities.

As the turnover rate for electron flow between the two photosystems is not likely to be subject to improvement, then the only plausible approach to increasing solar energy conversion efficiency is to reduce the photon capture by the photosynthetic apparatus at high light intensities. This can be accomplished by decreasing the number of so-called antenna chlorophylls in green algae (phycobiliproteins in cyanobacteria). Although fewer photons will be absorbed per individual cell in a culture with smaller photosynthetic antenna size, a larger proportion of the photons absorbed by the culture as a whole will be converted into useful chemical energy. Since each cell absorbs less of the incoming radiation, light penetration will proceed deeper into a culture and more cells will be productive. As reported by Professor T. Melis, the basic validity of this approach has been demonstrated and mutants are being developed that exhibit such high efficiencies at high solar insolation (Melis et al, 1998, 1999; Neidhardt et al, 1998). Clearly, such "antenna-size reduction" is a pre-condition

for any of the photobiological hydrogen production processes listed in Table I

INTERNATIONAL COLLABORATION

The ultimate goal of biohydrogen R&D is to provide a major source of sustainable and renewable energy free from hazardous greenhouse gas emissions. Realization of practical processes for photobiological hydrogen production from water by microalgae or cyanobacteria will require a long term, sustained and substantial investment in basic and applied research to achieve the scientific and technological advances required. The cost of these efforts, and their long-term nature, makes collaborative efforts at the international level, through the pooling of resources, personnel and skills, a cost effective and logical approach to this R&D effort. Applied research in biological hydrogen production is currently being supported by the United States and Japanese governments with European efforts mainly, though not exclusively, in more basic areas such as structure and mechanism of hydrogenases and microbial hydrogen metabolism and genetics.

Although the approaches, orientations, processes and objectives of the research carried out in these different countries are quite distinct, a number of common research and programmatic elements suggest several areas of mutual interest for future R&D collaborations:

- The demonstration and improvement of light-driven hydrogen evolution by reversible hydrogenases under anaerobic conditions in green algae and cyanobacteria;
- An increase in the photosynthetic efficiences for CO_2 fixation and H_2 production through accessory (antenna) pigment reduction;
- An increase in dark anaerobic hydrogen fermentation yields through metabolic engineering and other molecular and physiological techniques; and
- Systems integration and development including bioreactor design and testing.

A coordinated international collaborative R&D program offers the advantages of cost-sharing the overall research effort, of making information more readily available and avoiding unnecessary duplication while providing mechanisms for rapid validation of Hydrogen Production" under the IEA Agreement on the Production and Utilization of Hydrogen. Under the predecessor "Task 10", which included both photobiological and photoelectrochemical technologies, several collaborations were carried out, including:

- A joint effort between the United States, Japan and Norway to establish a data bank for hydrogen producing microorganisms;
- Collaborative research between Sweden, Japan and the United States on the genetics of cyanobacterial hydrogen production; and
- A United States - Italy collaboration on the development of photobioreactors for hydrogen production.

As detailed above, the United States and Japan share a long and productive history of

collaborative research in the mechanism of biological solar energy capture and utilization. There is strong support for such international activities in both of these countries. We can note the funding by RITE of several projects outside its own borders, including Italy, England and the United States. Dr. Neil Rossmeissl of the U. S. Department of Energy has expressed the interest of the United States in participating and supporting international collaborations in biohydrogen production (Rossmeissl, 1998). The new IEA "Annex15" specifically dealing with this topic and including European participation, highlights the importance placed by R&D authorities in these countries on the long-term technology. Both prior, current and novel mechanisms should be used and developed to further such international collaboration. The present meeting is one such mechanism to develop support from the R&D community for this field. Whatever the process or mechanism, the important objective is to help advance the science and technology as rapidly as possible, and international collaboration can play a central role.

REFERENCES

Benemann, J. R. (1998). The Technology of Biohydrogen, in "Biohydrogen", Zaborsky et al (Eds), Plenum Press, pp. 19-30.

Benemann. J. R. (1997). Process Analysis and Economics of Biophotolysis of Water: A Preliminary Assessment, Report to the IEA Agreement on the Production and Utilization of Hydrogen, Task 10B.

Benemann, J. R. (1996). Hydrogen Biotechnology: Progress and Prospects. *Nature Biotechnology* **14**, 1101-1103.

Lien, S. and San Pietro, A. (1975). An Inquiry Into Biophotolysis of Water to Produce Hydrogen, Report to the National Science Foundation.

McBride, A. C., Lien, S., Togasaki, R. and San Pietro, A. (1977). Mutational Analysis of Chlamysomonas reinhardti: Application to Biological Solar Energy Conversion, in Biological Solar Energy Conversion, Mitsui, A. et al (Eds.), Academic Press, New York, pp. 77-86.

Melis, A., Neidhardt, J. And Benemann, J. R. (1999). Dunaliella salina (Chlorphyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities an photon use efficiencies than normally pigmented cell. *J. App. Phycology* **10**, 515-528.

Melis, A., Neidhardt, J., Baroli, I. and Benemann, J. R., (1998). Maximizing Photosynthetic Productivity and Light Utilization in Microalgae by Minimizing the Light-Harvesting Chlorophyll Antenna Size of the Photosystems, in "Biohydrogen", Zaborsky et al, Eds, Plenum Press, pp. 41-52.

Miyamoto, K. and Benemann, J. R. (1988). Vertical Tubular Photobioreactor Design and Operation. *Biotechnology Letters* **10**, 703-710.

Neidhardt, J., Benemann, K. R. Zhang, L. And Melis, A. (1998). Photosystem II repair an chloroplast recovery from irrandiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in Dunaliella salina (green algae). *Photosynth. Res.* **56**, 175-184.

Rossmeissl, N. P. (1998) International Collaboration in Biohydrogen: An Opportunity in Biohydrogen, Zaborsky et al (Eds.), Plenum Press, pp. 447-450.

Seibert, M., Flynn, T., Bensen, D., Tracy, E. and Ghirardi, M. (1998) Development of selection and screening procedures for rapid identification of H_2-producing algal mutants with increased O_2-tolerance, in "Biohydrogen", Zaborsky et al (Eds.), Plenum Press, 227-234.

Strategic Plan for DOE Hydrogen Program, DOE/GO 10098-532, January 1998.

Tredicii, M. R., Zittelli, G. C. And Benemann, J. R. (1998). A tubular internal gas exchange photobioreactor for biological hydrogen production: Preliminary cost analysis in "Biohydrogen", Zaborsky et al (Eds.), Plenum Press, pp. 391-402.

Weaver, P. F., Maness, P. C., Rhodes, C, Seahill, J., Dundorf, S. and Martin, S. (1998). Biological Hydrogen from fuel Gases and From Water in Proceedings of the 1998 U. S. Hydrogen Program Review, Volume 1, pp. 1-8, NREL/CP-570-25315.

This Page Intentionally Left Blank

HYDROGENOTROPHY
–A NEW ASPECT OF BIOHYDROGEN–

Y. Igarashi

Department of Biotechnology, University of Tokyo,
Tokyo 113-8657, Japan,
E-mail; aigara@hongo.ecc.u-tokyo.ac.jp

ABSTRACT

It is well known that molecular hydrogen (H_2) is one of the products in the anaerobic metabolism of various microorganisms. At major the same time, molecular hydrogen can also serve as a suitable energy source for the growth of various microorganisms. Many kinds of microorganism can use molecular hydrogen as the sole energy source and carbon dioxide as the sole carbon source for their growth, which are known as hydrogen bacteria or hydrogenotroph. We have been working on taxonomy, physiology, biochemistry and molecular biology of hydrogenotrophs from various origin. In this presentation, I would like to summarize our studies on "hydrogenotrophy" and explain our new project, in which utilization of microbial metabolism on molecular hydrogen is used as the key technology.

1. UTILIZATION OF HYDROGENOTROPHS TO PRODUCE ORGANIC MATTERS FROM CO_2

Hydrogenophilus thermoluteolus (*Pseudomonas hydrogenothermophila*) isolated from hot spring in Japan is the fastest growing CO_2-fixing organism among any reported autotrophs including bacteria, algae and plants[1-3] (Fig. 1). The specific growth rate of *H. thermoluteolus* is around 0.7 (Fig. 2). *Hydrogenovibrio marinus* is also a hydrogen-oxidizing bacterium isolated fron sea water of Shonan Coast, Kanagawa, Japan[4] (Fig. 3). The specific growth rate of *H. marinus* in autotrophic culture is almost equal to that of *H. thermoluteolus*.

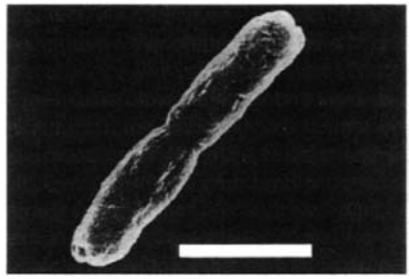


Figure 1: Scanning electronmicrograph of *Hydrogenophilus thermoluteolus*. Bar, 1 μm.

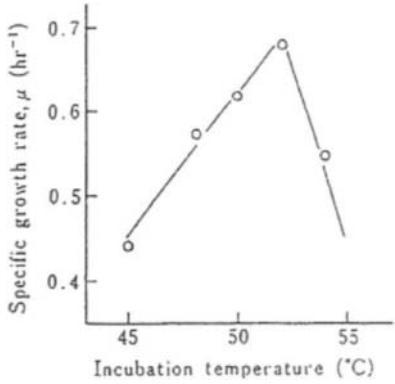


Figure 2: Effect of Growth Temperature on the Cell Growth of *H. thermoluteolus*.

Figure 3: Scanning electronmicrograph of *Hydrogenovibrio marinus*. Bar, 1 μm.

We have been studying production of organic matters from carbon dioxide using these hydrogenotrophs. Up to date, by using hydrogenotrophs including both of aerobic and anaerobic microorganisms, many kinds of organic matter has been produced carbon dioxide and molecular hydrogen as listed in Table 1.

TABLE 1
CURRENT SITUATION FOR THE MICROBIAL PRODUCTION OF ORGANIC MATERIALS
(FOCUS ON HYDROGEN BACTERIA)

Product		Strain	Productivity	Research Institute	Comment
Chemical	Alkohol				
	Ketone				
	Organic acid	Methanogen	Formate 31 g/l (50h)	Hiroshima Univ.	
		Acetogen *(Acetobacterium)*	Acetate 150 g/l/day	Daiseru Co.	
Biopolymer	PHB	*Alcalogenes eutrophus*	80% of cell mass	Kyushu Univ.	
Vitamin	Viramin B_{12}	Methanogen *(Methanosarcina)*	90 mg/l/day	Hiroshima Univ.	
Sugar	Polysacharide	*Pseudomonas hydrogenovora*	12 g/l	Tokyo Univ.	Possibility for the preparation of ^{13}C-labelled sugar(s)
	Glycogen	*Hydrogenovibrio marinus*		Tokyo Univ.	
Protein	SCP	*Pseudomonas hydrogenothermophila*	8 g/l (batch) 3 g/l (continuous)	Tokyo Univ.	
	L-Tryptophan	Analog resistant mutant of *P. hydrogenothermophila*			First example for the production from CO_2
Materials for high-value product	Hydrogenase	*Alcaligenes eutrophus*		Germany	
	Cytochrome *c*	*Hydrogenobacter*		Tokyo Univ.	

Autotrophic organisms can be divided to two categories, namely "photoautotrophs" using light energy to reduce CO_2 to organic matters and "chemoautotrophs" using chemical energy as the reductant. The advantage of using chemoautotrophs, especially using hydrogenotrophs, to reduce CO_2 to organic matters is that we can minimize the space the reaction by concentrating the reducing energy into a reactor. According to our calculation with the researchers of Central Research Institute of Electric Power Industry, we need more than 100 km^2 to fix CO_2 from a 1 MKW power plant using oil or natural gas when we use a phototrophs which can fix 100g CO_2 /m^2/day. On the other hand, when we use *Hydrogenophilus thermoluteolus* (*Pseudomonas hydrogenothermophila*) as the CO_2 fixing living catalyst, we need 105 m^3 bioreactor to fix the same amount of CO_2. Moreover this can be remarkably improved by developing a new reactor with more efficient energy supply system.

The marine hydrogenotroph, *H. marinus*, is known to produce glycogen in the cell under certain conditions[5,6] (Fig. 4).

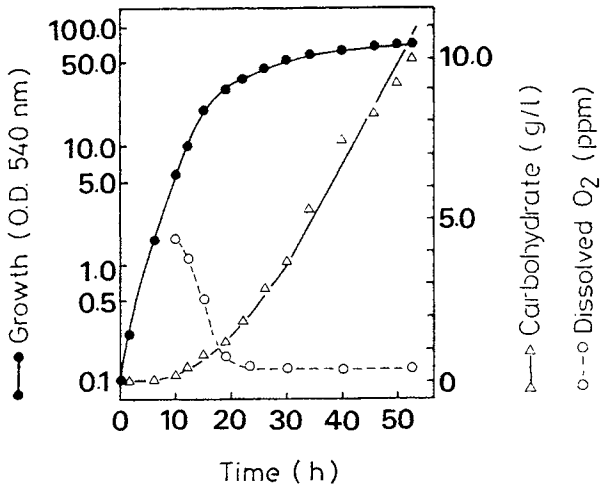


Figure 4: Production of plysaccharide (glycogen) by oxigen-limited culture of *H. marinus*.

This glycogen can be easily digested by digestive enzyme. The productive efficiency of polysaccharide production by using this microorganism is far higher than that by using plants.

In summary, production of organic resources from CO_2 and H_2 seems to me as the promising system in post-petroleum age, especially in Japan, a country with highly limited natural resources and land.

2. PROPOSAL OF A NEW PROJECT, BREEZE (BIOLOGICAL RECYCLING SYSTEM OF ENERGY AND ORGANIC MATERIALS FOR ZERO EMISSION.

According to above consideration, now we are proposing to construct a new industrial recycling system using microbiological processes (Fig. 5). The main concept of this process named *BREEZE* (Biological REcycling system of Energy and organic materials for Zero Emission) is to establish a new industrial or social system of recycling energy and organic materials and minimizing the amount of wasted materials and energy. The *BREEZE* system is just following been going on this planet, and various biological processes should be what has introduced into this system. Similar systems have been also proposed, but the most distinct feature of *BREEZE* project is putting molecular hydrogen in the center of the system as the key energy material.

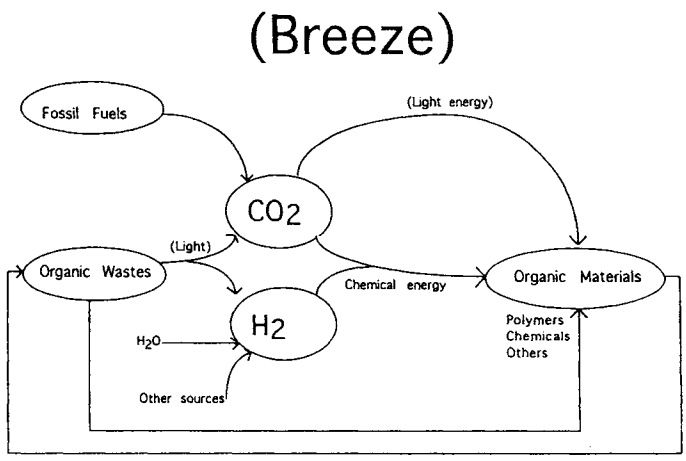


Figure 5: Biological Recycling System of Energy and Organic Substances for Zero-Emission of CO_2.

The main portion of the *BREEZE* Project is divided into two parts, namely production of molecular hydrogen by biological methods and reorganization of carbon dioxide by using hydrogenotrophs (Fig. 6). As a method for biological production of molecular hydrogen (biohydrogen), photobiological method using photosynthetic microorganisms is common, but at this moment, we are mostly thinking of the hydrogen production from organic waste by using anaerobic microorganisms in this system. The reason is available land for the utilization of solar energy is limited in Japan. By using condensed solar energy in organic waste, we can produce large amount of molecular hydrogen in a limited space. Our calculation revealed that the total combustion energy of various kinds of organic waste will reach to 10% of the total energy supply in this country.

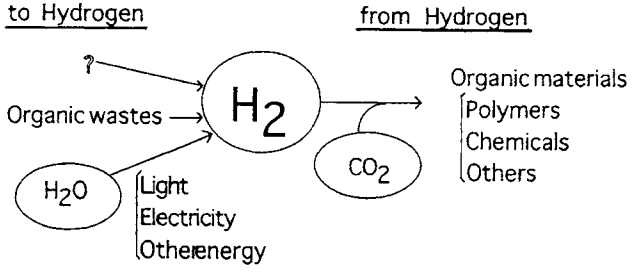


Figure 6: Biohydrgen (from Hydrogen and to Hydrogen). The major part of the BREEZE Project is production of hydrogen from organic waste by anaerobic bacteria and re-organization of CO_2 by using hydrogen as a reductant by hydrogenotrophic bacteria.

As organic materials produced by the reduction of carbon dioxide, now we are thinking of some chemicals such as acetic acid and biopolymers such as carbohydrates, proteins, and lipids. Some fine chemicals and biomaterials will be also producible, but the needs for those materials will not be so large.

At this moment, the *BREEZE* Project cannot be economically survive in Japan, because of surprisingly low price of petroleum, though it may survive in some part of Asian countries. But, we will never be able to receive benefits from low price of oil so long time. And we have almost no petroleum or natural gas resources in Japan. I believe, especially in this country, we should prepare for post petroleum era and think of the way for soft-landing to another new industrial or social system. I believe that will be biological recycling system. The *BREEZE* Project is the pioneer of the new system in the new era.

REFERENCES

1. E. Goto *et al.* (1977). Isolation and Culture Conditions of thermophilic Hydrogen Bacteria. *Agric. Biol. Chem.* **41**, 685.
2. Goto *et al*,. (1978). Growth and Taxonomy of thermophilic Hydrogen Bacteria. *Agric. Biol. Chem.* **42**, 1305.
3. N.R.Hayashi *et al.* (1999). Hydr*ogenophilus thermoluteolus* gen. nov., sp. nov., a thermophilic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacterium. *Int. J. System. Bacteriol.* **49**, 783.
4. Nishihara *et al.* (1991). *Hydrogenovibrio marihus*, gen. nov., sp. nov., a Marine Obligately Chemolithoautotrophic Hydrogen Oxidizing bacterium. *Int. J. System. Bacteriol.* **41**, 130.
5. Nishihara *et al.* (1991). Growth Characteristics and High Cell density Cultivation of a Marine Obligately Chemolithoautbtrophic Hydrogen-Oxidizing Bacterium, *Hydrogenovibrio marinus* Strain MH-110 under a Continuous Gas Flow System, *J. Ferment. Bioeng.* **72**, 358.
6. Nishihara *et al.* (1993). Production and Properties of Glycogen in the Marine Obligate Chemolithoautotroph, *Hydrogenovibrio marinus*. *J. Ferment. Bioeng.* **75**, 414.

III. Biochemical and Metabolic Systems

This Page Intentionally Left Blank

MAXIMIZING PHOTOSYNTHETIC EFFICIENCIES AND HYDROGEN PRODUCTION IN MICROALGA CULTURES

J. Polle, S. Kanakagiri, J. R. Benemann and A. Melis

Department of Plant and Microbial Biology,
411 Koshland Hall, University of California,
Berkeley, CA 94720-3102

ABSTRACT

For algal mass cultures and H_2 production, conditions that maximize photosynthetic productivity and solar conversion efficiency are important in determining sustainability and profit. We have shown (Melis et al. 1999) that photosynthetic efficiencies and hydrogen production by microalgal cultures can be increased upon minimizing the number of the light-harvesting chlorophyll (Chl) antenna pigments of photosynthesis. A highly truncated light-harvesting Chl antenna size in green algae could result in: (a) 6-7 times greater photosynthetic productivity (on a per Chl basis), compared to that of normally pigmented cells, and (b) ~3 times greater yields of photosynthesis and H_2 production under mass culture, compared to that of normally pigmented cells.

We report here the application of molecular genetic approaches for the generation of transformant green algae with a permanently truncated Chl antenna size. Upon generating and screening a library of 6,500 DNA insertional transformants in the green alga *Chlamydomonas reinhardtii,* 155 mutants aberrant in Chl fluorescence, i.e., possibly aberrant in Chl antenna size, have been isolated. Three distinct classes of mutants were identified: mutants aberrant in Chl *b* biosynthesis and mutants aberrant in the regulation of the Chl antenna size (both down-regulated and up-regulated). Initial biochemical characterization of some of these mutants is presented. The work provides evidence that a smaller and stable Chl antenna size in green algae can be achieved through the application of molecular genetic techniques. Moreover, some unique insights were gained from a detailed examination of the Chl *b*-less mutant. This mutation was partially overcome through a nearly quantitative substitution of Chl *b* with Chl *a* in

photosystem-I (PSI), and by a partial substitution by Chl *a* in PSII. These substitutions resulted in a PSI Chl antenna size almost as large in the mutant as in the control, but a PSII antenna size in the mutant that was less than half of that in the control. Genetically engineered algae with a *'truncated Chl antenna* 'can increase the productivity of the culture under moderate to high irradiance. Immediate future plans include the biochemical analysis of additional isolates in search of the smallest possible Chl antenna size for PSII and PSI, and the cloning and sequencing of the genes that regulate the Chl antenna size of photosynthesis.

INTRODUCTION

Microalgal mass cultures growing under high irradiance, such as direct sunlight, have significantly lower photon use efficiencies than when grown under low irradiance. The reason for this fundamental inefficiency is that, at moderate to high irradiance, the rate of photon absorption by the antenna chlorophylls far exceeds the maximal rate of photosynthesis. The excess absorbed photons are dissipated as fluorescence or heat. Thus, in algal mass cultures, the first few layers of cells absorb and waste a large proportion of the incident photons, while strongly attenuating the light received by cells deeper in the culture [Naus and Melis 1991, Neidhardt et al. 1998]. More than 90% of absorbed photons can thus be wasted [Melis et al. 1999], reducing photon use efficiencies and photosynthetic productivity.

Theoretically [Kok 1953, 1973, Myers 1957], a truncated chlorophyll (Chl) antenna size of the photosystems (PS) is expected to increase the photon use efficiency of microalgae in mass culture as it would minimize the wasteful dissipation of absorbed sunlight, diminish mutual cell shading, permit a greater transmittance of light through the culture and, thus, result in a more uniform illumination of the cells. Overall, this should result in a higher photosynthetic productivity of the microalgal culture [Kok 1960]. These theoretical considerations have been quantitatively tested in the laboratory, supporting the prediction that cells with a highly truncated Chl antenna size will exhibit superior photosynthetic productivity and solar use efficiency compared to that of normally pigmented control cells [Melis et al. 1999].

Thus, for purposes of industrial application, it would be necessary to develop microalgal mutants with a permanently truncated light-harvesting Chl antenna size. To achieve this goal, we took advantage of recent progress in the fields of the Chl antenna organization and regulation of assembly in chloroplasts. The work employed recently developed molecular genetic approaches to generate transformant green algae with a permanently truncated Chl antenna size. Preliminary results show that, indeed as expected, green algae with a permanently truncated Chl antenna size exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented control cells.

MATERIALS AND METHODS

Cell Cultures and Growth Conditions

Chlamydomonas reinhardtii, strains *cw15* and *CC425* (*arg7.8 cw15 mt*$^{+}$ *sr-u-60,* an arginine auxotroph; Chlamydomonas Genetics Center, Duke University), were cultivated in Tris-Acetate-Phosphate (TAP) [Gorman and Levine 1965] or high salt (HS) [Sueoka 1960] media. Cultures of strain *CC425* were supplemented with 50 μg mL^{-1} arginine. Liquid cultures were grown in flat Roux bottles upon stirring under continuous illumination (200 μmol photons·m^{-2}·s^{-1}) provided by cool-white fluorescence lamps.

E. coli cells transformed with plasmid pJD67 [Davies et al. 1996] were grown in a 37°C incubator/shaker in LB media supplemented with 100 μg mL^{-1} ampicillin. The plasmid DNA (pJD67), containing the argininosuccinate lyase gene, was isolated from liquid *E. coli* cultures using a Qiagen midiprep kit (Qiagen Inc, CA). Plasmids were linearized upon digestion with HindIII prior to been used for insertional mutagenesis of *C. reinhardtii* strain *CC425.*

Cell Count and Chlorophyll Determination

The cell density in the cultures was measured by counting with a Hemacytometer (improved Neubauer chamber) and an Olympus BH-2 compound microscope. Cells were immobilized and stained by addition of several μL of Lugol solution to a 1 mL aliquot of the culture. Pigments from cells or thylakoid membranes were extracted in 80% acetone and debris was removed by centrifugation at 10,000g for 5 min. The absorbance of the supernatant at 720, 663 and 645 nm was measured by a Shimadzu UV-visible spectrophotometer. The chlorophyll (*a* and *b*) concentration of the samples was determined according to Arnon [1949], with equations corrected as in Melis et al. [1987].

Thylakoid Membrane Isolation

Cells were harvested by centrifugation at 1,000g for 3 min at 4°C. Pellets were resuspended in 1-2 mL of growth medium and stored frozen at -80°C until all samples were ready for processing. Samples were thawed on ice and diluted with sonication buffer containing 100 mM Tris-HCl (pH 6.8), 100 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone-40, 0.2% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 μM phenyl-methylsulfonylfluoride (PMSF). Cells were broken by sonication in a Branson 200 Cell Disruptor operated at 4°C. The samples were sonicated three times for 30 s (pulse mode, 50% duty cycle, output power 5). Unbroken cells and starch grains were removed by centrifugation at 3,000g for 4 min at 4°C. Thylakoid membranes were collected by centrifugation of the supernatant at 75,000g for 30 min at 4°C. The thylakoid membrane pellet was resuspended in a buffer containing 250 mM Tris-HCl (pH 6.8), 20% glycerol, 7% SDS and 2 M urea. Solubilization of thylakoid proteins was carried out for 30 min at room temperature, a procedure designed to prevent the formation of protein aggregates during denaturation. Samples were centrifuged in a microfuge for 4 min to remove unsolubilized material, 2-mercaptoethanol was added to yield a final concentration of 10% and the samples were stored at -80°C.

SDS-PAGE and Western Blot Analysis

Samples were brought to room temperature prior to loading for electrophoresis and diluted accordingly to yield equal Chl concentrations. Gel lanes were loaded with an equal amount of Chl (1 nmol Chl per lane). SDS-PAGE was carried out according to Laemmli [1970], with the resolving gel containing 12.5% acrylamide, at a constant current of 9 mA for 16 h. Electrophoretic transfer of the SDS-PAGE resolved proteins onto nitrocellulose was carried out for 4 h at a constant current of 800 mA. The transfer buffer contained 50 mM Tris, 380 mM glycine (pH 8.5), 20% methanol and 1% SDS. Identification of thylakoid membrane light-harvesting proteins was accomplished with specific polyclonal antibodies kindly provided by Dr. R. Bassi [Di Paolo et al. 1990]. Cross-reaction with the antibodies was detected by a chromogenic reaction with anti Ig-G secondary antibodies conjugated with alkaline phosphatase (BioRad, Hercules, CA). The blots were scanned with an HP-scanner and quantified with an NIH Imaging program.

Photosynthetic Apparatus Activity Measurements

The concentration of functional PSI and PSII reaction centers was estimated from the amounts of P700 and Q_A, respectively, present in the various samples. The amounts of P700 and Q_A were determined from the amplitude of the light-*minus*-dark absorbance change at λ=700 nm (ΔA_{700}) and at λ =320 nm (ΔA_{320}), respectively [Melis 1989, Smith et al. 1990]. The functional Chl antenna size of PSI and PSII was measured from the kinetics of P700 photooxidation and Q_A photoreduction, respectively [Melis and Anderson 1983, Melis 1989].

The initial non-variable (F_o), variable (F_v) and maximum (F_{max}) yield of chlorophyll fluorescence was measured with intact cells suspended in their growth medium. Actinic excitation was provided in the green region of the spectrum by CS 4-96 and CS 3-69 Corning filters at an intensity of 35 μmol photons·m^{-2}·s^{-1}.

Photosynthetic activity of the cells was assessed from measurements of the light saturation curve of photosynthesis, obtained with a Clark-type oxygen electrode as described earlier [Melis et al. 1997]. Actinic excitation was provided in the yellow region of the spectrum by CS 3-69 Corning filter in combination with a 35-5453 VIQ 5-8 Ealing filter.

DNA Insertional Mutagenesis of C. reinhardtii

Strain *CC425* was used as the host strain to generate nuclear transformants of *Chlamydomonas reinhardtii*. *CC425* was grown in TAP medium supplemented with 50 μg mL^{-1} of arginine at ~50 μmol photons·m^{-2}·s^{-1}. Plasmid pJD67, containing the argininosuccinate lyase (*ARG7*) gene [Debuchy et al. 1989], was linearized with HindIII and subsequently used to transform strain *CC425* by a procedure similar to that described by Davies et al. [1994, 1996]. Arg^+ transformants were selected on TAP agar media lacking arginine. A library of 6,500 independent nuclear transformants were generated with the Arg^+ phenotype in *C. reinhardtii* and maintained on grid in 175 TAP agar index plates. Independent transformant colonies were streaked onto TAP agar index plates and grown to a size of ~10 mm^2 under cool-white fluorescent illumination

of ~50 μmol photons·m^{-2}·s^{-1} intensity. Subsequently, plates were transferred to weak light (5 μmol photons·m^{-2}·s^{-1}) until further processing.

Screening of Transformants by Fluorescence Video Imaging Analysis

C. reinhardtii transformants on TAP agar index plates were screened for aberrant chlorophyll fluorescence yield properties *via* a fluorescence video imaging apparatus [Niyogi et al. 1997]. Prior to screening, the index plates were kept under a light intensity of about 10 μmol photons·m^{-2}·s^{-1} for at least 18 h. Actinic illumination of ~ 2,000 μmol photons·m^{-2}·s^{-1}, sufficient to induce the F_{max} emission from the algal colonies, was employed in this fluorescence video imaging analysis. The actinic illumination was administered for a period of 1 s and the resulting fluorescence images were captured by the digital video camera of the apparatus. From the displayed color image, transformants with a yield of Chl fluorescence either lower or greater than the control were identified. Color images of chlorophyll fluorescence were calibrated with the *CC425* host strain prior to screening the mutant index plates.

Transformants showing fluorescence yields either lower or greater than the control were identified, isolated from the index plates and tested for photoautotrophic growth in HS media (agar plates as well as liquid media) under low illumination conditions.

RESULTS

Isolation of DNA Insertional Transformants with Aberrant Chlorophyll Fluorescence

Figure 1 shows a fluorescence video image of an index plate containing 24 *Chlamydomonas reinhardtii* DNA insertional transformants. All colonies, except one, displayed Chl fluorescence yields similar to that of the control (greenish color). The exception was a colony in position "g-3", which showed a substantially lower yield of Chl fluorescence (blue-violet color). From 6,500 transformants that were screened with this fluorescence video imaging technology, 129 transformant colonies displayed Chl fluorescence yield properties similar to that of the colony in position "g-3". The lower yield of Chl fluorescence from such colonies may be a consequence of a truncated Chl antenna size for the photosystems in these transformants. These colonies were selected for further study.

Figure 2 shows a fluorescence video image of an index plate containing 33 *Chlamydomonas reinhardtii* DNA insertional transformants. All colonies, except one, displayed chlorophyll fluorescence yields similar to that of the control. The exception in this case was a colony in position "c-4", which showed a substantially greater yield of Chl fluorescence (red color). From 6,500 transformants that were screened with this fluorescence video imaging technology, 26 transformant colonies displayed a high chlorophyll fluorescence yield, similar to that of the colony in position "c-4". The significantly greater yield of Chl fluorescence in these colonies may signify an unusually large Chl antenna size for the photosystems in these transformants. These transformants may be impaired in the regulation of the Chl antenna size in a way that causes the unregulated formation of large Chl antenna sizes in the cells. Such impairment is useful because it

may lead to the genes that regulate the Chl antenna size of photosynthesis. Colonies with a high yield of Chl fluorescence were also selected for further study.

Table 1 shows initial characterization of a small fraction of the isolated transformants. Strain # 1 was the "control", strains # 2-6 were DNA insertional transformants that displayed relatively low Chl fluorescence, and strain # 7 was a transformant with a relatively high Chl fluorescence. The Chl content of the cells and the Chl *a*/Chl *b* ratio were measured following strain cultivation in a small volume of liquid culture. A common feature of these transformants was the significantly lower than the control Chl content of the cells. Interestingly, this was the case for the five low-Chl fluorescence (strains # 2-6) as well as the sole high Chl fluorescence strain (# 7).

TABLE 1

CHLAMYDOMONAS REINHARDTII DNA INSERTIONAL TRANSFORMANTS WITH ABERRANT CHLOROPHYLL FLUORESCENCE PROPERTIES

Strain Number	Cell type/ Mutant No.	Fluorescence intensity	Chl/cell, $x10^{-15}$ mol/cell	Chl *a*/Chl *b* ratio
1	control	control	6.7	2.7/1
2	Chl *b*-less	very low	3.4	infinity
3	KS-061-16	very low	0.9	5.8/1
4	KS-032-18	low	2.3	2.8/1
5	KS-017-04	low	2.2	2.5/1
6	KS-009-23	low	3.3	2.4/1
7	KS-032-27	high	2.2	2.2/1

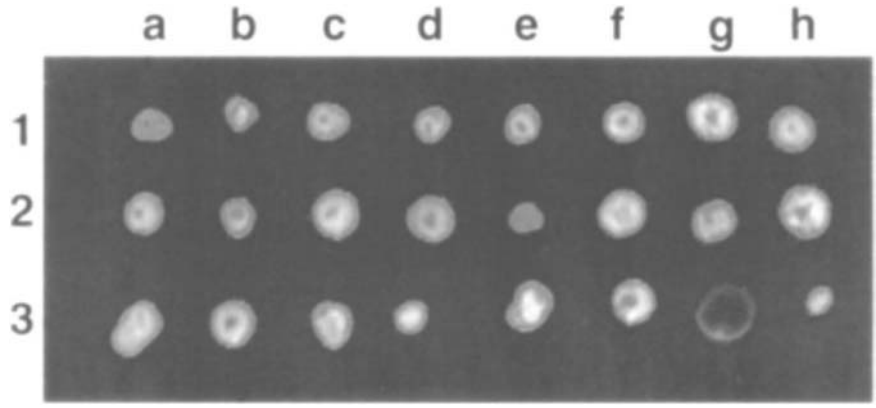


Figure 1: Fluorescence Video Imaging Analysis of *C. reinhardtii.*

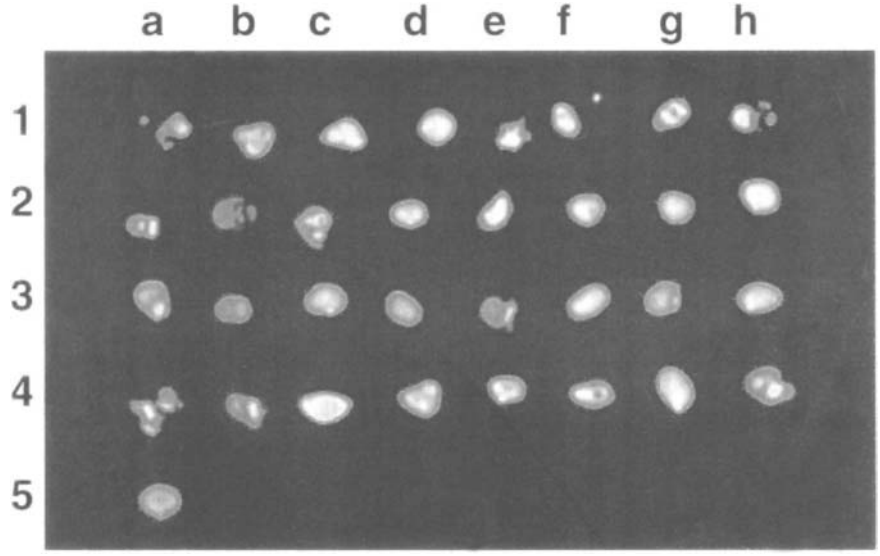


Figure 2: Fluorescence Video Imaging Analysis of *C. reinhardtii.*

The intensity of Chl fluorescence by the transformant strains was calibrated against that of the control. Green color signifies Chl fluorescence yield similar to that of the control, blue-violet is lower and red is higher yield of Chl fluorescence.

Measurements of the Chl *a*/Chl *b* ratio (Table 1) showed that strain # 2 [Tanaka et al. 1998] was aberrant in Chl *b* biosynthesis (Chl *b*-less mutant). Strain # 3 had a Chl *a*/Chl *b* ratio of 5.8/1 (Chl-deficient mutant), whereas strains # 4-7 had either similar or lower than the control Chl *a*/Chl *b* ratios. It is obvious that strains # 2 and # 3 are good candidates of a truncated Chl antenna size and, therefore, suitable for the objectives of the DOE H_2 program. The work below provides a more detailed characterization of the Chl *b*-less transformant (strain # 2, Table 1). Work currently in progress seeks to also characterize the remainder of the isolated transformants.

Photochemical Apparatus Organization in Control and Chl b-less Mutant

Table 2 shows the result of quantitative measurements of P700 and Q_A in isolated thylakoid membranes. Control *C. reinhardtii* exhibited a P700/Chl ratio of 2.14/1 (mmol/mol) and a Q_A/Chl ratio of 1.96/1. Relative to total Chl, the Chl *b*-less mutant had a greater content in P700 and Q_A (P700/Chl=3.1/1 and Q_A/Chl=2.95/1). This is consistent with a depletion of Chl from the Chl antenna of this mutant. The ratio of Q_A/P700 provided an estimate of PSII/PSI ratio in the thylakoid membrane of the two strains. This ratio was 0.92:1 for the control and 0.95:1 for Chl *b*-less (Table 2). The efficiency of PSII primary photochemistry was also estimated from the *in vivo* variable to maximal Chl fluorescence (F_v/F_{max}) yield ratio [Kitajima and Butler 1975]. This ratio was 0.65 for the control and 0.52 for the Chl *b*-less mutant.

TABLE 2

PHOTOCHEMICAL APPARATUS ORGANIZATION IN CONTROL AND CHL *B*-LESS MUTANT OF *C. REINHARDTII*. THE STANDARD DEVIATION OF THE MEAN IS GIVEN FOR N=3-5

Parameter measured	control	Chl *b*-less
P700/Chl (mmol/mol)	2.14±0.13	3.1±0.28
Q_A/Chl (mmol/mol)	1.96±0.03	2.95±0.22
PSII/PSI (mol/mol)	0.92	0.95
F_v/F_{max}	0.65±0.06	0.52±0.02

Determination of the Chl Antenna Size of PSII and PSI

Chlorophyll antenna sizes were estimated from the kinetics of the primary photochemical activity of PSII (chlorophyll fluorescence induction) and PSI (P700 photooxidation) upon illumination of the samples by continuous green actinic light of limiting intensity [Melis and Anderson 1983]. In this approach, functional Chl antenna sizes are assigned to each photosystem in direct proportion to the rate of the respective photochemical activity [Melis 1989]. Figure 3 (left panels) shows light-induced changes in the absorbance of the reaction center P700 at _=700 nm, occurring as a result of P700 photooxidation in thylakoid membranes of control and the Chl *b*-less mutant. Figure 3 (right panels) shows the respective semilogarithmic plots of the ΔA_{700} kinetics, revealing single exponential functions of time with rate constants K_{PSI} of 9.0 s^{-1} for control and 7.0 s^{-1} for the Chl *b*-less mutant. The slower P700 photooxidation kinetics for the Chl *b*-less mutant suggest a slightly smaller PSI Chl antenna size than in the control.

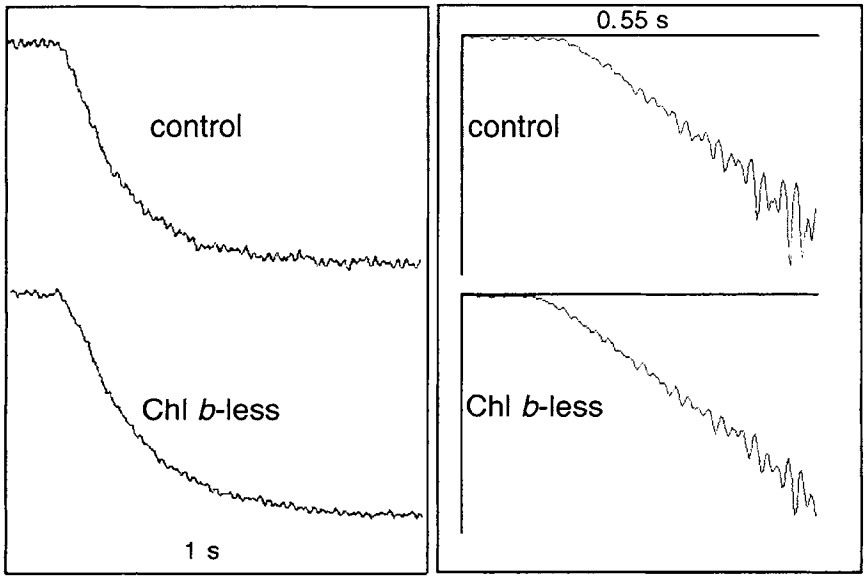


Figure 3: Light-induced absorbance change measurements (Left panels) Kinetics of P700 photooxidation(ΔA_{700}) with thylakoid membranes of control and a Chl *b*-less mutant of *C. reinhardtii*. Upper trace, control; lower trace, Chl *b*-less mutant. (Right panels) Corresponding semilogarithmic plot of theΔA_{700} kinetics.

Figure 4 (left panels) shows light-induced fluorescence induction kinetics, the variable part of which reflects the photoreduction of Q_A in the thylakoid membranes of control and the Chl *b*-less mutant [Melis and Duysens 1979]. The fluorescence induction kinetics of the control strain were faster than that of the Chl *b*-less mutant, suggesting a larger PSII Chl antenna size for the former.

Figure 4 (right panels) shows the respective semilogarithmic plots of the area over fluorescence induction kinetics. This parameter (area over fluorescence) is directly proportional to the amount of Q_A that becomes photoreduced [Melis and Duysens 1979, Melis 1989]. The analysis (Fig. 4, right panels) revealed biphasic Q_A reduction kinetics for the control, occurring with rate constants $K\alpha=9.7\ s^{-1}$ and $K\beta=4.0\ s^{-1}$. These biphasic kinetics reflect a PSII heterogeneity and the existence of two populations of PSII (PSIIα and PSIIβ) with significantly different Chl antenna sizes. In the Chl *b*-less mutant, Q_A photoreduction occurred as a single exponential function of time with rate constant $k_{PSII}=2.9\ s^{-1}$, suggesting lack of PSII heterogeneity and the occurrence of a uniform and small Chl antenna size for the mutant. Heterogeneity in the PSII Chl antenna size is well known in the literature [Melis 1991, Lavergne and Briantais 1996]. The relative amounts of PSIIα and PSIIβ centers in the control strain were 41% and 59%, respectively. In contrast to the control, the kinetic analysis of the fluorescence induction revealed only a slow, monophasic first order function of time for the Chl *b*-less mutant (Fig. 4, right panel). This is evidence for only one population of photosystem II with a uniform Chl antenna size in this mutant.

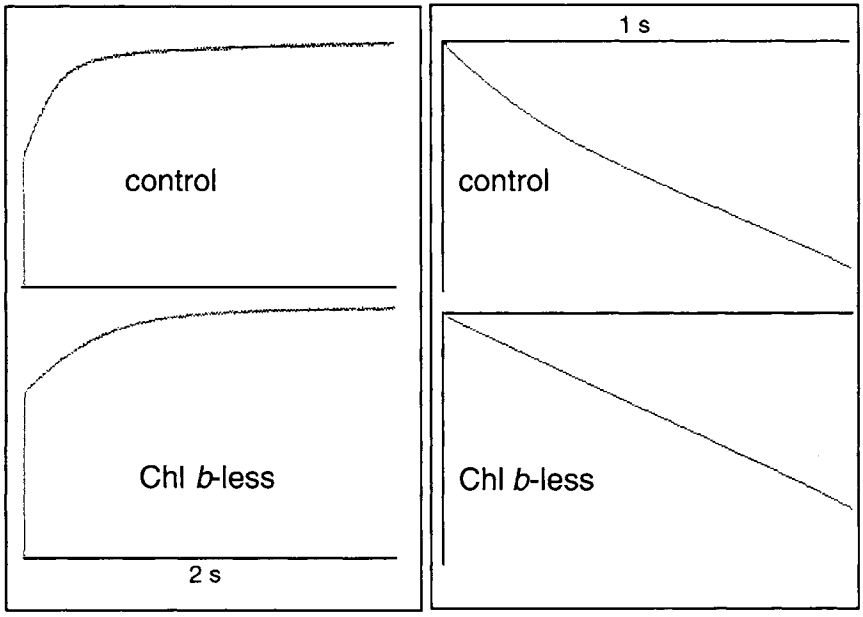


Figure 4: Chlorophyll fluorescence induction measurements
Left panels: Chl fluorescence induction kinetics of control (upper) and a Chl *b*-less mutant (lower). Right panels: Corresponding semilogarithmic plots of the area over the fluorescence induction curve.

From the measured kinetics of P700 photooxidation and "area over fluorescence induction" we determined the functional chlorophyll antenna size of PSI and PSII, respectively [Melis 1989], for the control and Chl *b*-less mutant (Table 3).

TABLE 3

CHL ANTENNA SIZE OF PSII AND PSI IN CONTROL AND A CHL *B*-LESS MUTANT
NUMBER OF CHLOROPHYLL MOLECULES PER REACTION CENTER

PS	control	Chl *b*-less	Minimal Chl antenna size*
PSIIα	322	---	
PSIIβ	127	---	
PSII	---	93	37
PSI	290	246	95

* from [Glick and Melis 1988]

The functional Chl antenna size of PSIIα and PSIIβ in the control was determined to be 322 and 127 Chl molecules, respectively, with 93 Chl molecules in PSII of the Chl *b*-less mutant. The drastic reduction in the PSII Chl antenna size of the mutant was evidently caused by the lack of Chl *b*. In contrast, the PSI Chl antenna size of 246 Chl molecules in the Chl *b*-less mutant was only slightly smaller than the 290 Chl measured in the control. The PSII and PSI Chl antenna size of the mutant are thus significantly larger than the minimal Chl antenna size of PSII-core and PSI-core complexes (Table 3, 4th column). It is concluded that the major portion of LHC-I complexes can assemble and functionally associate with PSI in the absence of Chl *b*.

Characterization of the Light-Harvesting Complex Proteins of the Photosystems in Control and Chl b-less Mutant

The results described above show a truncated Chl antenna size for PSI and PSII in the Chl *b*-less mutant. Consequently, the amount of light harvesting complex proteins should be accordingly reduced in the mutant relative to the control. The amount and composition of the LHC proteins was determined in western blots by using polyclonal antibodies that cross-react with the LHC proteins of both PSII and PSI [Bassi and Wollman 1991, Bassi et al. 1992]. Figure 5 shows the cross-reaction of at least 8 protein bands with these polyclonal antibodies. These bands originated either from LHC-II or LHC-I polypeptides.

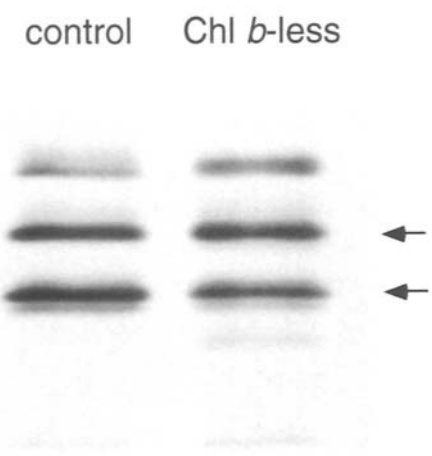


Figure 5: Western Blot Analysis of Chl Antenna Proteins Thylakoid membrane proteins were isolated from control and Chl *b*-less *C. reinhardtii.* Arrows mark the position of the major constituents of the LHC-II.

A comparison between control and Chl *b*-less in Fig. 5 revealed that the thylakoid membranes of the Chl *b*-less mutant contained all LHC proteins. However, loss of Chl *b* is correlated with a reduction in the amount of the major LHC-II proteins (shown by arrow in Fig. 5) [Webb and Melis 1995, Tanaka and Melis 1997]. Since the major LHC-II proteins form the peripheral antenna of PSII, the reduction in their amount is consistent with the spectrophotometric and kinetic results (Table 3) showing a truncated Chl antenna size for PSII in the Chl *b*-less strain.

Measurements of Photosynthetic Capacity

A measure of photosynthetic efficiency and productivity can be obtained from the light-saturation curve of photosynthesis. This type of analysis is necessary and sufficient for the measurement of the vital signs of photosynthesis [Melis et al. 1999]. In such measurements, the rate of O_2 evolution, when plotted as a function of irradiance, first increases linearly and then levels off as the saturating irradiance (I_s) is approached [Neale et al. 1993]. The slope of the

initial linear increase provides information about the photon use efficiency of photosynthesis (estimated from the number of O_2 evolved per photon absorbed [Björkman and Demmig 1987, Neale et al. 1993]. The rate of photosynthesis is saturated at irradiances greater than I_s. This light-saturated rate (P_{max}) provides a measure of the capacity of photosynthesis for the particular sample [Powles and Critchley 1980]. Figure 6 shows the light saturation curve of photosynthesis for control and the Chl *b*-less mutant. Control cells showed a light-saturated rate of photosynthesis (P_{max}) of ~30 mmol O_2 (mol Chl)$^{-1}$ s^{-1} with I_s = ~ 400 µmol photons m^{-2} s^{-1}. The Chl *b*-less mutant reached a P_{max} of ~90 mmol O_2 (mol Chl)$^{-1}$ s^{-1}, i.e., ~3 times greater than that of the control. This difference is attributed to the smaller Chl antenna size for the HL-grown cells, translating into higher per Chl productivity of the culture. Consistent with this interpretation is also the difference in the I_s values which was ~3 times greater for the Chl *b*-less mutant than for the control.

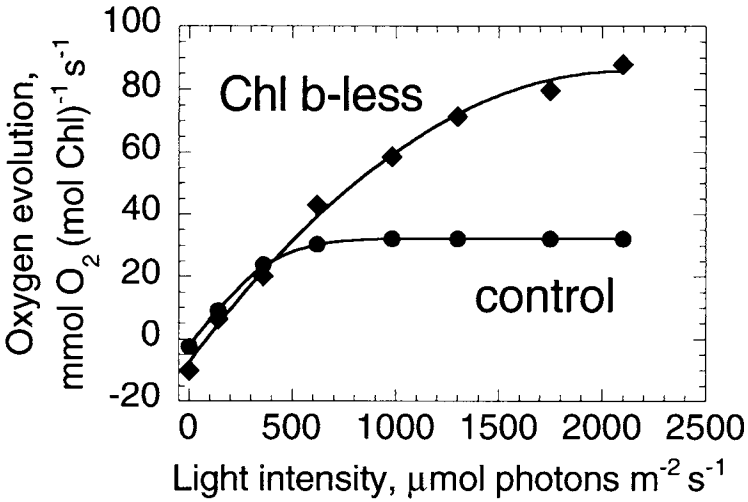


Figure 6: The light-saturation curve of photosynthesis Rates of oxygen evolution on a *per Chl* basis. Note the similar initial slopes and the different light-saturated rates between control and Chl *b*-less mutant.

Fig. 6 also compares the initial linear portion of the light-saturation curves for the two strains. It is obvious that the initial slopes, which provide a measure of the photon use efficiency of photosynthesis, are similar in the two samples, suggesting that both samples exhibit a similar 'photon use efficiency' of photosynthesis. It is concluded that the smaller Chl antenna size in the Chl *b*-less mutant does not introduce an adverse effect on the efficiency of photosynthesis under low light-intensity conditions.

DISCUSSION

Work in several laboratories established that the size and composition of the light-harvesting Chl antenna of the photosystems is adjusted and optimized depending on the prevailing growth and irradiance conditions (reviewed in [Anderson 1986, Melis 1991, Melis 1996]). In general, growth under low light promotes larger Chl antenna size for both PSI and PSII (larger photosynthetic unit size). High-light growth conditions elicit a smaller Chl antenna size. This adjustment in the Chl antenna size of the photosystems comes about because of regulated changes in the size and composition of the auxiliary Chl *a-b* light-harvesting complex (LHC-II and LHC-I) [Leong and Anderson 1984, Larsson et al. 1987, Sukenik et al. 1988, Morrissey et al. 1989, Smith et al. 1990, Mawson et al. 1994]. The response appears to be highly conserved in all photosynthetic organisms examined, suggesting the existence of a highly conserved regulatory mechanism that controls the development of the Chl antenna size in the photosystems.

Mechanistic details of this regulatory mechanism are not known. The regulation could occur at several different steps in the pathway of chlorophyll biosynthesis [Matters and Beale 1995, Reinbothe et al. 1996, Fujita 1996, Falbel et al. 1996, Ohtsuka et al. 1997] resulting in less tetrapyrrole biosynthesis under high irradiance than under low irradiance. In turn, Chl availability may determine the priority of Chl-protein assembly in the chloroplast. According to Greene et al. [1988], Chl-protein assembly occurs with the following distinct hierarchy: PSII-core>PSI-core>LHC-monomers>LHC-inner trimers>LHC-peripheral trimers. A limited availability of Chl under moderate or high irradiance may permit the assembly of the PSII- and PSI-core complexes. However, lack of sufficient Chl will not be conducive to the assembly of LHC-peripheral trimers, resulting in a smaller Chl antenna size.

The present work employed a mutagenesis approach, based on the random insertion of tagged DNA into *C. reinhardtii* cells, by which to impair the Chl antenna size regulation mechanism. This procedure, along with the stringent screening employed (Fig.1 and Fig. 2), will help to unlock the "black box" of the developmental regulation of the Chl antenna size in microalgae. Thus, it is expected that mutants with a permanently truncated Chl antenna size, as well as mutants with a permanently large Chl antenna, will be isolated (Table 1). The advantage of this molecular genetic approach is that it will lead to the identification of genes responsible for the operation of this highly conserved regulatory mechanism. Identification of these genes in *C. reinhardtii* will permit the direct manipulation of the Chl antenna size in other microalgae that may be of equal interest to the DOE Hydrogen Program.

In green algae, the largest Chl antenna configurations reported contain about 500 Chl (*a* and *b*) for PSII and 350 Chl (*a* and *b*) for PSI [Melis 1996]. The smallest stable Chl antenna configurations for the photosystems are the so-called PSII-core complex (containing 37 Chl *a* molecules) and PSI-core complex (containing 95 Chl *a* molecules). These core-complexes with a minimal Chl antenna size are necessary and sufficient for the stable assembly of functional PSII and PSI in thylakoids [Glick and Melis 1988]. The goal of this project is to generate, through the application of molecular genetic approaches, green algae with Chl antenna configurations that are as close to the "core" antennae as possible.

Earlier work showed that *Dunaliella salina* (green alga), grown under continuous illumination of high intensity, had a highly truncated Chl antenna size where PSII contained ~ 60 Chl and PSI contained 105 Chl molecules [Smith et al. 1990, Neidhardt et al. 1998]. Functional analysis of these algae provided a "proof of concept", i.e., the ability of the internal chloroplast regulatory mechanism to generate highly truncated Chl antenna sizes [Neidhardt et al. 1998], and the optimization of photosynthetic productivity and solar conversion efficiency in microalgae by minimizing the light-harvesting chlorophyll antenna size of the photosystems [Melis at al. 1999].

The present work illustrates in some detail the result of a unique mutation, one that impaired the biosynthesis of Chl *b* and resulted in a truncated Chl antenna size for the photosystems [Tanaka et al. 1998]. In the present Chl *b*-less mutant, PSII contained 93 Chl *a* molecules and PSI contained 246 Chl *a* molecules (Table 3). These antenna sizes are significantly larger than the PSII-core and PSI-core antennae, suggesting that Chl *b* may not be absolutely essential for the assembly of all Chl *a*-*b* light-harvesting complexes (see also [Ghirardi et al. 1986]). This was especially true for PSI which, in the Chl *b*-less mutant, had a Chl antenna size almost as large as that of the control (Table 3). It may be concluded that the Chl *b*–less mutation can be overcome by a nearly quantitative substitution of Chl *b* with Chl *a* in the Chl antenna of PSI, and by a partial substitution by Chl *a* in the antenna of PSII.

An explanation of the peculiar features of the PSII and PSI antenna configuration in the Chl *b*-less mutant may be provided upon consideration of the role of Chl *b* in these complexes. This pigment is associated exclusively with the LHC proteins of the two photosystems. Since the core complex of PSII contains only about 37 Chl *a* molecules [Glick and Melis 1988], it follows that the remaining ~56 Chl *a* molecules in PSII of the Chl *b*-less mutant must be associated with LHC-II proteins. Based on the assumption of ~12 Chl molecules per Lhcb protein in *C. reinhardtii* [Thornber et al. 1988, Morrissey et al. 1989, Bassi and Wollman 1991, Harrison and Melis 1992], we estimated that 4-5 LHC-II proteins are assembled and functionally associated with PSII.

Conversely, the core complex of PSI contains about 95 Chl *a* molecules [Glick and Melis 1988]. Since PSI in the Chl *b*-less mutant contains 246 Chl *a* molecules, it follows that about 150 Chl *a* molecules must be associated with LHC-I proteins. Based on the assumption of ~10 Chl molecules per Lhca protein in PSI [Thornber et al. 1988], we estimated that about 15 LHC-I proteins must be assembled and functionally associated with PSI in the absence of Chl *b*. Consistent with these conclusions are the western blot results with polyclonal LHC antibodies (Fig. 5) which showed the presence of significant amounts of LHC proteins in thylakoid membranes isolated from Chl *b*-less cells.

In summary, the work clearly shows that a permanently truncated Chl antenna size in green algae can be achieved through the application of DNA insertional mutagenesis and related molecular genetic techniques. It is shown that cells with a permanently truncated Chl antenna size of the photosystems are capable of higher rates of light-saturated oxygen evolution than the wild type. From the preliminary results presented in this report, it is also concluded that a Chl *b*–less mutation does not lead to the maximum truncation of the PSII and PSI Chl antenna size in the green alga *C. reinhardtii*. Rather, given the stable assembly of the LHC without Chl *b*, it

appears that the absence of Chl *b* can be overcome, presumably through a nearly quantitative substitution of Chl *b* by Chl *a* in PSI, and through a limited substitution by Chl *a* in PSII [Sukenik *et al.* 1987, Tanaka and Melis 1997].

Since the absence of Chl *b* does not lead to the minimum possible Chl antenna size of the photosystems, it is important to continue to test and analyze transformants in which impairment in the regulation of the Chl antenna size has brought about a highly truncated Chl antenna size for the two photosystems. Accordingly, plans for future work include the analysis of additional transformants in search of the smallest possible Chl antenna size for PSII and PSI, and the cloning and sequencing of the genes that regulate the Chl antenna size of photosynthesis.

ACKNOWLEDGEMENTS

The work was supported by the US DOE Hydrogen R&D Program under DOE-UC Berkeley Cooperative Agreement Number DE-FC36-98GO10278. The authors wish to thank Dr. Kris Niyogi for making available instrumentation for the Fluorescence Imaging Analysis.

REFERENCES

Anderson JM. (1986). Photoregulation of the composition, function and structure of thylakoid membranes. Annu. *Rev. Plant Physiol.* **37**, 93-136.

Arnon D .(1949). Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* **24**, 1-15.

Bassi R and Wollman F-A. (1991). The chlorophyll-*a/b* proteins of photosystem II in *Chlamydomonas reinhardtii*. *Planta* **183**, 423-433.

Bassi R, Soen SY, Frank G, Zuber H and Rochaix J-D. (1992). Characterization of chlorophyll *a/b* proteins of photosystem I from *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **267**, 25714-25721.

Björkman O and Demmig B. (1987). Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* **170**, 489-504.

Davies JP, Yildiz F and Grossman AR. (1994). Mutants of *Chlamydomonas* with aberrant responses to sulfur deprivation. *The Plant Cell* **6**, 53-63.

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

Debuchy R, Purton S and Rochaix J-D. (1989). The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: An important tool for nuclear transformation and for correlating the genetic and molecular maps of the *ARG7* locus. *EMBO Journal* **8**, 2803-2809.

Di Paolo ML, Peruffo dal Belin A and Bassi R. (1990). Immunological studies on chlorophyll *a/b* proteins and their distribution in thylakoid membrane domains. *Planta* **181**, 275-286.

Falbel T, Meehl JB and Staehelin LA. (1996). Severity of mutant phenotype in a series of chlorophyll-deficient wheat mutants depends on light intensity and the severity of the block in chlorophyll synthesis. *Plant Physiol.* **112**, 821-832.

Fujita Y. (1996). Protochlorophyllide reduction: A key step in the greening of plants. *Plant Cell Physiol.* **37**, 411-421.

Ghirardi ML, McCauley SW and Melis A. (1986). Photochemical apparatus organization in the thylakaid membrane of *Hordeum vulgare* wild-type and chlorophyll *b*-less chlorina f2 mutant. *Biochim. Biophys. Acta* **851**, 331-339.

Glick RE and Melis A. (1988). Minimum photosynthetic unit size in system-I and system-II of barley chloroplasts. *Biochim. Biophys. Acta* **934**, 151-155.

Gorman DS and Levine RP. (1965). Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA* **54**, 1665-1669.

Greene, B.A., Allred, D.R., Morishige, D. and Staehelin, L.A. (1988). Hierarchical response of light-harvesting chlorophyll-proteins in a light-sensitive chlorophyll *b*-deficient mutant of maize. *Plant Physiol.* **87**, 357-364

Harrison MA and Melis A. (1992). Organization and stability of polypeptides associated with the chlorophyll *a-b* light-harvesting complex of photosystem-II. *Plant Cell Physiol.* **33**, 627-637.

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

Kok B. (1953). Experiments on photosynthesis by *Chlorella* in flashing light. In JS Burlew (ed), Algal Culture: From Laboratory to Pilot Plant. Carnegie Institution of Washington, Washington DC: 63-75.

Kok B. (1960). Efficiency of photosynthesis. In W Ruhland (ed), Encyclopedia of Plant Physiology. Springer Verlag, Berlin: 566-633.

Kok B. (1973). Photosynthesis. In: Gibbs M et al. Eds), Proceedings of the Workshop on Biosolar Hydrogen Conversion, pp. 22-30. Bethesda, MD.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Larrson UK, Sundby C and Andersson B. (1987). Characterization of two different subpopulations of spinach light-harvesting chlorophyll *a-b*-protein complex (LHC-II): polypeptide composition, phosphorylation pattern and association with photosystem-II. *Biochim. Biophys. Acta* **894**, 59-68.

Lavergne J and Briantais J-M. (1996). Photosystem-II heterogeneity. In: Ort DR and Yocum CF (Eds), Oxygenic photosynthesis: The Light Reactions, pp. 265-287. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Leong TA and Anderson JM. (1984). Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. I. Study on the distribution of chlorophyll-protein complexes. *Photosynth. Research* **5**, 105-115.

Matters GL and Beale SI. (1995). Blue-light-regulated expression of genes for two early steps of chlorophyll biosynthesis in *Chlamydomonas reinhardtii*. *Plant Physiol.* **109**, 471-479.

Mawson BT, Morrissey PJ, Gomez A and Melis A. (1994). Thylakoid membrane development and differentiation: assembly of the chlorophyll *a-b* light-harvesting complex and evidence for the origin of Mr=19, Mr=17.5 and 13.4 kDa proteins. *Plant Cell Physiol.* **35**, 341-351.

Melis A. (1989). Spectroscopic methods in photosynthesis: photosystem stoichiometry and chlorophyll antenna size. *Phil. Trans. R. Soc. Lond.* **B 323**, 397-409.

Melis A. (1991). Dynamics of photosynthetic membrane composition and function. *Biochim. Biophys. Acta* (Reviews on Bioenergetics) **1058**, 87-106.

Melis A. (1996). Excitation energy transfer: Functional and dynamic aspects of *Lhc* (*cab*) proteins. In Ort DR, Yocum CF (eds), Oxygenic Photosynthesis: The Light Reactions. Kluwer Academic Publishers, Dordrecht, The Netherlands: 523-538.

Melis A. (1998). Photostasis in plants: mechanisms and regulation. In Williams TP, Thistle A (eds), Photostasis. Plenum Publishing Corporation, New York, NY: 207-221.

Melis A. and Anderson J.M. (1983). Structural and functional organization of the photosystems in spinach chloroplasts: antenna size, relative electron transport capacity, and chlorophyll composition. *Biochim. Biophys. Acta* **724**, 473-484.

Melis A and Duysens LNM. (1979). Biphasic energy conversion kinetics and absorbance difference spectra of photosystem II of chloroplasts. Evidence for two different system II reaction centers. *Photochem. Photobiol.* **29**, 373-382

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

Melis A, Neidhardt J, Baroli I, Benemann JR. (1997). Maximizing photosynthetic productivity and light utilization in microalgae by minimizing the light-harvesting chlorophyll antenna size of the photosystems. In Zaborsky OR (ed), BioHydrogen '97. Proceedings of the International Conference on Biological Hydrogen Production. Waikoloa-Hawaii, Plenum Publishing Corporation, New York, NY: 41-52.

Melis A, Spangfort M and Andersson B. (1987). Light-absorption and electron transport balance between photosystem-II and photosystem-I in spinach chloroplasts. *Photochem. Photobiol.* **45**, 129-136.

Morrissey PJ, Glick RE and Melis A. (1989). Supramolecular assembly and function of subunits associated with the chlorophyll *a-b* light-harvesting complex II (LHCII) in soybean chloroplast. *Plant Cell Physiol.* **30**, 335-344.

Myers J. (1957). Algal Culture. In Kirk RE, Othmer DE (eds), Encyclopedia of Chemical Technology. Interscience, New York, NY: 649-680.

Naus J, Melis A. (1991). Changes of photosystem stoichiometry during cell growth in *Dunaliella salina* cultures. *Plant Cell Physiol.* **32**, 569-575.

Neale PJ, Cullen JJ, Lesser MP, Melis A. (1993). Physiological bases for detecting and predicting photoinhibition of aquatic photosynthesis by PAR and UV radiation. In Yamamoto HY, Smith CM (eds), Photosynthetic Responses to the Environment. Current Topics in Plant Physiology. American Society of Plant Physiologists Publication Series, Volume 8: 61-77.

Neidhardt J, Benemann JR, Zhang L, Melis A. (1998). Photosystem II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae). *Photosynth. Res.* **56**, 175-184.

Niyogi KK, Bjorkman O and Grossman AR. (1997). *Chlamydomonas* xanthophyll cycle mutants identified by video imaging of chlorophyll fluorescence quenching. *Plant Cell* **9**, 1369-1380.

Ohtsuka T, Ito H and Tanaka A. (1997). Conversion of chlorophyll *b* to chlorophyll *a* and the assembly of Chl with apoproteins by isolated chloroplasts. *Plant Physiol.* **113**, 137-147.

Powles SB and Critchley C. (1980). Effect of light intensity during growth on photoinhibition of intact attached bean leaflets. *Plant Physiol.* **65**, 1181-1187.

Reinbothe S, Reinbothe C, Lebedev N and Apel K. (1996). PORA and PORB, two light-dependent protochlorophyllide-reducing enzymes of angiosperm chlorophyll biosynthesis. *Plant Cell* **8**, 763-769.

Smith BM, Morrissey PJ, Guenther JE, Nemson JA, Harrison MA, Allen JF and Melis A. (1990). Response of the photosynthetic apparatus in *Dunaliella salina* (green algae) to irradiance stress. *Plant Physiol.* **93**, 1433-1440.

Sueoka N. (1960). Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **46**, 83-91.

Sukenik A, Bennett J, Falkowski PG. (1988). Changes in the abundance of individual apoproteins of light-harvesting chlorophyll *a/b*-protein complexes of photosystem I and II with growth irradiance in the marine chlorophyte *Dunaliella tertiolecta*. *Biochim. Biophys. Acta* **932**, 206-215.

Sukenik A, Wyman KD, Bennett J and Falkowski PG. (1987). A novel mechanism for regulating the excitation of photosystem II in a green alga. *Nature* **327**, 704-707.

Tanaka A and Melis A. (1997). Irradiance-dependent changes in the size and composition of the chlorophyll *a-b* light-harvesting complex in the green alga *Dunaliella salina*. *Plant Cell Physiol.* **38**, 17-24.

Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshida K and Okada K. (1998). Chlorophyll *a* oxygenase (CAO) is involved in chlorophyll *b* formation from chlorophyll *a*. *Proc. Natl. Acad. Sci. USA* **95**, 12719-12723.

Thornber JP, Peter GF, Chitnis PR, Nechushtai R and Vainstein A. (1988). The light-harvesting complex of photosystem II of higher plants. In: Stevens SE Jr, Bryant DA (eds), Light-energy Transduction in Photosynthesis: Higher Plant and Bacterial Models, pp. 137-154 The American Society of Plant Physiologists, Rockville, Maryland.

Webb MR and Melis A. (1995). Chloroplast response in *Dunaliella salina* to irradiance stress. Effect on thylakoid membrane assembly and function. *Plant Physiol.* **107**, 885-893.

A SCHEME FOR DEVELOPING THE YIELD OF HYDROGEN BY FERMENTATION

S. Tanisho

Department of Environmental Sciences
Yokohama National University
Hodogaya-ku, Yokohama 240-8501, JAPAN
E-mail: tanisho@chemeng.bsk.ynu. ac. jp

ABSTRACT

It is necessary to understand the yield of hydrogen from various substrates in order to produce hydrogen efficiently by fermentation. The yield differs depending on the bacteria utilized. If we use strict anaerobic bacteria, for example, we expect a maximum yield of 4 mol H_2 from 1 mol glucose by directing the bacteria's metabolism to acetate formation. In the case of facultative anaerobic bacteria, we may expect a maximum 10 mol H_2 from 1 mol glucose by using the TCA cycle and re-oxidizing the $FADH_2$ generated in the electron transport system. In this presentation, these possibilities are discussed from the point of reduction-oxidation reactions.

1. CONVERSION EFFICIENCY OF ENERGY PRODUCTION BY HYDROGEN FERMENTATION

There are several ways to produce energy by fermentation, such as methane fermentation, ethanol fermentation and hydrogen fermentation. Of these, methane fermentation is rather organic waste treatment than energy production, while ethanol fermentation is of practical importance under certain conditions as demonstration in Brazil. Hydrogen fermentation is still not in practical use because the energy conversion efficiency from substrates is fairly low (Table 1), and also is not estimated from a suitable point of view for utilization.

TABLE 1
IDEAL YIELD AND ENERGY RECOVERY FROM GLUCOSE

Material	yield	heating value [kJ/mol]	recovery [%]
Methane	3	2,646	94
Ethanol	2	1,645*	58
Hydrogen	2	572	20
	4	1,144	41
	6	1,716	61
	8	2,288	81
	10	2,860	102

*; estimated the distillation energy at 40% of the product.

Although, ethanol fermentation (equation 1) is known to have high conversion efficiency (97% estimated from the ideal enthalpy recovery), the real efficiency falls to around 50% if we consider the distillation energy (Table 1). This is the non improvable efficiency.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \text{(Eq. 1)}$$

In contrast, since hydrogen fermentation (Eq. 2) does not require a distillation step, the real efficiency will ideally be comparable to ethanol fermentation. Moreover, it may become more effective than ethanol fermentation if we utilize the hydrogen in a fuel of fuel cell.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2C\,H_3COOH + 2CO_2 \quad \text{(Eq. 2)}$$

As stated above, we can expect a higher energy conversion efficiency for hydrogen fermentation by improving the yield of hydrogen. In this paper, I will present a scheme for increasing the yield of hydrogen.

2. PATHWAY OF HYDROGEN PRODUCTION

2.1. Three Pathways of Hydrogen Production

Many pathways have been suggested for hydrogen production by bacteria. Among these, three representative pathways are shown in Figure 1.

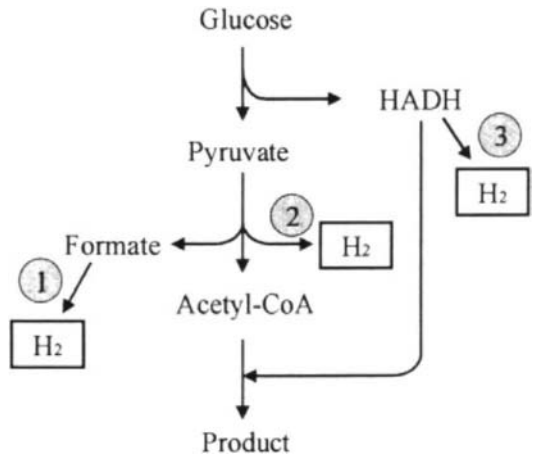


Figure 1: Three ways of the hydrogen pathway.

One is the formate pathway characteristic of the mixed acid fermentation by bacteria such as *Escherichia coli*. The pathway was proposed from stoichiometric considerations, such as the molar amount of formate at pH around 7 is equal to the molar amount of CO_2 and H_2 at pH below 6.

$$HCOOH \rightarrow H_2 + CO_2 \qquad \text{(Eq. 3)}$$

This pathway is closely related with acetate production, therefore, it may become an important pathway for the improvement of hydrogen yield through acetate fermentation.

$$CH_3COCOOH + H_2O \rightarrow CH_3COOH + HCOOH \qquad \text{(Eq. 4)}$$

The second is the direct production pathway characteristic of the acetone-butanol fermentation by bacteria such as *Clostridium butyricum*. In this pathway, hydrogen is produced directly without formate production. This pathway, however, may be unified with NADH pathway, because the mass balance of NADH (Nicotinamide Adenine Dinucleotide, reduced form) shows the same result with NADH pathway.

The third pathway is the NADH pathway which was proposed from a concept that *Clostridium butyricum* re-oxidizes residual NADH to NAD^+ by producing H_2 during butyrate fermentation.

$$NADH + H^+ \rightarrow NAD^+ + H_2 \qquad \text{(Eq. 5)}$$

In the butyrate fermentation, 2 mol of H_2 and 2 mol of NADH are produced ideally from 1 mol of glucose. Reoxidation of NADH produces H_2.

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2NADH + 2H^+ \quad \text{(Eq. 6)}$$

$$2NADH + 2H^+ \rightarrow 2NAD^+ + 2H_2 \quad \text{(Eq. 7)}$$

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2H_2 + 2CO_2 \quad \text{(Eq. 8)}$$

For example, *C. butyricum* produced 235 mmol of H_2 from 100 mmol of glucose. In this case, 76 mmol of butyrate and 42 mmol of acetate were also produced from 100 mmol of glucose. The volume of evolved hydrogen was in good agreement with the calculated volume from the mass balance of analysis. Moreover, the hydrogen yield (2.35) from glucose was the largest ever reported. This is a known example for increased yield by increasing acetate or residual NADH and suggests the direction for future yield improvement.

2.2. Mechanism of Hydrogen Production Through NADH Pathway and Redox Potential

For hydrogen production through the NADH pathway, the membrane-bound hydrogenase accepts electrons from NADH inside the cell and transfers them to protons outside the cell to evolve molecular hydrogen (Fig. 2). This mechanism is feasible from electrochemical considerations as follows:

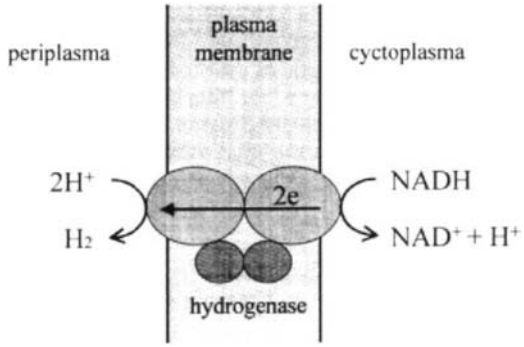


Figure 2: Mechanism of hydrogen production of NADH pathway.

The reactions producing H_2 from NADH are expressed as two electrochemical reaction equations as follows:

$$NADH + H^+ \rightarrow NAD^+ + 2H^+ + 2e \quad E_0'_{NAD} = -0.320V \quad \text{(Eq. 9)}$$

$$2H^+ + 2e \rightarrow H_2 \quad E_0'_{NAD} = -0.414V \quad \text{(Eq. 10)}$$

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad E_0'_{NAD} = -0.094V \quad \text{(Eq. 11)}$$

Redox potentials of these reactions are expressed by functions of pH as follows,

NAD:

$$E = E_0 + (RT/2F)\ \mathrm{In}([NAD^+][H^+]/[NADH]) \quad \text{(Eq. 12)}$$
$$= E_0 + (2.303RT/2F)(pH) + (2.303RT/2F)\log([NAD^+]/[NADH])$$
$$= -0.113 - 0.0296\ pH + 0.0296\ \log([NAD^+]/[NADH])$$

H_2:

$$E = (RT/2F)\ \mathrm{In}([H^+]^2/p_{H2}) \qquad \text{(Eq. 13)}$$
$$= -0.0592\ \mathrm{pH} - 0.0296\ \log(p_{H2})$$

These relations are shown in Figure 3.

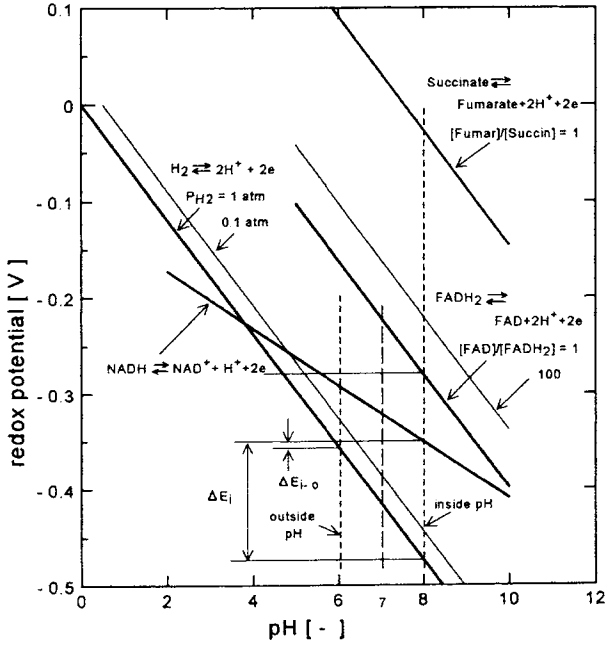


Figure 3: Culture pH vs. redox potential.

If H_2 is produced inside the cell, ΔE_0' is negative the Gibbs's free energy change, ΔG_0'=-nFΔE_0', becomes positive, 23.9 kJ/mol at pH 8 (well known inside pH of *Escherichia coli*), and the equilibrium constant, K'=[NAD^+] [H_2]/[NADH], becomes 6.5×10^{-5}. This means that if H_2 is produced inside the cell, only 10^{-4} atm of partial H_2 pressure will stop H_2 production. But, if H_2 is produced outside the cell at pH 6 and NADH is oxidized inside the cell at pH 8, ΔE_0' becomes nearly 0 and therefore H_2 production is possible even at approx. 1 atm of partial pressure of H_2. In fact, bacteria produced H_2 continuously under a partial pressure 0.6 atm.

3. ANAEROBIC OR FACULTATIVE ANAEROBIC BACTERIA

There are two kinds of hydrogen producing bacteria, one is facultative anaerobic bacteria and facultative anaerobic bacteria. We have to evaluate on the metabolic pathways of these bacteria to determine for which bacteria an improved hydrogen yield is possible.

3.1. Glycolisys

There are two pathways degrading glucose to pyruvate; i.e., the Embden-Meyerhof(EM) pathway and the Entner-Doudoroff(ED) pathway. By the EM pathway, 2 mol of NADH are produced while 1 mol each of NADH and NADPH (Nicotinamide Adenine Dinucleotide Phosphate, reduced form) is produced by the ED pathway as follows.

EM pathway:

$$C_6H_{12}O_6 + 2NAD^+ + 2ADP \rightarrow 2CH_3COCOOH + 2NADH + H_+ + 2ATP \quad \text{(Eq. 14)}$$

ED pathway:

$$C_6H_{12}O_6 + NAD^+ + NADH \rightarrow 2CH_3COCOOH + NADH + NADPH + 2H^+ \quad \text{(Eq. 15)}$$

Utilized pathway differs by bacterium, though NADP as well as NAD is the coenzyme participating in the reduction-oxidation reaction of organic substance and also has the structural resemblance. It is, therefore, not too much to say that the glycolytic pathway of the bacteria producing hydrogen by NADH pathway is the EM pathway (Figure 4).

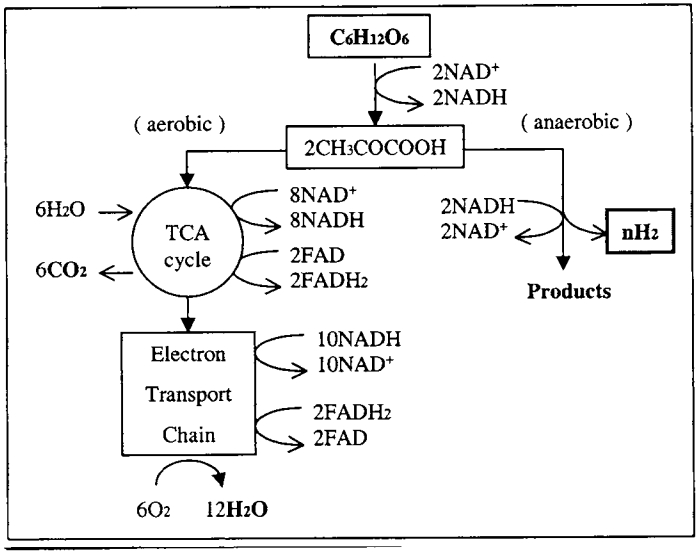


Figure 4: Aerobic and anaerobic degradation of glucose.

3.2. Metabolic Pathway of Anacrobic Bacteria

As seen in the above, NAD works as the coenzyme in glucose metabolism, therefore, NADH has to be re-oxidized to NAD^+ to support continued glucose oxidation. Anaerobic bacteria such as Clostridia re-oxidizes NADH by producing organic acids like lactate, acetate and butyrate as well as alcohols like ethanol, butanol and butanediol, from pyruvate. Many bacteria have multiple metabolic pathways, and change the pathway in accordance with metabolite availability and/or pH in the culture liquid. Therefore, in general, it is very

difficult to restrict metabolism to a single pathway not only by fermentation but also by genetic.

3.3. Metabolic Pathway of Facultative Anaerobic Bacteria and the Amount of NADH Produced Under Aerobic Conditions

Facultative anaerobic bacteria such as *Enterobacteriaceae* and *Bacillus* species produce organic acids and alcohols from pyruvate and NADH under anaerobic conditions in a way similar to anaerobic bacteria. However, the facultative anaerobes possess the pathways for aerobic metabolism, such as the TCA cycle and electron transport system (even under anaerobic condition), and quickly respond to the presence of oxygen to oxidize NADH via the by electron transport system. NADH are also produced in the TCA cycle as seen in Figure 4. In the presence of oxygen, hydrogen production stops.

$$NADH + H^+ + 1/2O_2 \rightarrow NAD^+ + H_2O \quad \text{(Eq. 16)}$$

ATP is produced effectively by the electron transport system under aerobic conditions.

$$CH_3COCOOH + 4NAD^+ + FAD + 3H_2O + ADP \rightarrow 4NADH + 4H^+ + FADH_2 + 3CO_2 + ATP \quad \text{(Eq. 17)}$$

Therefore, if we can utilize the NADH produced in the TCA cycle as a source of hydrogen production, the yield should increase. Moreover, metabolites from glucose should become only H_2 and CO_2, the treatment of waste water, therefore, will be lightened.

4. HOW TO MODIFY THE TCA CYCLE

Why does the TCA cycle works only under aerobic conditions and not under anaerobic conditions. We need to utilize the TCA cycle as a NADH supplier and to increase H_2 yield.

4.1. Gibbs's Free Energy Change of TCA Cycle

The TCA cycle starts from citrate synthesis by acetyl-CoA and oxaloacetate as seen in Figure 5. This reaction, however, has a negative free energy change (ΔG_0'= -32.3 kJ); therefore, oxaloacetate must react very quickly with acetyl-CoA and be kept nearly 0 concentration normally.

Detecting the free energy change of the cycle after citrate to succinate synthesis, ΔG_0' of the reactions are all 0 or very negative values, therefore, it must easily proceed to succinate synthesis. However, ΔG_0' of reactions from succinate to fumarate and from malate to oxaloacetate incline extremely to positive value, i.e., +36.0 kJ and +29.7 kJ, respectively. Since the equilibrium constant for these reactions becomes very small, such as 4.90×10^{-7} and 6.22×10^{-6}, respectively, these reactions therefore easily stop running by a slight amount of products. These reactions run with NAD or FAD (Flavin Adenine Dinucleotide) shown as follows:

$$Malate + AND^+ \rightarrow Oxaloacetate + NADH + H^+ \quad \text{(Eq. 18)}$$

$$Succinate + FAD \rightarrow Fumarete + FADH_2 \quad \text{(Eq. 19)}$$

Reminding that the concentration of oxaloacetate must be kept nearly 0 for the synthesis of citrate, malate-to-oxaloacetate reaction may run perpetually even if the concentration ratio [NADH]/[NAD$^+$] is approximately 1 at anaerobic condition.

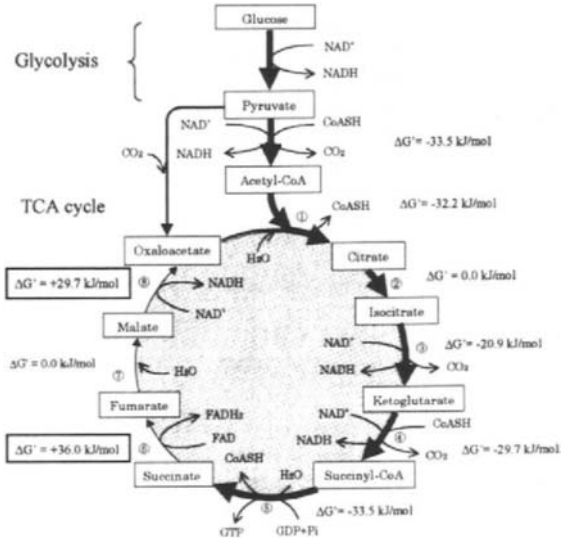


Figure 5: TCA cycle with Gibbs's free energy.

4.2. Redox Potential of FAD

In contrast to malate-to-oxaloacetate reaction, succinate-to-fumarate reaction is the reaction which FAD takes part in, and not only the equilibrium constant is very small but also the standard redox potentials of Fumarate/Succinate and FAD/$FADH_2$ are very high as seen in Table 2 and Figure 3. Consequently, if the concentration of $FADH_2$ becomes a little high by accumulation, concentration of fumarate becomes unsuitably small to keep the TCA cycle, for example, the concentration ratio of [Fumarete]/[Succinate] becomes approximately 10^{-7} at [FAD]/[$FADH_2$]≅1. Therefore, $FADH_2$ must be reoxidized immediately and the concentration ration of [FAD]/[$FADH_2$] must be kept extremely high. Living organisms seem to solve this problem by using very high redox potential substance, i.e. oxygen, as the electron acceptor of $FADH_2$. In other words, the concentration ratio [FAD]/[$FADH_2$] must be the switch of revolution of the TCA cycle.

From the above consideration, it has become clear that the oxidation of $FADH_2$ the most important problem to keep the TCA cycle.

TABLE 2
REDOX POTENTIAL

Redox compound	E_0' [mV]
O_2/H_2O	818
NO^{3-}/NO^{2-}	433
Ubiquinon	113
Fumarate/succinate	33
Oxaloacetate/malate	-172
$FAD/FADH_2$	-220
NAD/NADH	-320
H^+/H_2	-414

5. ELECTRON TRANSPORT CHAIN AND INHIBITION

Electrons of $FADH_2$ and NADH are transported through the Electron transport chain to oxygen. Although aerobic or facultative anaerobic bacteria have the electron transport chain, but different microorganisms in general have different enzyme systems. In the mitocondrial electron transport system as shown in Figure 6, $FADH_2$ transports electrons at a different transport site with NADH, since FAD has a higher redox potential than NAD.

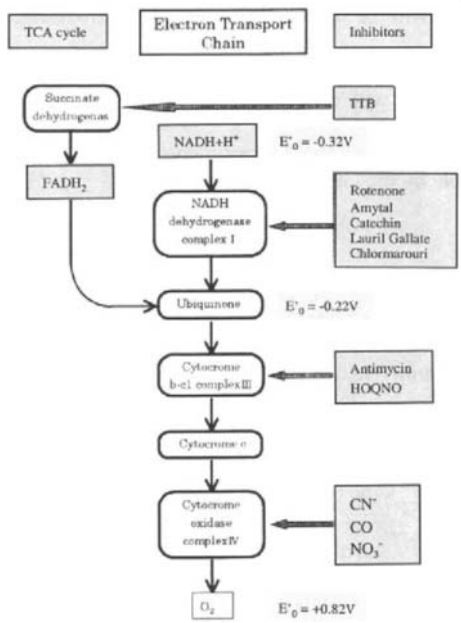


Figure 6: Electron transport chain and inhibitors.

This is a very lucky fact for us, because if we can oxidize $FADH_2$ by oxygen and separately NADH by H_2 production, then TCA cycle must run continuously. Such condition, we may realize by inhibiting the NADH active site of the electron transport chain under aerobic condition and culture pH lower than 6.
The over all reaction equation will be like follows, and we may expect a hydrogen yield of 10.

$$C_6H_{12}O_6 + 10NAD^+ + 2FAD + 4ADP + 6H_2O \rightarrow 10NADH + 10H^+ + 2FADH_2 + 6CO_2 + 4ATP$$

$$2FADH_2 + O_2 + 4ADP \rightarrow 2FAD + 2H_2O + 4ATP$$

$$10NADH + 10H^+ \rightarrow 10NAD^+ + 10H_2$$

$$C_6H_{12}O_6 + 8ADP + O_2 + 4H_2O \rightarrow 10H_2 + 6CO_2 + 8ATP$$

6. RESULTS OF THOUGHT EXPERIMENT

Then what will happen if NADH dehydrogenase complex (NADH active site) is inhibited and TCA cycle is still running. We can guess probable results by thought experiment as follows:
1) Because of higher ATP productivity, glucose consumption rate will be slow.
2) Because of higher ATP productivity, cell mass yield will be large.
3) Gas producing rate will increase owing to higher H_2 and CO_2 productivity.
4) CO_2 yield from glucose becomes larger.
5) H_2 yield from glucose will be larger, if NADH dehydrogenase do not concern H_2 production.
6) Volume ratio of H_2 to CO_2 will change in the produced gas.
7) Amount of liquid products will decrease in comparison with the amount of glucose consumption.
From the above results, we can prepare real experiments and judge whether the Improvement of hydrogen yield is possible by the inhibition or not.

7. CONCLUSION

A scheme improving hydrogen yield was proposed for the hydrogen production by fermentation and the improvement using facultative anaerobic bacteria is shown to be expectable to get the maximum yield. But actual experiments are still under preparation. Although we have to wait the decision of feasibility in future experiments, it is need to pay efforts to find strains lacking the NADH dehydrogenase complex in the wild or mutant strains.

IV. Molecular Biology

This Page Intentionally Left Blank

CYANOBACTERIAL HYDROGENASES AND BIOHYDROGEN: PRESENT STATUS AND FUTURE POTENTIAL

P. Lindblad[1] and P. Tamagnini[1,2]

[1]Department of Physiological Botany, EBC, Uppsala University,
Villavägen 6, SE-752 36 Uppsala, Sweden
Tel & Fax: + 46 - 18 471 28 26;
E-mail: Peter.Lindblad@Fysbot.uu.se

[2]Departamento de Botânica and Instituto de Biologia Molecular e Celular,
Universidade do Porto, P-4150 Porto, Portugal
Tel: +351 -22 607 49 00; Fax: +351 - 22 609 91 57;
E-mail: pmtamagn@gaia.ibmc.up.pt

ABSTRACT

Molecular hydrogen (H_2) is an environmentally clean energy carrier that may be a valuable alternative to the limited fossil fuel resources of today. For photobiological H_2 production, photosynthetic cyanobacteria are among the ideal candidates since they have the simplest nutritional requirements: they can grow in air (N_2 & CO_2), water (electrons & reductant), and mineral salts with light (solar energy) as the only source of energy. In N_2-fixing cyanobacteria, H_2 is mainly produced by nitrogenases, but its partial consumption is quickly catalyzed by a unidirectional uptake hydrogenase. In addition, a bidirectional (reversible) enzyme may also oxidize some of the molecular hydrogen. The same enzyme will, under certain conditions, evolve H_2. Filamentous cyanobacteria have been used in bioreactors for the photobiological conversion of water to hydrogen. However, the conversion efficiencies achieved are low because the net H_2 production is the result of H_2 evolution via a nitrogenase and H_2 consumption mainly via an uptake hydrogenase. Consequently, the improvements of the conversion efficiencies are for example achieved through the optimization of the conditions for H_2 evolution by nitrogenase, through the production of mutants deficient in H_2 uptake activity, and by an increased H_2 evolution by a bidirectional enzyme. Symbiotic cells are of fundamental interest since *in situ* they "function as a bioreactor"; high metabolism, transfer of metabolite(s) from symbiont to host but almost no growth. In this communication we present general knowledge about hydrogen metabolism/hydrogenases in filamentous cyanobacteria focusing on recent advances using molecular techniques, outline strategies for

improving the capacity of H_2 production by filamentous strains, and stress the importance of international cooperations and networks.

1. INTRODUCTION

Molecular hydrogen (H_2) is an energy carrier that may in future be a valuable alternative to the limited fossil fuel resources of today. Its advantages as a fuel are numerous: It is environmentally clean, efficient, renewable, no CO_2 is released during its generation and, at the most, only small amounts of NOx are produced. An attractive possibility is the direct splitting of water for generation of H_2 using solar radiation. This splitting can be achieved either in photochemical fuel cells, or by applying photovoltaics which directly utilizes solar radiation for electrolysis of water into H_2 and O_2. The third and most challenging option is the production of hydrogen by photosynthetic microorganisms which will, under natural conditions, produce (and evolve) no, or very small amounts of, H_2. However, under specific incubations and/or treatments a substantial induction of H_2 production may occur. Previous studies, using small scale bioreactors, demonstrated a capacity for photoproduction of H_2 by several filamentous heterocystous cyanobacteria but the conversion efficiencies were low. In order to achieve significant H_2 production rates over long time the following need to be considered: (1) the strains used must be selected for their specific hydrogen metabolism; (2) the selected strains must be genetically engineered in order to produce large amounts of H_2; and (3) the overall conditions for cultivation in bioreactors must be improved. For photobiological H_2 production, cyanobacteria are among the ideal candidates since they have minimal nutritional requirements: They require only air (N_2 and CO_2), water (electrons and reductant) and mineral salts with light as the single energy source. Cultivation is therefore simple and relatively inexpensive.

2. CYANOBACTERIAL HYDROGENASE

Cyanobacteria may possess several enzymes directly involved in hydrogen metabolism: nitrogenase(s) catalyzing the production of H_2 concomitantly with the reduction of N_2 to NH_4^+, an uptake hydrogenase, catalyzing the consumption of H_2 produced by the nitrogenase, and a reversible/bidirectional hydrogenase, which has the capacity to both take up and produce H_2 (Fig. 1; see Appel & Schulz, 1998; Bergman et al., 1997; Bothe et al., 1991; Flores & Herrero 1994; Hansel & Lindblad, 1998; Houchins, 1984; Lambert & Smith, 1981; Lindblad, 1999; Lindblad et al., 1998; Papen et al., 1986; Smith, 1990). The latter enzyme was called "reversible hydrogenase" until the respective structural genes were sequenced and characterized by Schmitz et al. (1995). These authors, to avoid confusion with the 42 kDa subunit cloned by Ewart et al. (1990), have chosen to call the enzyme "bidirectional hydrogenase", a name that is used throughout this presentation.

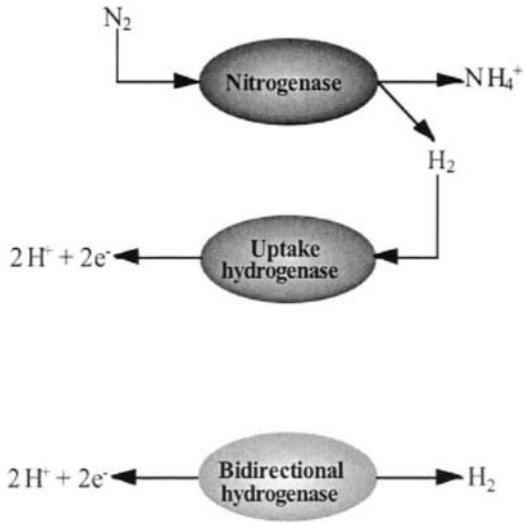


Figure 1: Enzymes directly involved in hydrogen metabolism in cyanobacteria. While the uptake hydrogenase is present in all N_2-fixing strains tested so far, the reversible/bidirectional enzyme seems to be present, although not universially, in both N_2-fixing and non-N_2-fixing cyanobacteria.

2.1. Nitrogenase(s)

In spite of the fact that nitrogenase is certainly a key enzyme in cyanobacterial hydrogen metabolism/production, it is not the object of this work and, consequently, its features are only mentioned briefly here.

The activity of nitrogenase, the enzymatic complex for N_2-fixation, is essential for the maintainance of the nitrogen cycle on earth, since this element is often limiting for the growth of all living organisms (Burris, 1991; Postgate, 1987; Gallon, 1992; Sta, 1995). The ability to fix N_2 is restricted to prokaryotic organisms. However, a representative number of eukaryotes, notably green plants, can establish symbiosis with diazotrophs (N_2 fixers). A broad range of microorganisms, including both archae and eubacteria, has been found to have the capacity to fix N_2. This diversity of organisms contrasts to the remarkable conservation of nitrogenase itself (Burris & Roberts, 1993; Flores & Herrero, 1994; Haselkorn & Buikema, 1992).

The nitrogenase complex consists of two proteins: the dinitrogenase (MoFe-protein or protein I) and the dinitrogenase reductase (Fe-protein or protein II). The dinitrogenase is a $\alpha_2\beta_2$ heterotetramer of about 220-240 kDa, which binds four 4Fe4S clusters, organized into two "P-clusters", and two FeMo cofactors (Flores & Herrero, 1994). It is generally accepted that the FeMo-cofactors constitute the active site in the dinitrogenase. The α and the β subunits are encoded by the genes *nifD* and *nifK*, respectively. The Fe-protein, encoded by the gene *nifH*, is a homodimer of about 60-70 kDa and has the specific role of mediating the

transfer of electrons from the external electron donors (a ferredoxin or a flavodoxin) to the P clusters of dinitrogenase. Together, the subunits bind one intersubunit 4Fe4S center (Orme-Johnson, 1992; Flores & Herrero, 1994). In addition to the three structural genes mentioned above, many other genes are involved in the nitrogen fixation process and its regulation (for a recent review see Böhme, 1998).

The reduction of nitrogen to ammonium, catalyzed by nitrogenase, is a highly endergonic reaction requiring metabolic energy in the form of ATP. First, the dinitrogenase reductase is reduced by a ferredoxin or a flavodoxin (Flores & Herrero, 1994; Masepohl et al., 1997) and binds MgATP, which lowers its potential. At this lower potential (around -400 mV) the dinitrogenase reductase transfers electrons to dinitrogenase. The transfer is accompanied by the hydrolysis of MgATP to MgADP + Pi. As two MgATP are required for each electron transferred from dinitrogenase reductase to dinitrogenase, the reaction requires a minimum of 16 MgATP until the dinitrogenase has accumulated enough electrons to reduce N_2 to NH_3. This reaction is also accompanied by an obligatory reduction of protons to H_2. Apparently, at infinite pN_2 75% of the electrons would be allocated for N_2 reduction and 25% for H^+ reduction. The overall reaction can be written as follows:

$$N_2 + 8\ e^- + 8H^+ + 16\ MgATP \text{---> } 2\ NH_3 + H_2 + 16\ MgADP + 16\ P_i$$

Nitrogenase is very oxygen labile, hence, all diazotrophs must protect they enzymatic complex from the deleterious effects of O_2. Cyanobacteria and Prochlorophytes are the only organisms known to be able to perform oxygenic photosynthesis (Flores & Herrero, 1994; Matthijs et al., 1994); cyanobacteria must, therefore, protect their N_2 fixing machinery not only from atmospheric O_2 but also from the intracellularly generated O_2. In fact, cyanobacteria have evolved diverse mechanisms/strategies ranging from temporal separation of nitrogen fixation and oxygen evolution, in unicellular and filamentous non-heterocystous strains, to cellular differentiation into N_2-fixing heterocystous in filamentous strains (for reviews see Bergman et al., 1997; Böhme, 1998; Fay, 1992; Wolk, 1996; Wolk et al., 1994).

Recently, the non-heterocystous cyanobacteria have particularly attracted the attention of scientists because of the apparent paradox of being able of both fix N_2 and evolve O_2. However, a very restricted number of strains are able to perform N_2 fixation under aerobic conditions, and there is no evidence that two processes, oxygenic photosynthesis and N_2 fixation, may occur simultaneously within a single cell (see Bergman et al., 1997). Temporal separation between photosynthetic O_2 evolution and N_2 fixation, seems to be the most common strategy adopted by non-heterocystous cyanobacteria. However, not all strains fix N_2 exclusively during the dark phase of light/darkness cycle. It is important to remember that N_2 fixation is process that requires ATP and reductant, and that the fermentation of stored carbohydrates may not be sufficient to cover the energy demand of nitrogenase activity. Interestingly, it has been shown that in natural populations of *Oscillatoria limosa* the nitrogenase activity coincides with the transitions from dark to light and light to dark with a maximum at sunrise. The last event could be explained by light energy driven N_2 fixation in an initially low O_2environment. Within a short time, the nitrogenase activity will be inhibited by the photosynthetically evolved O_2. The situation in the marine filamentous non-heterocystous *Trichodesmium* is far more complex. In this strain a spatial separation between the N_2 fixation and the photosynthesis probably occurs, without any obvious cellular differentiation. It is known that, in contrast with the permanent changes occurring during heterocysts differentiation, those occurring in non-heterocystous cyanobacteria can be reversed (Fredriksson & Bergman, 1997; Bergman et al., 1997).

Filamentous cyanobacteria are able to differentiate 5-10% of their vegetative cells into cells specialized in N_2 fixation - the heterocysts or heterocytes. Heterocyst formation does not take place randomly within the filament. Most probably it is a dynamic selection in response to nitrogen deprivation but neither the existence of predetermination nor a combination of both events can be ruled out (Wolk, 1996).

The heterocyst provides a virtually anaerobic environment suitable for the functioning of nitrogenase since: it lacks photosystem II activity (does not produce O_2 as a by product of photosynthesis), it has a higher rate of respiratory O_2 consumption, and is surrounded by a thick envelope that limits the diffusion of O_2 through the cell wall (Fay, 1992; Wolk et al., 1994). The connection between the vegetative cell and the heterocyst is narrow and occurs via microplasmodesmata. Heterocysts import carbohydrates and in return export glutamine to the vegetative cells. ATP formation in heterocysts is not completely understood. ATP can be produced in the light by either cyclic photophosphorylation (PSI) or oxidative phosphorylation, the latter process consumes oxygen and uses pyridine nucleotides or hydrogen as electron sources. It has been suggested that NADPH provides electrons for nitrogenase via a heterocyst-specific ferredoxin (FdxH) and ferredoxin: $NADP^+$ oxidoreductase (Böhme, 1998). The hydrogen, produced as a by product of N_2 fixation, is taken up by an uptake hydrogenase, and reacts with oxygen contributing to the ATP pool required for biosynthetic reactions such as N_2 fixation.

An interesting feature of heterocyst differentiation in cyanobacteria is the occurrence of developmentally regulated genome rearrangements (Carrasco et al., 1994; Carrasco et al., 1995; Carrasco & Golden, 1995; Golden et al., 1985; Golden et al., 1987; Golden et al., 1988; Matveyev et al., 1994; Meeks et al., 1994; Mulligan et al., 1988; Mulligan & Haselkorn, 1989). These rearrangements occur late during heterocyst differentiation at about the same time as the nitrogen fixation genes are transcribed. In the vegetative cells of *Anabaena* sp. strain PCC 7120 the genes *fdxN*, *nifD* and *hupL* are interrupted by a 55-kb, an 11-kb, and a 10.5-kb element, respectively. These elements are excised, during heterocyst differentiation, by site-specific recombinases encoded by the genes *xisF*, *xisA*, and *xisC*, respectively. Recently, it was shown that the excision of the 55-kb element (*fdxN*) requires also the products of two other genes named *xisH* and *xisI* (Ramaswamy et al., 1997).

2.1.1. "Alternative nitrogenases"

In the heterocystous cyanobacterium *Anabaena variabilis* four different nitrogenases have been identified and characterized (for details see Kentemich et al., 1988; Kentemich et al., 1991; Thiel, 1993; Thiel et al., 1995; Thiel et al., 1997). Two are Mo-dependent enzymes but while one (the so-called conventional nitrogenase, encoded by the *nif1* gene cluster) functions only in heterocysts and under both aerobic or anaerobic growth conditions, the other (encoded by the *nif2* gene cluster) functions strictly under anaerobic conditions in both the vegetative cells and the heterocysts. Furthermore the differences between the two *nif* clusters suggest that the conventional nitrogenase is developmentally regulated while the other is regulated by environmental factors. The occurrence of a V-containing nitrogenase was first reported by Kentemich et al. (1988) and, subsequently, confirmed by Thiel (1993). This enzyme is encoded by the *vnfDGK* gene cluster, that is transcribed in the absence of molybdenum, and in which *vnfDG* are fused in a single ORF (Thiel, 1993). The fourth nitrogenase is believed to be an Fe-enzyme similar to the one encoded by the *anf* gene cluster in *Azotobacter vinelandii* (Bishop & Premakumar, 1992; Kentemich et al., 1991). At the present, alternative nitrogenases have been found in other cyanobacterial strains (Bishop & Premakumar, 1992),

and it is known that they differ from the conventional enzyme physically, chemically, and by their catalytic properties. For example, both the V- and Fe-nitrogenases are repressed by Mo and characteristically produce ethane as well as ethylene from acetylene. It is important to keep in mind that all the alternative enzymes investigated so far seem to allocate a higher proportion of electrons to the reduction of H^+ to H_2 when compared to the conventional Mo-enzyme complex. However, hydrogen produced by nitrogenase is generally taken up by a hydrogenase, so that net H_2 evolution by nitrogen-fixing cyanobacteria is hardly observed, at least under aerobic conditions (Almon & Böger, 1988).

2.2. Uptake Hydrogenase

An uptake hydrogenase, with the evident function of catalyzing the uptake of H_2 produced by nitrogenase, has been found in all N_2-fixing unicellular and filamentous cyanobacteria examined so far (Lambert & Smith, 1981; Houchins, 1984). The enzyme seems to be membrane-bound and, in some filamentous strains, is particularly expressed in the N_2-fixing heterocysts with no or minor activity in the photosynthetic vegetative cells (Papen et al., 1986; Houchins, 1984; Houchins & Burris, 1981a; Carrasco et al., 1995). Some physiological data, together with the recent analysis of a mutant of the bidirectional hydrogenase gene *hoxH*, indicate that the unicellular non- N_2-fixing *Synechococcus* sp. strain PCC 6301 (= *Anacystis nidulans*) may also possesses an uptake enzyme (Eisbrenner et al., 1978; Peschek, 1979a; Peschek, 1979b; Boison et al., 1996). However, the presence of an uptake hydrogenase in non- N_2-fixing cyanobacteria is still a controversial subject and further work is needed to clarify this question.

The first molecular data concerning cyanobacterial hydrogenases appeared in 1995. Carrasco et al. described a novel developmental genome rearrangement for *Anabaena* sp. strain PCC 7120, which is present in addition to the known *nifD* and *fdxN* rearrangements (see above) occurring during the differentiation of a photosynthesizing vegetative cell into a nitrogen-fixing heterocyst. This third rearrangement occurs within a gene (*hupL*) that exhibits homology to the genes encoding the large subunit of membrane-bound uptake hydrogenases (Fig. 2).

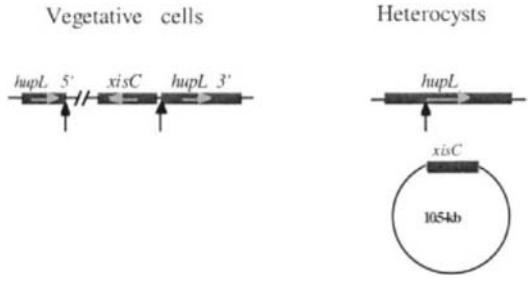


Figure 2: Schematic representation of the *hupL* rearrangement in *Anabaena* sp. strain PCC 7120. Horizontal grey arrows show the orientation of the *hupL* and the *xisC* open reading frames. Vertical black arrows indicate the recombination site (adapted from Carrasco et al., 1995).

The excision of a 10.5-kb DNA element occurs late during the heterocyst differentiation process indicating that HupL, in *Anabaena* sp. strain PCC 7120, is expressed only in heterocysts. The excision occurs by a site-specific recombination between short directly repeated sequences and the putative recombinase gene *xisC* was found 115-bp inside the right border of the *hupL* element. At present, it is known that the occurrence of the *hupL* rearrangement it is not ubiquous in filamentous heterocyst-forming cyanobacteria (Axelsson et al., 1999; Oxelfelt et al., 1998; Tamagnini et al., 2000), and *hupSL* homologues have been found and sequenced in *Nostoc* sp. strain PCC 73102 (Oxelfelt et al., 1998), and *Anabaena variabilis* (Happe et al., unpublished; GenBank Y13216).

Although the above mentioned molecular studies helped to elucidate discrepancies arising from previous works, the exact subunit composition of cyanobacterial uptake hydrogenase and, consequently, the molecular mass of the holoenzyme are still unknown. The data concerning filamentous heterocystous cyanobacteria point out to the existence of, at least, two dissimilar subunits of 60 kDa and 35 kDa (Carrasco et al., 1995; Houchins & Burris, 1981a; Houchins & Burris, 1981b, Lindblad & Sellstedt, 1990; Oxelfelt et al., 1998; Tamagnini et al., 1995). It is tempting to infer that those cyanobacterial strains possess a heterodimeric uptake hydrogenase. However, at present, it is premature to exclude the possibility of the occurrence of other subunits or/and the repetition of the known ones in the active form of the enzymes since no cyanobacterial uptake hydrogenase has yet been purified to homogeneity. The existence of a membrane anchoring polypeptide(s) is almost certain, although, it has not been yet sequenced/characterized.

The cellular and subcellular localization of hydrogenases in cyanobacteria it is another source of controversy. It is commonly accepted that the uptake hydrogenase is bound to a membrane, but while some authors suggest that the enzyme is located in the thylakoid membranes of heterocysts (Eisbrenner et al., 1981), others reported its presence in both the vegetative cells and the heterocysts and its association with the cytoplasmic membrane (Houchins & Burris, 1981a; Lindblad & Sellstedt, 1990; Rai et al., 1992). It should be pointed out that some of these interpretations are based in immunological studies and, in this case, one can not exclude the possibility that the antibodies recognize both uptake and bidirectional hydrogenase antigens or other, at present, unknown proteins. Moreover, there is evidence for the occurrence of an uptake hydrogenase only in heterocysts of aerobically grown cells of *Anabaena* spp. but a small amount of activity was detected in the vegetative cells of filaments grown microaerobically/anaerobically (Houchins & Burris, 1981a; Peterson & Wolk, 1978; Troshina et al., 1996). Recently, transcriptional studies demonstrated the induction of a *hupL* transcript approximately 24h after a shift from non-nitrogen-fixing to nitrogen-fixing conditions, occurring concomitatly with the induction of an *in vivo* light-dependent H_2 uptake activity, in *Nostoc muscorum* (Axelsson et al., 1999). The authors also showed the presence of a contiguous *hupL* gene in both vegetative cells and heterocysts of this strain.

The uptake hydrogenase activity versus reversible/bidirectional hydrogenase activity was well characterized by Houchins & Burris (1981b) for *Anabaena* sp. strain PCC 7120. In whole filaments, the uptake hydrogenase is resistant to atmospheric O_2 levels but, an inactivation that increased with the disruption of the system was observed. The two hydrogenases differ in their thermal stabilities, the uptake enzyme also being much more sensitive with a half-life of 12 minutes at 70°C. In comparison, the uptake hydrogenase is less sensitive to carbon monoxide competitive inhibition versus H_2 than the bidirectional enzyme. Both enzymes have low K_ms for H_2 but only the uptake hydrogenase activity was

shown to be elicited by addition of H_2 to the gas phase. A comparative study of the growth and nitrogen fixation of nickel-depleted and nickel-supplemented cultures of *A. cylindrica* showed that in each case nickel-containing cells had an active hydrogen uptake capacity whereas nickel-depleted cells did not. These differences in hydrogenase activities did not correlate with differences in acetylene reduction and growth rate, or fixed nitrogen, phycocyanin or chlorophyll contents. It was concluded that under the growth conditions used the capacity of cells to consume hydrogen gas confers no advantage to the organisms in terms of their growth rates and nitrogen fixation (Daday et al., 1985). Uptake hydrogenases of cyanobacteria, as well as uptake hydrogenases of other bacteria, have been shown to be dependent on the availability of Ni^{2+} in the growth medium (Xiakong et al., 1984; Daday et al., 1985; Shravan Kumar & Polasa, 1991; Oxelfelt et al., 1995). In *Oscillatoria subbrevis*, Ni ions are required for an active uptake hydrogenase activity, and the repression of the hydrogenase activity by EDTA was specifically released by Ni^{2+}. Moreoer, H_2 production and uptake activities decreased in the presence of copper (Shravan Kumar & Polasa, 1991).

Combining all the physiological and immunological works with the recent molecular data, notably with the presence/absence of the rearrangement within the gene *hupL* in the filamentous strains, it is impossible to establish one single pattern of cellular/subcellular localization and regulation of the uptake hydrogenase, even for closely related strains.

2.3. Bidirectional Hydrogenase

In contrast with the uptake hydrogenase, the soluble/loosely membrane associated bidirectional hydrogenase was believed to be a constitutive enzyme (Kentemich et al., 1989; Kentemich et al., 1991; Serebriakova et al., 1994) widely distributed among N_2-fixing and non- N_2-fixing cyanobacteria. The activity of this enzyme, in heterocystous strains, increases considerably under anaerobic or microaerobic conditions (Serebriakova et al., 1994), whereas in the unicellular non- N_2-fixing *Gloeocapsa alpicola* the partial pressure of oxygen does not seem to have any significant influence (Serebryakova et al., 1998). The hydrogenase activity in vegetative cells of *Anabaena variabilis* has been suggested to be subjected to a form of "redox control" whereby the enzyme is only activated upon removal of light and oxygen. This mechanism may involve a thioredoxin (Spiller et al., 1983).

The bidirectional hydrogenase of *Anabaena variabilis* has been partially purified and characterized (Serebryakova et al., 1996). In 1995, Schmitz et al. sequenced a set of structural genes (*hox* genes) encoding a bidirectional hydrogenase in the filamentous heterocystous *Anabaena variabilis.* These authors suggested that the bidirectional enzyme is a heterotetrameric enzyme consisting of a hydrogenase part (encoded by *hoxYH*) and a diaphorase part (encoded by *hoxFU*) (Fig. 3). In subsequent work, *hox* genes have been sequenced and characterized in the unicellular cyanobacteria *Synechocystis* sp. strain PCC 6803 and *Anacystis nidulans* (=*Synechococcus* sp. strain PCC 6301) (Appel & Schulz, 1996; Boison et al., 1996; Boison et al., 1998; Nakamura et al., 1998; Schmitz & Bothe, 1996a). Nucleotide sequence comparisons showed that there is a high degree of homology between the *hox* genes of cyanobacteria and genes encoding the NAD^+-reducing hydrogenase from the chemolitotrophic H_2-metabolizing bacterium *Alcaligenes eutrophus* as well as methyl viologen-reducing hydrogenases from species of the archae genera *Methanobacterium*, *Methanococcus*, and *Methanothermus.*

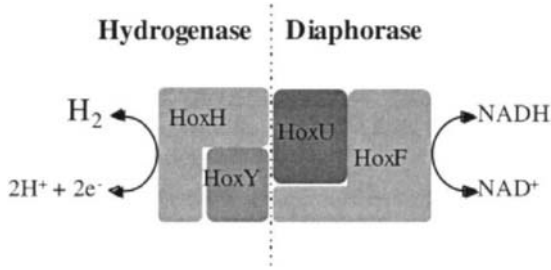


Figure 3: Schematic representation of the heterotetrameric bidirectional hydrogenase of *Anabaena variabilis* (adapted from Schmitz et al., 1995).

As for the uptake hydrogenase, the molecular studies helped to clarify the picture of the subunit composition/molecular mass of the bidirectional hydrogenase. In agreement with the molecular data, previous works referred to large subunits of about 50-56 kDa in *Anabaena variabilis*, *Anacystis nidulans*, *Microcystis aeruginosa*, and *Spirulina plantensis* (Asada et al., 1987; Llama et al., 1979; Kentemich et al., 1989), and small subunits of about 17 kDa in *Anabaena variabilis* and *Anacystis nidulans* (Kentemich et al., 1989). In *Anabaena variabilis* the genes *hoxH* and *hoxY* encode predicted polypetides of 54.8 kDa and 22.5 kDa, respectively.

Concerning the holoenzyme, earlier chromatographic studies reported an apparent molecular mass of 230 kDa for the hydrogenase of *Anabaena cylindrica* (Hallenbeck & Benemann, 1978). However, as pointed out by the authors, the enzyme was probably bound in a complex with other proteins. Considerably lower molecular masses (165 kDa and 113 kDa) were reported for the bidirectional hydrogenase of *Anabaena* sp. strain PCC 7120, with two/thirds of the activity in the 165 kDa peak (Houchins & Burris, 1981b). Native-PAGE followed by *in vitro* activity staining demonstrated the presence of a functional enzyme of about 118 kDa in induced cells of *Anabaena variabilis* (Serebriakova et al., 1994). It is believed that in *Anabaena variabilis* the four predicted polypetides (HoxH - 54.8 kDa, HoxY - 22.5 kDa, HoxU - 26.9 kDa, and HoxF - 57.9 kDa) are assembled together forming a tetrameric enzyme, which is consistent with previous work. However, transcriptional studies are required to evaluate if, in cyanobacteria, the four genes form a single transcriptional unit as it happens in the facultative chemolithoautotrophic bacterium *Alcaligenes eutrophus* (Tran-Betcke et al., 1990). Interestingly, a variety of mutations were introduced into the four structural genes of *Alcaligenes eutrophus* to obtain mutant enzymes composed of monomeric and dimeric forms (Massanz et al., 1998). The resulting mutant proteins, HoxHY, HoxH, HoxFU and HoxF indicate two independent functional modules for the hydrogenase and the diaphorase moieties. However, the HoxHY module displayed a reduced H_2-dependent dye-reducing activity indicating that the diaphorase moiety may contribute to a more stable configuration.

The cellular/subcellular localization of the bidirectional hydrogenase is a topic needing further investigation. Earlier works indicate that the enzyme was present in both the vegetative cells and the heterocysts, and that it was located in the cytoplasm since it was easily solubilized by gentle cell disruption procedures (Hallenbeck & Benemann, 1978; Houchins & Burris, 1981a; Houchins & Burris, 1981b). However, recent works point to an association of the bidirectional hydrogenase with cell membranes. The enzyme of the

unicellular *Anacystis nidulans* seems to be loosely associated with the cytoplasmic membrane (Kentemich et al., 1989; Kentemich et al., 1991), whereas in *Anabaena variabilis* an association with the thylakoid regions was reported (Serebriakova et al., 1994).

2.4. Accessory Genes

At present, very little information is available about cyanobacterial accessory genes; e.g., *hyp* genes. In the unicellular non- N_2-fixing *Synechocystis* sp. strain PCC 6803 the *hyp* genes are scattered throughout the genome, while in the heterocystous *Anabaena* sp. strain PCC 7120 a cluster consisting of the *hupBAED* genes (homologous to the corresponding *hyp* genes known in other bacteria) was identified (Gubili & Borthakur, 1996; Gubili & Borthakur, 1998). These genes are located upstream of *hupSL* and, although clustered, their organization is different from those of other bacteria - the positions of the genes with respect to each other are different and there is no *hupC* in *Anabaena* sp. strain PCC 7120 on either side of *hupA* or *hupB*. Very little is known about the functions of all these genes but it was demonstrated that, in *Anabaena* sp. strain PCC 7120, the *hupB* gene is expressed under nitrogen-fixing conditions only (Gubili & Borthakur, 1998).

3. CYANOBACTERIAL HYDROGENASES FOCUSING ON A SINGLE FILAMENTOUS HETEROCYSTOUS STRAIN - *NOSTOC* SP. STRAIN PCC 73102

3.1. Immunological Characterization

Nostoc sp. strain PCC 73102 was examined for the presence of hydrogenases by using antisera directed against proteins purified from other microorganisms (Tamagnini et al., 1995). Native-PAGE/immunoblots demonstrated that, in N_2-fixing cells of *Nostoc* sp. strain PCC 73102, two proteins (or two forms of a single protein) with apparent molecular masses of approximately 200 kDa and 215 kDa, were immunologically related to the hydrogenases purified from *Azotobacter vinelandii* (uptake hydrogenase), *Bradyrhizobium japonicum* (uptake hydrogenase), and *Methanosarcina barkeri* (coenzyme F_{420}-reducing hydrogenase). Moreover, one protein with a molecular mass of about 200 kDa was strongly recognized by the antiserum directed against the stable hydrogenase purified from *Tiocapsa roseopersicina*. Occasionally, a faint recognition towards the 215 kDa protein was also observed with the latter antiserum. SDS-PAGE/immunoblots showed that in *Nostoc* sp. strain PCC 73102 one polypeptide, with a molecular mass of about 58 kDa, was immunologically related to all the hydrogenases mentioned previously. In addition to the 58 kDa polypeptide, two other polypeptides were recognized: one of about 34 kDa, immunologically related to the hydrogenase purified from *T. roseopersicina*, and another of approximately 70 kDa, immunologically related to the F_{420}-reducing hydrogenase purified from *M. barkeri*. Immunogold labeling/TEM, using the same polyclonal antisera as above, showed that hydrogenase proteins are present in both the nitrogen-fixing heterocysts and in the photosynthetic vegetative cells of *Nostoc* sp. strain PCC 73102. However, the cellular localization data differ substantially depending on the antisera used. Primary antisera directed against the holoenzyme purified from *A. vinelandii*, the large and the small subunits of hydrogenase purified from *B. japonicum*, and the F_{420}-reducing hydrogenase purified from *M. barkeri* revealed 1.6 to 2.7 times higher labeling intensity in the vegetative cells compared to the heterocysts. Interestingly, the results were identical when antisera against either the large or the small subunit of the hydrogenase purified from *B. japonicum* were used, implying that they recognize similar protein(s). In contrast, when the primary antiserum against the

hydrogenase purified from *T. roseopersicina* was used, the label was much higher (7.7 times) in the vegetative cells than in the heterocysts.

3.2. Physiological Characterization

Using a H_2-electrode it was possible to demonstrate an *in vivo*, light dependent uptake of hydrogen in nitrogen-fixing cell suspensions of *Nostoc* sp. strain PCC 73102 (Oxelfelt et al., 1995). In darkness, no net uptake or evolution of H_2 occurred. Addition of Ni^{2+}, up to a concentration of 5.0 μM, to the growth medium resulted in a significant stimulation of the *in vivo* H_2 uptake in *Nostoc* sp. strain PCC 73102. In contrast, 10 μM Ni^{2+} had no stimulatory effect and the appearance of the culture changed remarkably during the incubation. All further experiments were performed using cells grown with the addition of 0.5 μM $NiSO_4$ to the growth medium. Adding 9% H_2 to the air continuously bubbling the light-grown *Nostoc* sp. strain PCC 73102 cultures significantly enhanced the observed light-dependent uptake of H_2. When the *Nostoc* sp. strain PCC 73102 cultures were incubated in darkness, addition of 9% H_2 induced a temporary stimulation of the light-dependent hydrogen uptake, whereas in a culture without addition of H_2, the activity went down to zero within a few hours. Addition of organic carbon (a combination of 30 mM glucose and 30 mM fructose) to the growth medium stimulated both light-dependent H_2 uptake as well as nitrogenase activities. Interestingly, maximal *in vivo* light-dependent uptake hydrogenase activity occurred in cells grown heterotrophically in darkness, about four times higher than in cells grown photoautotrophically. Organic carbon also induced significantly higher uptake of H_2 in photoautotrophically grown cells. When cells were grown heterotrophically in darkness (as above), addition of the protein synthesis inhibitor, chloramphenicol, abolished the significant increase observed with organic carbon. Similarly, chloramphenicol prevented the stimulation observed by light and extra nickel. Combined nitrogen, added as ammonium chloride, reduced both *in vivo* uptake hydrogenase and nitrogenase activities, suggesting a strong correlation between hydrogen uptake and nitrogenase activity in *Nostoc* sp. strain PCC 73102.

Cell suspensions of *Nostoc* sp. strain PCC 73102, *N. muscorum*, *Anabaena* sp. strain PCC 7120, and *A. variabilis* were assayed for the presence of an active bidirectional hydrogenase (Tamagnini et al., 1997). With three different growth media, an *in vivo* functional bidirectional enzyme was observed in *N. muscorum*, *Anabaena* sp. strain PCC 7120, and *A. variabilis*. *Nostoc* sp. strain PCC 73102 consistently lacked any detectable in vivo activity. Moreover, similar results were obtained when assaying for the presence of an enzyme functional *in vitro*. Native PAGE followed *in situ* hydrogenase activity staining was used to demonstrate the presence or absence of a functional enzyme in partially purified extracts from the four cyanobacteria. Again, bands corresponding to hydrogenase activity and with apparent molecular masses of about 560, 130, and/or 117 kDa were observed for *N. muscorum*, *Anabaena* sp. strain PCC 7120, and *A. variabilis* but not for *Nostoc* sp. strain PCC 73102.

3.3. Molecular Characterization

To continue the characterization of H_2 metabolism in *Nostoc* sp. strain PCC 73102, oligonucleotide primers were designed from conserved sequences within the *hoxY* and *hoxH* genes (bidirectional hydrogenase) of *Anabaena variabilis* ATCC 29413, and within the *hupL* gene (uptake hydrogenase) of *Anabaena* sp. strain PCC 7120. These primers were used in PCRs with genomic DNA from either *A. variabilis* (*hox*) or *Anabaena* sp. strain PCC 7120 (*hup*) as the template. PCR products of the expected sizes were obtained, and their respective

identities were established by sequencing (Tamagnini et al., 1997). Subsequently, these DNA fragments were used as probes. Low-stringency Southern hybridizations revealed the occurrence of *hox* sequences in *A. variabilis* (control), *Anabaena* sp. strain PCC 7120 and *N. muscorum* but not in *Nostoc* sp. strain PCC 73102 (Tamagnini et al., 1997). As a control, Southern hybridizations demonstrated the presence of *hup* sequences in all the cyanobacteria tested, including *Nostoc* sp. strain PCC 73102. PCRs using primer pairs within both the *hox*- and the *hup*-genes and genomic DNA of *Nostoc* sp. strain PCC 73102 resulted in either no amplification (*hox*) or amplification (*hup*) of the corresponding part of the gene.

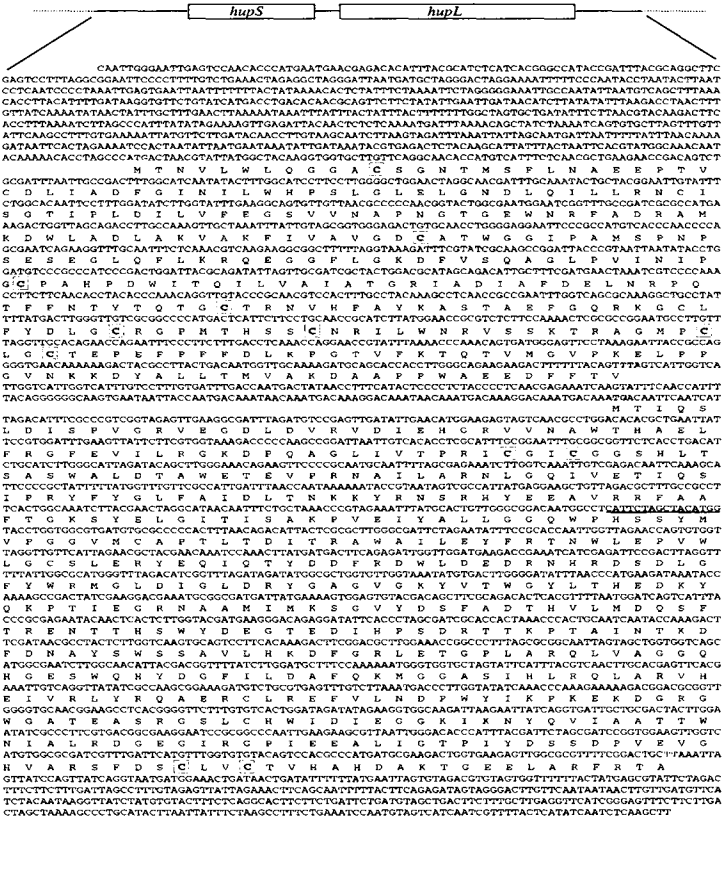


Figure 4: Nucleotide sequence (3940-bp) of genomic DNA of *Nostoc* sp. strain PCC 73102 containing *hupS* and *hupL*. Conserved cysteines are boxed, and the potential recombination site is underlined. GenBank accession no. AF030525 (Oxelfelt et al., 1998).

To identify the structural gene encoding the large subunit of an uptake hydrogenase (*hupL*) in *Nostoc* sp. strain PCC 73102, partial genomic libraries were constructed and screened. Two clones, pNfo01 and pNfo02, together revealed the complete sequence of a *hupS* and *hupL* homologue with upstream and downstream regions in *Nostoc* sp. strain PCC 73102 (Fig. 4; Oxelfelt et al., 1998). The genes encoding the small and the large subunit (*hupSL*) in *Nostoc* sp. strain PCC 73102 encode two proteins with calculated molecular masses of 34,917 Da and 60,157 Da, respectively.

The HupS protein of *Nostoc* sp. strain PCC 73102 contains 11 Cys residues 8 of which clearly correspond to the residues that are proposed to be involved in the formation of Fe-S clusters. HupS in *Nostoc* sp. strain PCC 73102 lacks the signal peptide at the N terminus present in many other organisms with membrane-bound or periplasmic hydrogenases. Moreover, the motif located at the C terminus of the small subunit of class I NiFe-hydrogenases (Wu & Mandrand, 1993), the unique feature of membrane-bound hydrogenases for anchoring the protein to the membrane was not found in the HupS protein of *Nostoc* sp. strain PCC 73102. The HupL protein of *Nostoc* sp. strain PCC 73102 contains the putative Ni-binding site present in NiFe-hydrogenases large subunits at the N-terminal end (RxCGxC). At the C-terminal end the Ni-binding site is also present but the second amino acid (Pro) is exchanged for a Ser (Wu & Mandrand, 1993; Albracht, 1994). In HupL of *Nostoc* sp. strain PCC 73102 the putative amino acid sequence that is proteolytically removed when the protein undergoes maturation, can also be recognized (Menon & Robson, 1994). Moreover, high-stringency Southern hybridizations using different probes covering or flanking the potential recombination site in *Nostoc* sp. strain PCC 73102, revealed the presence of a contiguous *hupL* in both nitrogen-fixing and non-nitrogen-fixing cells of *Nostoc* sp. strain PCC 73102. This together with sequence data show that *Nostoc* sp. strain PCC 73102 does not exhibit the same type of rearrangement within the structural *hupL* gene as previously shown in *Anabaena* sp. strain PCC 7120 (Carrasco et al., 1995). A potential rearrangement site was found in *Nostoc* sp. strain PCC 73102 but the sequence differs in 6 positions out of 16 on the nucleotide level (i.e. only 62.5% identical) in comparison with *Anabaena* sp. strain PCC 7120.

The present knowledge about genes related to hydrogenases in filamentous cyanobacteria is summarized in Table 1.

TABLE 1
GENES RELATED TO HYDROGENASES IN FILAMENTOUS CYANOBACTERIA

Organism	Gene	Accession number[1]	Gene product	Function/feature	Reference
***Anabaena* PCC 7120**	*hupL*	U08013	531 aa	uptake hydrogenase large subunit	Carrasco et al., 1995
	xisC	U08014	498 aa	rearrangement within *hupL*	Carrasco et al., 1995
	hupD	AF006594	199 aa	hydrogenases accessory gene	Gubili & Borthakur 1998
	hupE	AF006594	372 aa	hydrogenases accessory gene probably a membrane-associated protein	Gubili & Borthakur 1998

	hupA	AF006594	113 aa	hydrogenases accessory gene probably a cytoplasmic protein	Gubili & Borthakur 1998
	hupB	AF006594	262 aa	hydrogenases accessory gene only expressed in heterocyst-induced cultures	Gubili & Borthakur 1998
Anabaena variabilis	*hupS*	Y13216	320 aa	uptake hydrogenase small subunit	Happe et al., unpublished
	hupL	Y13216	482 aa[2]	uptake hydrogenase large subunit	Happe et al., unpublished
	hoxF	X79285	535 aa (57.9 kDa)	Bidirectional hydrogenase, diaphorase part, large subunit	Schmitz et al., 1995
	hoxU	X79285	238 aa (26.9 kDa)	bidirectional hydrogenase, diaphorase part, small subunit	Schmitz et al., 1995
	hoxY	X79285	205 aa (22.5 kDa)	bidirectional hydrogenase, hydrogenase part, small subunit	Schmitz et al., 1995
	hoxH	X79285	487 aa (54.8 kDa)	bidirectional hydrogenase, hydrogenase part, large subunit	Schmitz et al., 1995
Anabaena cylindrica	-	P16421	383 aa (41.1 kDa)	tritium exchange 42 kDa subunit	Ewart et al., 1990
***Nostoc* PCC 73102**	*hupS*	AF030525	320 aa (34.9 kDa)	uptake hydrogenase small subunit	Oxelfelt et al., 1998
	hupL	AF030525	531 aa (60.2 kDa)	uptake hydrogenase large subunit	Oxelfelt et al., 1998

[1] GenBank; http://www.ncbi.nlm.nih.gov/
[2] Only partly sequenced

3.3.1. Transcriptional Analyses in Nostoc spp.

Non N_2-fixing cultures of *Nostoc muscorum* and *N.* PCC 73102 (cells grown with the addition of ammonia) exhibit no *in vivo* light-dependent H_2-uptake activity (Axelsson et al., 1999; Oxelfelt et al., 1995). Cells of *N. muscorum* were further analyzed in detail since they contain both an uptake hydrogenase and a bidirectional enzyme (Axelsson et al., 1999). A transfer to media lacking combined nitrogen induced the presence of an *in vivo* light-dependent H_2-uptake activity after 24-41 h (Fig. 5A). Similarly, a *hupL* transcript (visualized using RT-PCR) could be detected after 24 h, and the relative quantity increased in parallel

with an increase in the *in vivo* light-dependent H_2-uptake activity (Fig. 5B). This is in agreement with an earlier observation using the heterocystous strain *A.* PCC 7120 in which a 10.5 kb DNA fragment prevents the transcription of *hupL* in vegetative cells (Carrasco et al., 1995). However, in *N. muscorum hupL* is contiguous in both vegetative cells and heterocysts (Axelsson et al., 1999). Recently, the cellular pools of transcripts for both hydrogenases in *Alcaligenes eutrophus* were shown to correlate with the activities of the respective promotors (Schwartz et al., 1997). Also, an immediate and drastic increase in transcript pool levels occurred upon derepression of the hydrogenase system. *hoxH* transcripts (structural gene encoding the large subunit of the bidirectional hydrogenase) were detected throughout the induction of the *in vivo* light-dependent H_2-uptake activity with no apparent increase, or decrease, during nitrogen-fixing conditions (Fig. 5C).

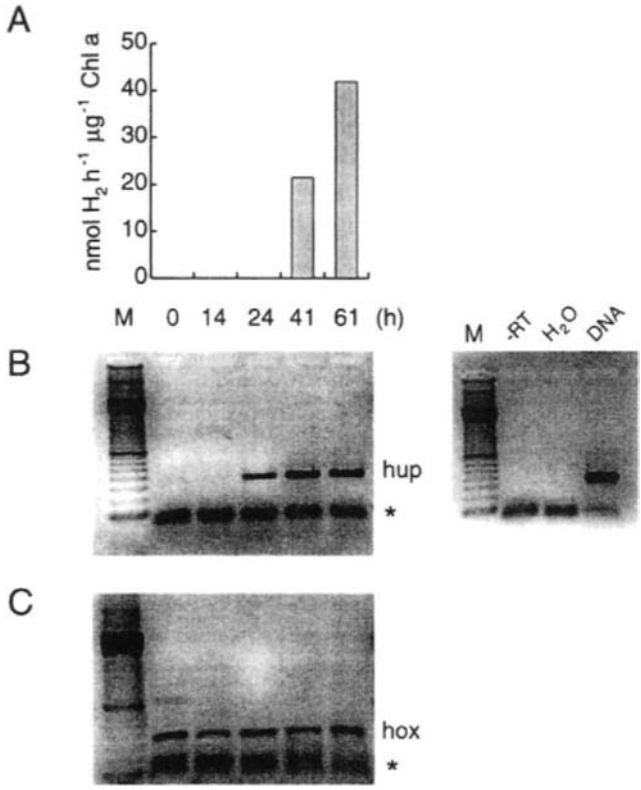


Figure 5: Induction of an *in vivo* light-dependent H_2-uptake in non N_2-fixing cells of *Nostoc muscorum* transferred to a medium lacking combined nitrogen. Ammonia grown cells were transferred to N_2-fixing conditions (time = 0) and analyzed for the appearance of an *in vivo* light-dependent H_2-uptake (A), and the presence of *hupL* (B) and *hoxH* (C) transcripts after 14, 24, 41, and 61 hours, respectively. (A) *In vivo* light-dependent H_2-uptake measured using an H_2-electrode. (B, C) Presence of *hupL* (B, left panel) and *hoxH* (C) transcripts visualized using RNA prepared from cells sampled at different time intervals (0, 14, 24, 41, and 61 h)

and RT-PCR (negative images). M - Marker, 100 bp DNA-ladder. * - Primer artefacts. Controls (right panel) include only a PCR (-RT; all RNA samples, only t = 0 h shown, negative control), replacing RNA with water (H_2O; negative control), and using genomic DNA instead of RNA (DNA; positive control). From Axelsson et al., 1999.4.

4. CYANOBACTERIAL BIOHYDROGEN

In cyanobacteria two types of enzymes are capable of H_2 production: the nitrogenase(s) and the bidirectional hydrogenase (see above). Most of the research has been carried out with N_2-fixing strains (Famiglietti et al., 1993; Kumazava & Asakawa, 1995; Lichtl et al., 1997; Reddy et al., 1996; Tsygankov et al., 1998a; Tsygankov et al., 1998b). In these organisms, the net H_2 production is the result of H_2 evolution catalyzed by nitrogenase and H_2 consumption mainly catalyzed by the uptake hydrogenase. Consequently, the production/selection of mutants deficient in H_2 uptake activity is necessary (Mikheeva et al., 1995). Moreover, the nitrogenase has a high ATP requirement and this lowers considerably their potential solar energy conversion efficiencies. On the other hand, the reversible hydrogenase requires much less metabolic energy, but it is extremely sensitive to oxygen (Asada & Kawamura, 1984; Asada et al., 1987).

An efficient photoconversion of water to hydrogen by cyanobacteria is certainly influenced by many other factors, and only an extensive knowledge of this field can lead to the improvement of the cyanobacterial H_2 production rates. For example, it is already known that immobilized cells produce more H_2 than free-living cultures (Markov et al., 1995), that non-Mo-containg nitrogenases allocate more electrons to the production of hydrogen (Tsygankov et al., 1998b), and that the age and density of the culture, the composition, pH, and temperature of the growth medium are crucial for the final result. The selection/construction of a suitable strain(s) is also a major field of interest in which the screening of natural populations of cyanobacterial may play an important role. For recent reviews on the potential, problems, and prospects of H_2 production by cyanobacteria, see Appel & Schulz, 1998; Benemann, 1996; Benemann, 1997; Hansel & Lindblad, 1998; Lindblad, 1999; Markov et al., 1995; Rao & Hall, 1996; Schulz, 1996; Schulz et al., 1998.

5. STRATEGIES FOR IMPROVING CYANOBACTERIAL STRAINS FOR PHOTOBIOLOGICAL H_2-PRODUCTION

5.1. Additional Strains

Thorough studies on H_2 uptake and/or evolution have until now focused on only a few filamentous cyanobacteria, e.g. different *Anabaena* and *Nostoc* strains. However, other cyanobacteria, e.g. *Oscillatoria*, are able fix N_2 without forming heterocysts with the strategy of temporal separation of the O_2-sensitive nitrogen fixation and the O_2-evolving photosynthesis. Such strains deserve a thorough examination concerning their O_2-metabolism. Considering the versatility of cyanobacteria and their ability to survive under many different environmental conditions, more strains originating from different habitats have to be studied with respect to their applicability in biohydrogen production. Of specific interest might be isolates originating from nitrogen-fixing associations. The situation of the symbiotic cyanobacteria is similar to steady state cultures in bioreactors: The cells almost do not grow, they have a high nitrogen fixation and thus a high H_2-production rate, and they export metabolite(s) to the host.

5.1.1. Molecular diversity of cyanobacterial hydrogenases

The natural molecular variation of hydrogenases in different cyanobacteria is a field to explore, both to understand the physiological functions of the respective enzymes, and to identify/find/construct a strain suitable of photobiological H_2 production in a bioreactor.

Cyanobacterial strains, from a broad range of sources, were screened for the presence of DNA sequences similar to *hupS* and *hupL* (uptake hydrogenase), *xisC* (rearrangement within *hupL*), and *hoxY*, *hoxH*, and *hoxF* (bidirectional hydrogenase) by heterologous Southern hybridizations (Tamagnini et al., 2000).

DNA sequences similar to the genes encoding an uptake hydrogenase are present in all N_2-fixing strains tested (Tamagnini et al., 2000). Screening for *xisC* revealed that sequences similar to the gene responsible for the rearrangement within *hupL* are present in about half of the filamentous heterocystous strains tested. DNA sequences similar to the bidirectional hydrogenase genes (*hox*) seem to be absent not only in *Nostoc* sp. strain PCC 73102 (Tamagnini et al., 1997) but also in other filamentous strains (Axelsson et al., 1999; Tamagnini et al., 2000). Although *hup* genes/homologues are present in all *Anabaena* and *Nostoc* strains examined, all kinds of combinations can be detected concerning the presence/absence of sequences homologues to *xisC* and to the bidirectional hydrogenase genes (Table 2). It should be pointed out that, although tempting, it is not possible to establish a correlation between the presence/absence of the bidirectional hydrogenase, and the occurrence of the gene responsible for the rearrangement of *hupL*.

TABLE 2

MOLECULAR DIVERSITY OF HYDROGENASES IN SELECTED *ANABAENA* AND *NOSTOC* STRAINS DEMONSTRATED BY THE PRESENCE (+), OR ABSENCE (-), OF SEQUENCES HOMOLOGOUS TO *HUP* GENES (UPTAKE HYDROGENASE), *XISC* (REARRANGEMENT WITHIN *HUPL*), AND *HOX* GENES (BIDIRECTIONAL HYDROGENASE)

Strain	***hup***	***xisC***	***hox***	**Reference(s)**
Anabaena PCC 7120	+	+	+	Carrasco et al., 1995, Tamagnini et al., 1997
Anabaena variabilis	+	-	+	Schmitz et al., 1995, Tamagnini et al., 1997
Nostoc Mitsui 56111	+	+	+	Tamagnini et al., 2000
Nostoc Mutsui 38901	+	+	-	Tamagnini et al., 2000
Nostoc Mitsui 91911	+	-	+	Tamagnini et al., 2000
Nostoc muscorum	+	-	+	Tamagnini et al., 1997, Axelsson et al., 1999
Nostoc PCC 73102	+	-	-	Tamagnini et al., 1997, Oxelfelt et al., 1998

One consequence of the above data is that the bidirectional enzyme is not as widely distributed as believed before. Furthermore, these data have implications for the theoretical model that links the diaphorase part of the bidirectional hydrogenase with the respiratory

complex I in cyanobacteria (see Appel & Schulz, 1996; Appel & Schulz, 1998; Boison et al., 1998; Schmitz & Bothe, 1996b; Schulz et al., 1998). Since the bidirectional hydrogenase is absent in a significant set of strains, it seems unlikely that the diaphorase subunits play a central role in cyanobacterial respiratory complex I, unless different cyanobacteria have adopted different strategies. In fact, the conserved sequence motifs of the diaphorase subunits are similar in the two corresponding complex I subunits, but apart from them there are only low sequence similarities (see Friedrich & Weiss, 1997). These authors proposed that the cyanobacterial and chloroplastidial complex, which both have eleven subunits in common with the respiratory complex I, might work as a NADPH: Plastoquinone oxidoreductase possibly being involved in the cyclic photosynthetic electron transport. Moreover, they also suggest that the sequence similarities observed between the NADH dehydrogenase part of complex I and the diaphorase part of the NAD^+-reducing hydrogenase are due to a common ancestor. However, it should be pointed out that, at present, the existence of a respiratory complex I in cyanobacteria can not be ruled out. Recently, hybridizations with all *hoxEFU* genes have been performed with negative results for *Nostoc* PCC 73102 (Boison et al., 1999). In addition, *Nostoc* PCC 73102 respires with rates comparable to those of other cyanobacteria. Moreover, it was also shown that *hox*$^-$ mutants of *Synechocystis* PCC 6803 (lacking *hoxE* or *hoxF*) exhibited growth and respiration rates comparable to those of the wild-type (Howitt & Vermaas, 1999). Altogether these data are against the theoretical model that links the diaphorase part of the bidirectional hydrogenase with the respiratory complex I in cyanobacteria.

5.2. Genetic Engineering

Genetic engineering has become possible with the establishment of molecular biological tools and techniques for cyanobacteria. A few unicellular strains, including *Synechoccus* PC 6301 and PCC 7942 as well as *Synechocystis* PCC 6803, are naturally transformable. Protocols and vector systems useful for the transfer of DNA into different cyanobacteria are available for non-transformable strains. These methods have been used with success in filamentous genera such as *Anabaena* and *Nostoc* which might be interesting candidates for future photobiotechnological applications (Hansel & Lindblad, 1998; Hansel et al., 1998).

Several strategies are available for improving existing cyanobacterial strains for the biotechnological production of H_2. Inactivation of a gene encoding an uptake hydrogenase might lead to mutants which are not able to recycle the H_2 evolved by nitrogenase under N_2-fixing conditions. As a consequence H_2 produced through the action of a nitrogenase will either be oxidized by some other hydrogenase or, if not present, evolved from the cells. The absence of a bidirectional enzyme in *Nostoc* PCC 73102 makes this an interesting candidate for such inactivation experiments. Identification/engineering of an oxygen-stable H_2-evolving hydrogenase might result in a photosynthesizing microorganism evolving H_2. Moreover, by providing genes encoding a selected hydrogenase on an expression vector overproducing mutants might be obtained. Coupling the genes to a promotor of a gene strongly expressed in heterocysts, such as the *nif* genes, might lead to an increased amount of the hydrogenase and thus increased levels of H_2 produced by the organism. Similarly, overexpression might also be used for increasing the nitrogenase activity. A thorough examination of the genes involved in the regulation of hydrogenase expression might generate knowledge leading to further strategies for improving H_2 production rates in cyanobacteria. In the completely sequenced genome of *Synechocystis* PCC 6803 (Nakamura et al., 1998) several ORFs homologous to regulatory genes of other bacterial hydrogenases were identified. However, nothing is known

about their interaction with the structural genes in this strain or in any other cyanobacterial strain.

6. INTERNATIONAL COOPERATIONS AND NETWORKS

Besides national programs and projects, two major international initiatives can be recognized; (1) In the International program IEA (http://www.iea.org) Agreement of the Production and Utilization of Hydrogen, Annex 15 *Photobiological Hydrogen Production* the main objectives are to investigate and to develop processes and equipment for photobiological production of hydrogen by direct conversion of solar energy. The research is organised 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. (2) In the European program COST 8.41 *Biological and Biochemical Diversity of Hydrogen Metabolism* the main objective is to pool interrelated European expertise in order to understand the structural and molecular basis of the functions as well as the factors that influence the activity and stability of hydrogenase enzymes.

7. CONCLUSION

Cyanobacterial biohydrogen is a rapidly developing scientific field. Since the introduction of molecular techniques a few years ago, significant advances have occurred. However, in order to utilize the potential of a photobiological hydrogen production by cyanobacteria both fundamental and applied research and development are needed. Moreover, the strains used must be selected for their specific hydrogen metabolism; we need more knowledge (both detailed and general) about cyanobacterial hydrogenases, corresponding genes, and how they are regulated. The selected strains must be genetically engineered in order to produce large amounts of H_2 (these techniques must be developed, and many genetically engineered strains should be created and tested), and the overall conditions for cultivation in bioreactors must be improved. The present status is that we have the general knowledge on how to proceed in order to explore the future potential of bioreactors containing genetically modified photosynthetic cyanobacteria producing large amounts of H_2 without any significant growth.

8. ACKNOWLEDGEMENTS

Presented work is part of the IEA Agreement of the Production and Utilization of Hydrogen, Annex 15 *Photobiological Hydrogen Production.* The authors research was financially supported by PRAXIS XXI (Portugal), the Royal Swedish Academy of Sciences (*Kungliga Vetenskapsakademien*), the Swedish National Energy Administration (*Energimyndigheten*), and the Swedish Natural Science Research Council (*NFR*).

9. REFERENCES

Albracht, S.P.J. (1994). Nickel hydrogenases: in search of the active site. *Biochim. Biophys. Acta* **1188**, 167-204.

Almon, H. and Böger, P. (1988). Nitrogen and hydrogen metabolism: induction and measurement. *In* Methods in Enzymology 167: Cyanobacteria, pp. 459-467. Edited by L. Packer and A. N. Glazer. Academic Press, London, Great Britain.

Appel, J. and Schulz, R. (1996). Sequence analysis of an operon of NAD(P)-reducing nickel hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803 gives additional evidence for direct coupling of the enzyme to NADP(H)-dehydrogenase (complex I). *Biochim. Biophys. Acta* **1298**, 141-147.

Appel, J. and Schulz, R. (1998). Hydrogen metabolism in organisms with oxygenic photosynthesis: hydrogenases as important regulatory devices for a proper redox poising? *J. Photochem. Photobiol. B: Biol.* **47**, 1-11.

Asada, Y. and Kawamura, S. (1984). Hydrogen evolution by *Microcystis aeruginosa* in darkness. *Agric. Biol. Chem.* **48**, 2595-2596.

Asada, Y., Kawamura, S. and Ho, K.-K. (1987). Hydrogenase from the unicellular cyanobacterium *Microcystis aeruginosa*. *Phytochem.* **26**, 637-640.

Axelsson, R., Oxelfelt, F. and Lindblad, P. (1999). Transcriptional regulation of *Nostoc* uptake hydrogenase. FEMS *Microbiol. Lett.* **170**, 77-81.

Benemann, J.R. (1996). Hydrogen biotechnology: progress and prospects. *Nature Biotechnol.* **14**, 1101-1103.

Benemann, J.R. (1997). Feasibility analysis of photobiological hydrogen production. *Int. J. Hydrogen Energy* **22**, 979-988.

Bergman, B., Gallon, J.R., Rai, A.N. and Stal, L.J. (1997). N_2 fixation by non-heterocystous cyanobacteria. FEMS *Microbiol. Rev.* **19**: 139-185.

Bishop, P.E. and Premakumar, R. (1992). Alternative nitrogen fixation systems. *In* Biological nitrogen fixation, pp. 736-762. Edited by G. Stacey, R. H. Burris and H. J. Evans. Chapman and Hall, New York, USA.

Böhme, H. (1998). Regulation of nitrogen fixation in heterocyst forming cyanobacteria. *Trends Plant Sci.* **3**, 346-351.

Boison, G., Bothe, H., Hansel, A. and Lindblad, P. (1999). Evidence against a common use of the diaphorase subunits by the bidirectional hydrogenase and by the respiratory complex I in cyanobacteria. FEMS *Microbiol. Lett.* **174**, 159-165.

Boison, G., Schmitz, O., Mikheeva,, L. Shestakov, S. and Bothe, H. (1996). Cloning, molecular analysis and insertional mutagenesis of the bidirectional hydrogenase genes from the cyanobacterium *Anacystis nidulans*. FEBS *Lett.* **394**, 153-158.

Boison, G., Schmitz, O., Schmitz, B. and Bothe, H. (1998). Unusual gene arrangement of the bidirectional hydrogenase and functional analysis of its diaphorase subunit HoxU in respiration of the unicellular cyanobacterium *Anacystis nidulans Curr. Microbiol.* **36**, 253-258.

Bothe, H., Kentemich, T. and Heping, D. (1991). Recent aspects on the hydrogenase-nitrogenase relationship in cyanobacteria. In: Polsinelli M, Materassi R, Vincenzini M (eds) Nitrogen fixation, Kluwer Academic Publishers, Dordrecht, pp. 367-375.

Burris R.H. (1991). Nitrogenases. *J. Biol. Chem.* **266**, 9339-9342.

Burris R.H. and Roberts, G.P. (1993). Biological nitrogen fixation. *Annu. Rev. Nutr.* **13**, 317-335.

Carrasco C.D. and Golden J.W. (1995). Two heterocyst-specific DNA rearrangments of *nif* operons in *Anabaena cylindrica* and *Nostoc* sp. strain Mac. *Microbiol.* **141**, 2479-2487.

Carrasco, C.D., Buettner, J.A. and Golden J.W. (1995). Programed DNA rearrangment of a cyanobacterial *hup*L gene in heterocysts. *Proc. Natl. Acad. Sci.* (USA) 92, 791-795.

Carrasco, C.D., Ramaswamy, K.S., Ramasubramanian, T.S. and Golden J.W. (1994). *Anabaena xis*F gene encodes a developmentally regulated site-specific recombinase. *Genes Develop.* **8**, 74-83.

Daday, A. and Smith, G.D. (1987). The hydrogenase-nitrogenase relationship in a symbiotic cyanobacterium isolated from *Macrozamia communis* L. Johnson. *Aust. J. Plant Physiol.* **14,**: 319-324.

Eisbrenner, G., Distler, E., Floener, L. and Bothe, H. (1978). The occurrence of the hydrogenase in some blue-green algae. *Arch. Microbiol.* **118**, 177-184.

Eisbrenner, G., Roos, P. and Bothe, H. (1981). The number of hydrogenases in cyanobacteria. *J. Gen. Microbiol.* **125**, 383-390.

Ewart, G.D., Reed, K.C. and Smith, G.D. (1990). Soluble hydrogenase of *Anabaena cylindrica*. Cloning and sequencing of a potential gene encoding the tritium exchange subunit. *Eur. J. Biochem.* **187**, 215-223.

Famiglietti, M., Hochkoeppler, A. and Luisi, P.L. (1993). Surfactant-induced hydrogen production in cyanobacteria. *Biotechnol. Bioeng.* **42**, 1014-1018.

Fay, P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol. Rev.* **56**, 340-373.

Flores, E. and Herrero, A. (1994). Molecular evolution and taxonomy of the cyanobacteria. *In* The molecular biology of cyanobacteria, pp. 487-517. Edited by D.A. Bryant. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Fredriksson, C. and Bergman, B. (1997). Ultrastructural characterisation of cells specialized for nitrogen fixation in a non-heterocystous cyanobacterium *Trichodesmium* spp. *Protoplasma* **197**, 76-85.

Fredrich, T. and Weiss, H. (1997). Modular evolution of the respiratory NADH:ubiquinone oxidoreductase and the origin of its modules. *J. Theor. Biol.* **187**: 529-540.

Gallon, J.R. (1992). Reconciling the incompatible: N_2 fixation and O_2. *New Phytol.* **122**, 571-609.

Golden, J.W., Carrasco, C.D., Mulligan, M.E., Schneider G.J. and Haselkorn, R. (1988). Deletion of a 55-kilobase-pair DNA element from the chromosome during heterocyst differentiation of *Anabaena.* sp. strain PCC 7120. *J. Bacteriol.* **170**, 5034-5041.

Golden J.W., Mulligan M.E. and Haselkorn, R. (1987). Different recombination site specificity of two developmentally regulated genome rearrangements. *Nature* **327**, 526-529.

Golden J.W., Robinson S.J. and Haselkorn R. (1985). Rearrangement of nitrogen-fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena. Nature* **314**, 419-423.

Gubili, J. and Borthakur, D. (1996). The use of a PCR cloning and screening strategy to identify lambda clones containing the *hupB* gene of *Anabaena* sp. strain PCC 7120. *J. Microbiol. Meth.* **27**, 175-182.

Gubili, J. and Borthakur, D. (1998). Organization of the *hupDEAB* genes within the hydrogenase gene cluster of *Anabaena* sp. strain PCC 73102. *J. Appl. Phycol.* **10**, 163-167.

Hallenbeck, P.C. and Benemann, J.R. (1978). Characterization and partial purification of the reversible hydrogenase of *Anabaena cylindrica.* FEBS *Lett.* **94**: 261-264.

Hansel, A. and Lindblad, P. (1998). Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. *Appl. Microbiol. Biotechnol.* **50**, 153-160.

Hansel, A., Oxelfelt, F. and Lindblad, P. (1998) Construction of transconjugable plasmids for use in the insertion mutagenesis of *Nostoc* PCC 73102 uptake hydrogenase. In: Biohydrogen. Pages 209-218. Editor: Zaborsky, O.R. Plenum Press, New York, USA. ISBN 0-306-46057-2.

Haselkorn R. and Buikema, W.J. (1992). Nitrogen fixation in cyanobacteria. *In* Biological nitrogen fixation, pp. 166-190. Edited by G. Stacey, R. H. Burris, and H. J. Evans. Chapman & Hall, London, Great Britain.

Houchins, J.P. (1984). The physiology and biochemistry of hydrogen metabolism in cyanobacteria. *Biochim. Biophys. Acta* **768**, 227-255.

Houchins, J.P. and Burris, R.H. (1981a). Occurrence and localization of two distinct hydrogenases in the heterocystous cyanobacterium *Anabaena* sp. strain 7120. *J. Bacteriol.* **146**, 209-214.

Houchins, J.P. and Burris, R.H. (1981b). Comparative characterization of two distinct hydrogenases from *Anabaena* sp. strain 7120. *J. Bacteriol.* **146**, 215-221.

Howitt, C.A. and Vermaas, W.F.J. (1999). Subunits of the NAD(P)-reducing nickel-containing hydrogenase do not act as part of the type-1 NAD(P)H-dehydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803. *In* The Phototrophic Prokaryotes pp. 595-601. Edited by Peschek, G.A. Kluwer Academic/Plenum Publishers, New York, USA.

Kentemich, T., Bahnweg, M., Mayer, F. and Bothe, H. (1989). Localization of the reversible hydrogenase in cyanobacteria. *Z. Naturforsch.* **44c**, 384-391.

Kentemich, T., Danneberg, G., Hundeshagen, B. and Bothe, H. (1988). Evidence for the occurrence of the alternative, vanadium-containing nitrogenase in the cyanobacterium *Anabaena variabilis*. FEMS *Microbiol. Lett.* **51**, 19-24.

Kentemich, T., Haverkamp, G. and Bothe, H. (1991). The expression of a third nitrogenase in the cyanobacterium *Anabaena variabilis*. *Z. Naturforsch.* **46c**, 217-222.

Kumazawa, S. and Asakawa, H. (1995). Simultaneous production of H_2 and O_2 in closed vessels by marine cyanobacterium *Anabaena* sp. TU37-1 under high-cell-density conditions. *Biotechnol. Bioeng.* **46**, 396-398.

Lambert, G.R. and Smith, G.D. (1981). The hydrogen metabolism of cyanobacteria (blue-green algae). *Biol. Rev.* **56**, 589-660.

Lichtl, R.R., Bazin, M.J. and Hall, D.O. (1997). The biotechnology of hydrogen production by *Nostoc flagelliforme* grown under chemostat conditions. *Appl. Microbiol. Biotechnol.* **47**, 701-707.

Lindblad, P. (1999). Cyanobacterial H_2-metabolism: Knowledge and potential/strategies for a photobiological production of H_2. *Biotechn. Aplicada* **16**, 141-144.

Lindblad, P. and Sellstedt, A. (1990). Occurrence and localization of an uptake hydrogenase in the filamentous heterocystous cyanobaterium *Nostoc* PCC 73102. *Protoplasma* **159**, 9-15.

Lindblad, P., Hansel, A., Oxelfelt, F., Tamagnini, P. and Troshina, O. (1998). *Nostoc* sp. strain PCC 73102 and H_2: Knowledge, research, and biotechnological challenges. In: Zaborsky, O.R. (ed) Biohydrogen, Plenum Press, New York, USA, pp. 53-63.

Llama, M.J., Serra, J.L., Rao, K.K. and Hall, D.O. (1979). Isolation and characterization of the hydrogenase activity from the non-heterocystous cyanobacterium *Spirulina maxima*. FEBS *Lett.* **98**, 342-346.

Markov, S.A., Bazin, M.J. and Hall, D.O. (1995). The potential of using cyanobacteria in photobioreactors for hydrogen production. *In* Advances in biochemical engineering biotechnology vol. 52, pp. 59-86. Edited by A. Fiechter. Springer-Verlag, Berlin, Germany.

Masepohl, B., Schoelisch, K., Goerlitz, K., Kutzki, C. and Böhme, H. (1997). The heterocyst-specific *fdxH* gene product of the cyanobacterium *Anabaena* sp. PCC 7120 is important but not essential for nitrogen fixation. *Mol. Gen. Genetics* **253**, 770-776.

Massanz, C., Schmidt, S. and Friedrich, B. (1998). Subforms and in vitro reconstitution of the NAD-reducing hydrogenase of *Alcaligenes eutrophus*. *J. Bacteriol.* **180**, 1023-1029.

Matthijs van der Staay, G.W.M. and Mur, L.R. (1994). Prochlorophytes: the other cyanobacteria? *In* The molecular biology of cyanobacteria, pp. 49-64. Edited by D.A. Bryant. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Matveyev, A.V., Rutgers, E., Söderbäck, E. and Bergman, B. (1994). A novel rearrangement involved in heterocyst differentiation of the cyanobacterium *Anabaena* sp. PCC7120. FEMS *Microbiol. Lett.* **116**, 201-208.

Meeks J.C., Campbell E.L. and Bisen P.S. (1994). Elements interrupting nitrogen fixation genes in cyanobacteria: presence and absence of a *nifD* element in clones of *Nostoc* sp. strain Mac. *Microbiol.* **140**, 3225-3232.

Menon, A.L. and Robson, R.L. (1994). In vivo and in vitro nickel-dependent processing of the [NiFe] hydrogenase in *Azotobacter vinelandii*. *J. Bacteriol.* **176**, 291-295.

Mikheeva, L.E., Schmitz, O., Shestakov, S.V. and Bothe, H. (1995). Mutants of the cyanobacterium *Anabaena variabilis* altered in hydrogenase activities. *Z. Naturforsch.* 50c, 505-510.

Mulligan, M.E., Buikema, W.J. and Haselkorn, R. (1988). Bacterial-type ferredoxin genes in the nitrogen fixation regions of the cyanobacterium *Anabaena* sp. strain PCC 7120 and *Rhizobium meliloti*. *J. Bacteriol.* **170**, 4406-4410.

Mulligan M.E. and Haselkorn R. (1989). Nitrogen-fixation (*nif*) genes of the cyanobacterium *Anabaena* sp. strain PCC 7120: the *nifB-fdxN-nifS-nifU* operon. *J. Biol. Chem.* **26**, 19200-19207.

Nakamura, Y., Kaneko, T., Hirosawa, M., Miyajima, N. and Tabata, S. (1998). CyanoBase, a www database containing the complete genome of *Synechocystis* strain PCC 6803. *Nucleic Acids Res.* **20**, 63-67.

Orme-Johnson, W.H. (1992). Nitrogenase structure: where to now. *Science* **257**, 1639-1640.

Oxelfelt, F., Tamagnini, P., Salema, R. and Lindblad, P. (1995). Hydrogen uptake in *Nostoc* strain PCC 73102: effects of nickel, hydrogen, carbon and nitrogen. *Plant Physiol. Biochem.* **33**, 617-623.

Oxelfelt, F., Tamagnini, P. and Lindblad, P. (1998). Hydrogen uptake in *Nostoc* sp. strain PCC 73102. Cloning and characterization of a *hupSL* homologue. *Arch. Microbiol.* **169**, 267-274.

Papen, H., Kentemich, T., Schmülling, T. and Bothe, H. (1986). Hydrogenase activities in cyanobacteria. *Biochimie* **68**, 121-132.

Peschek, G.A. (1979a). Aerobic hydrogenase activity in *Anacystis nidulans*. The oxyhydrogen reaction. *Biochim. Biophys. Acta* **548**, 203-215.

Peschek, G.A. (1979b). Evidence for two functionally distinct hydrogenases in *Anacystis nidulans*. *Arch. Microbiol.* **123**, 81-92.

Peterson, R.B. and Wolk, C.P. (1978). Localization of an uptake hydrogenases in *Anabaena*. *Plant Physiol.* **61**, 688-691.

Postgate, J. (1987). Nitrogen fixation. 2nd edition. New studies in biology. Edward Arnold Publishers, London, Great Britain.

Rai, A.N., Borthakur, M., Söderbäck, E. and Bergman, B. (1992). Immunogold localization of hydrogenases in the cyanobacterial-plant symbioses *Peltigera canina*, *Anthoceros punctatus*, and *Gunnera magellanica*. *Symbiosis* **12**, 131-144.

Ramaswamy, K.S. Carrasco, C.D., Fatma, T. and Golden, J.W. (1997). Cell-type specificity of the *Anabaena fdxN* element rearrangement requires *xisH* and *xisI*. *Mol. Microbiol.* **23**, 1241-1249.

Rao, K.K. and Hall, D.O. (1996). Hydrogen production by cyanobacteria: potential, problems and prospects. *J. Mar. Biotechnol.* **4**, 10-15.

Reddy, P.M., Spiller, H., Albrecht, S.L. and Shanmugam, K.T. (1996). Photodissimilation of fructose to H_2 and CO_2 by a dinitrogen-fixing cyanobacterium, *Anabaena variabilis*. *Appl. Environ. Microbiol.* **62**, 1220-1226.

Schmitz, O., Boison, G., Hilscher, R., Hundeshagen, B., Zimmer, W., Lottspeich, F. and Bothe, H. (1995). Molecular biological analysis of a bidirectional hydrogenase from cyanobacteria. *Eur. J. Biochem.* **233**, 266-276.

Schmitz, O. and Bothe, H. (1996a). $NAD(P)^+$-dependent hydrogenase activity in extracts from the cyanobacterium *Anacystis nidulans*. FEMS *Microbiol. Lett.* **135**, 97-101.

Schmitz, O. and Bothe, H. (1996b). The diaphorase subunit HoxU of the bidirectional hydrogenase as electron transferring protein in cyanobacterial respiration? *Naturwissenschaften* **83**, 525-527.

Schulz, R. (1996). Hydrogenases and hydrogen production in eukaryotic organisms and cyanobacteria. *J. Mar. Biotechnol.* **4**, 16-22.

Schulz, R., Appel, J., Phunpruch, K., Stangier, K. and Wünschiers, R. (1998). Biotechnological approach for future use of solar energy: photohydrogen production by the green algae *Scenedesmus obliquus* and the cyanobacterium *Synechocystis* sp. PCC 6803. *In* Hydrogen Energy progress XXI, vol. 3, pp. 2069-2078. Edited by J.C. Bolcich and T.N. Veziroglu. Asociacion argentina del hidrogenio, Buenos Aires, Argentina.

Schwartz, E., Gerischer, U. and Friedrich, B. (1998). Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. *J. Bacteriol.* **180**, 3197-3204.

Serebriakova, L., Zorin, N.A. and Lindblad P. (1994). Reversible hydrogenase in *Anabaena variabilis* ATCC 29413, Presence and localization in non- N_2-fixing cells. *Arch. Microbiol.* 161, 140-144.

Serebryakova, L.T., Medina, M., Zorin, N.A., Gogotov, I.N. and Cammack, R. (1996). Reversible hydrogenase of *Anabaena variabilis* ATCC 29413: catalytic properties and characterization of redox centres. FEBS *Lett.* **383**, 79-82.

Serebryakova, L.T., Sheremetieva, M. and Tsygankov, A.A. (1998). Reversible hydrogenase activity of *Gloeocapsa alpicola* in continuous culture. *FEMS Microbiol. Lett.* **166**, 89-94.

Shravan Kumar, C. and Polasa, H. (1991). Infuence of nickel and copper on photobiological hydrogen production and uptake in *Oscillatoria subbrevis* strain 111. *Proc. Indian Natn. Sci. Acad.* **B57**, 281-285.

Smith, G.D. (1990). Hydrogen metabolism in cyanobacteria. In: Kumar HD (ed) Phycotalk vol 2, Rastogi and Company, Meerut, India, pp. 131-143.

Stal, L.J. (1995) Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytol.* **131**, 1-32.

Tamagnini, P., Oxelfelt, F., Salema, R. and Lindblad, P. (1995). Immunological characterization of hydrogenases in the nitrogen-fixing cyanobacterium *Nostoc* sp. strain PCC 73102. *Curr. Microbiol.* **31**, 102-107.

Tamagnini, P., Oxelfelt, F., Salema, R. and Lindblad, P. (1997). Hydrogenases in *Nostoc* sp. strain PCC 73102, a strain lacking a bidirectional enzyme. *Appl. Environ. Microbiol.* **63**, 1801-1807.

Tamagnini, P., Costa, J.-L., Almeida, L., Oliveira, M.-J., Salema, R. and Lindblad, P. (2000). Diversity of cyanobacterial hydrogenases, a molecular approach. *Curr. Microbiol.* (in press).

Thiel, T. (1993). Characterization of genes for an alternative nitrogenase in the cyanobacterium *Anabaena variabilis*. *J. Bacteriol.* **175**, 6276-6286.

Thiel, T., Lyons, E.M., Erker, J.C. and Ernst, A. (1995). A second nitrogenase in vegetative cells of a heterocyst forming cyanobacterium. *Proc. Natl. Acad. Sci.* (USA) **92**. 9358-9362.

Thiel, T., Lyons, E.M. and Erker, J.C. (1997). Characterization of genes for a second Mo-dependent nitrogenase in the cyanobacterium *Anabaena variabilis*. *J. Bacteriol.* **179**, 5222-5225.

Tran-Betcke, A., Warnecke, U., Böcker, C., Zaborosch, C. and Friedrich, B. (1990). Cloning and nucleotide sequence of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. *J. Bacteriol.* **172**, 2920-2929.

Troshina, O.Y., Serebryakova, L.T. and Lindblad, P. (1996). Induction of H_2-uptake and nitrogenase activities in the cyanobacterium *Anabaena variabilis* ATCC 29413: effects of hydrogen and organic substrate. *Curr. Microbiol.* **33**, 11-15.

Tsygankov, A.A., Serebryakova, L.T., Rao, K.K. and Hall, D.O. (1998a). Acetylene reduction and hydrogen photoproduction by wild-type and mutant strains of *Anabaena* at different CO_2 and O_2 concentrations. FEMS *Microbiol. Lett.* **167**, 13-17.

Tsygankov, A.A., Serebryakova, L.T., Sveshnikov, D.A., Rao, K.K., Gogotov, I.N. and Hall, D.O. (1998b). Hydrogen photoproduction by three different nitrogenases in whole cells of *Anabaena variabilis* and the depedence on pH. *Int. J. Hydrogen Energy* **22**, 859-867.

Wolk, C.P. (1996). Heterocyst formation. *Annu. Rev. Genet.* **30**, 59-78.

Wolk, C.P., Ernst, A. and Elhai, J. (1994). Heterocyst metabolism and development *In* The molecular biology of cyanobacteria, pp. 769-823. Edited by D. A. Bryant. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Wu, L.-F. and Mandrand, M.A. (1993). Microbial hydrogenases: primary structure, classification, signatures and phylogeny. FEMS *Microbiol. Reviews* **104**, 243-270.

Xiankong, Z., Tabita, F. and Van Baalen, C. (1984). Nickel control of hydrogen production and uptake in *Anabaena* spp. strains CA and 1F. *J. Gen. Microbiol.* **130**, 1815-1818.

This Page Intentionally Left Blank

INTEGRATION OF HYDROGEN EVOLVING SYSTEMS WITH CELLULAR METABOLISM: THE MOLECULAR BIOLOGY AND BIOCHEMISTRY OF ELECTRON TRANSPORT FACTORS AND ASSOCIATED REDUCTASES

Patrick C. Hallenbeck

Department of Microbiology and Immunology, University of Montreal
Tel: 514 343 6278, E-mail: patrick.hallenbeck@umontreal.ca

ABSTRACT

The molecular machinery responsible for hydrogen evolution in a variety of organisms is reviewed. Hydrogen evolving enzymes are considered first, nitrogenase, NiFe hydrogenase and Fe hydrogenase. A consideration of their recently described structures shows that a variety of oxygen labile complex metallocomplexes are necessary for proton reduction. The catalytic efficiency of the various enzymes differs considerably (100x). However, at least under some conditions catalytic activity for proton reduction may not be limiting for hydrogen evolution. Instead, other factors may need to be considered; electron transport to the hydrogenase enzyme, or rates of reductant generation by the cellular machinery. The structure and function of electron transport proteins to hydrogenase are discussed. Finally, various pathways leading to the formation of hydrogen are reviewed with emphasis placed on the potential energy efficiency of these processes and their potential for driving the various hydrogen evolving activities. This forms a basis for discussing possible means for increasing the energy flow to the hydrogen producing processes.

1. INTRODUCTION

Biological hydrogen has been advanced as a potential technology for producing clean energy and has been studied in a number of model and small-scale systems over the last twenty-five years. Despite the many man-years of efforts devoted to this problem, the presently presented rates and efficiencies of hydrogen production by any system fall far

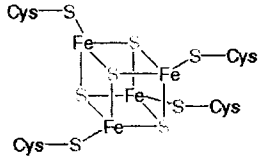
short of economic feasibility. During the same time period, much has been learned about the molecular biology and biochemistry of the hydrogen producing enzymes, reductant generating systems, and attendant electron transfer factors, as well as the physiology of many hydrogen producing organisms. Some of the relevant details are herein reviewed in the hopes of identifying potentially limiting factors and therefore indicating directions for future research aimed at increasing production rates and conversion efficiencies to economically feasible levels.

2. HYDROGEN PRODUCING ENZYMES

Hydrogen producing enzymes catalyze what is arguably the simplest chemical reaction: $2H^+ + 2e^- \leftrightarrow H_2$. However, a survey of all presently known enzymes capable of hydrogen evolution shows that they contain complex metallo-clusters as active sites and that the active enzyme units are synthesized in a complex processes involving auxiliary enzymes and protein maturation steps. At present three enzymes are known; nitrogenase, Fe-hydrogenase, and NiFe hydrogenase.

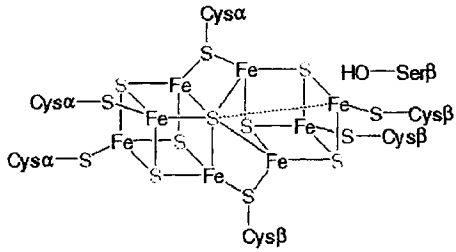
Nitrogenase

Nitrogenase is a two component protein system that uses MgATP (2ATP/e^-) and low potential electrons derived from reduced ferredoxin or flavodoxin to reduce a variety of substrates. In the absence of other substrates, nitrogenase continues to turnover, reducing protons to hydrogen. The smaller of the two proteins, Fe-protein, contains a [4Fe-4S] cluster and acts as a specific reductase of the larger protein MoFe-protein. The [4Fe-4S] cluster is unique in that it bridges the two subunits of the Fe-protein.

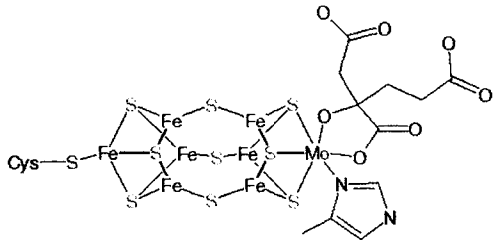


The [4Fe-4S] cluster found in the nitrogenase Fe-protein component and ferredoxin.

At each cycle, MgATP complexed Fe-protein associates with MoFe-protein, 2ATP are hydrolyzed with the transfer of one electron to MoFe-protein and the complex dissociates. Turnover is extremely slow, 6.4 s^{-1} (estimated from in vitro rapid kinetic measurements), necessitating the biosynthesis of enormous quantities of the two proteins. Electrons are presumably accumulated in the P-center before transfer to the FeMo cofactor where substrate reduction is thought to occur.



The P-cluster of the nitrogenase MoFe-protein component



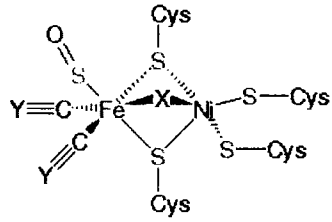
The MoFe cofactor of the nitrogenase MoFe-protein component

Obviously, biosynthesis of these complex metal centers requires a great deal of additional enzymatic machinery, energy, and time. Probably at least an additional 20 gene products are necessary for cofactor synthesis and insertion as well as metal metabolism (transport and activation). Alternative nitrogenases exist, but given their lower catalytic activity and even lower stability they are even poorer candidates for a hydrogen evolution system. Thus, considering the low turnover number, the considerable energy inputs necessary for biosynthesis and the requirement for ATP for catalysis, nitrogenase does not appear to be a very cost-effective way to produce hydrogen. Indeed, because of the energy burden, nitrogenase synthesis and activity is tightly regulated with respect to fixed nitrogen. Despite these considerations, nitrogenase activity (i.e. its potential for hydrogen evolution), at least in some cultures (1), does not appear to be limited by the amounts catalytically active protein but rather by reductant or possibly ATP which as discussed is required in large amounts. Thus, there is potential for increasing activity by understanding the factors that limit catalysis.

NiFe and NiFeSe Hydrogenases

Many organisms have been shown to contain a NiFe or NiFeSe hydrogenase which is usually thought of as functioning as an "uptake" hydrogenase, that is a hydrogenase whose normal metabolic function is to derive reductant from H_2 (although see the FHL complex below). Electrons derived from hydrogen are used, either directly, or indirectly through the quinone pool, to reduce NAD(P). The NiFe hydrogenases are heterodimeric

proteins consisting of both small (S) and large (L) subunits. The small subunit contains three iron-sulfur clusters, two [4Fe-4S] and one [3Fe-4S]. The large subunit contains a unique, complex nickel iron center:

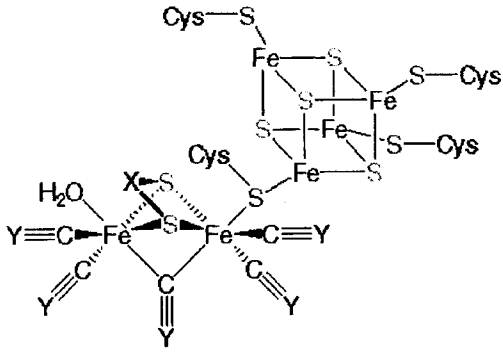


The NiFe center of one class of hydrogenases (2,3)

Fourier transform infrared spectroscopy as well as chemical analysis has been used to establish that the Fe in this center is coordinated to 2 CN and one CO, forming a biologically unique metallocenter (4). Obviously, the synthesis of NiFe hydrogenase is a highly complicated process requiring a number of accessory gene products that are required for metal (nickel and iron) capture, synthesis (CO and CN^-) as well as cluster insertion and protein maturation (proteolytic cleavage at the C-terminus of the L subunit). Activities in the "uptake" direction are usually in the order of 300-400 μmol min^{-1} mg^{-1} and rates of hydrogen evolution by the purified enzyme are lower, 65 μmol min^{-1} mg^{-1} (5), which corresponds to a turnover rate of 98 s^{-1}. Thus even working in reverse this class of hydrogenases is a better catalyst for hydrogen evolution than nitrogenase.

Fe Hydrogenase

Hydrogen evolving enzymes have been known for over 30 years following the isolation of hydrogenase from *Clostridium pasteurianum.* Both homodimeric soluble cytoplasmic (*C. pasteurianum* and *Megasphaera elsdenii*) and periplasmic heterodimeric (*Desulfovibrio* spp) Fe hydrogenases have been described. In general the cytoplasmic enzyme functions to remove excess reducing equivalents during fermentations carried out by strict anaerobic bacteria and the periplasmic enzyme function normally in hydrogen oxidation. Correspondingly, they interact with different intermediate electron carriers; ferredoxin in the case of the cytoplasmic enzyme and cytochrome c3 in the case of the periplasmic enzyme from *Desulfovibrio* spp. These enzymes have extremely high turnover numbers; 6,000 s^{-1} (*C. pasteurianum*), 9,000 s^{-1} (*Desulfovibrio* spp) (**Note:** this is 10^3 higher than the turnover number of nitrogenase!) and contain a unique complex Fe-S center in which one of the Fe atoms is complexed with CO and CN (6,7):



The H-cluster of the Fe hydrogenase from *C. pasteurianum*

The highly reactive nature of this cluster together with the proposed formation of an iron-hydride intermediate during proton reduction (6) may make the search for an oxygen stable hydrogenase a rather elusive goal.

3. ELECTRON CARRIERS

Typically, hydrogen evolving enzymes, which for the most part appear to be soluble, are connected to cellular reductant generating processes via soluble, low molecular weight electron carriers capable of operating at low potentials, approximately that of the hydrogen electrode. Two types of carriers have been extensively studied; ferredoxin, containing Fe-S center(s) and flavodoxin, containing FMN (flavin mononucleotide).

Ferredoxin

Perhaps the best studied ferredoxin is the so called "bacterial" type ferredoxin containing 2 [4Fe-4S] clusters and normally coupling the reductant generated during fermentation with either nitrogen fixation or hydrogen evolution:

Structure of a 2 [4Fe-4S] ferredoxin

Flavodoxin

In some cases flavodoxins are expressed under iron limiting conditions to compensate for the lack of functional ferredoxin. In other cases, for example NifF, flavodoxins are expressed as a necessary component of a specific pathway. The low molecular weight (~ 20kDa) proteins contain FMN which theoretically has three oxidation states available, oxidized, the 1 e^- reduced semiquinone, and the 2 e^- reduced hydroquinone. In practice, these proteins are thought to function in hydrogen metabolism or nitrogen fixation by cycling between the semiquinone and hydroquinone states.

Three dimensional structure of a long chain flavodoxin (*Anabaena*)

Relatively little is known about the electron transfer rates of electron carriers in driving hydrogen evolution processes. Reduction of ferredoxin by PFOR (see below) may be rather sluggish (~40 s^{-1}, (8)). In some cases multiple electron carriers may be involved, but this has not been well studied. In the case of NifF and FdI of *R. capsulatus*, a rapid kinetic study showed that: 1) there was no evidence for transfer between the two electron carriers, 2) NifF formed a tight complex with the nitrogenase Fe-protein, and 3) FdI even though capable of reducing nitrogenase, did not form a tight complex with Fe-protein (9). It was concluded that NifF and FdI act in vivo in parallel to reduce Fe-protein and not in series. However, it is not possible from these studies to determine in the in vivo concentration of electron carriers is limiting for nitrogenase activity.

4. REDUCTANT GENERATING MECHANISMS

Hydrogen Production by Degradation of Carbon Substrates

For the purposes of discussion, we will examine the maximum hydrogen obtainable from carbon substrates (sugars) by currently known biological mechanisms by regarding the fate of pyruvate. In fact, in general most fermentations proceed by conversion of the substrate to pyruvate, generating various amounts of NADH and ATP depending upon the substrate oxidation state and the pathway involved. Pyruvate can be further catabolized by two different enzyme systems that can both ultimately fuel hydrogen evolution:

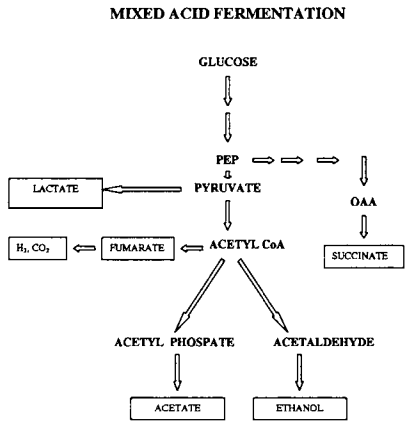
1. Pyruvate:formate lyase (PFL)

$$\text{Pyruvate} + \text{CoA} \xrightarrow[\text{PFL}]{} \text{acetyl-CoA} + \text{formate}$$

Formate can then, under the appropriate conditions, be further metabolized to CO_2 and H_2 by a formate hydrogen lyase complex (enteric bacteria). The acetyl-CoA can be used to produce ATP and acetate. Therefore under ideal (theoretical) conditions:

$$1\ \text{Glucose} \longrightarrow 2\ \text{pyruvate} \longrightarrow 2H_2 + 2CO_2 + 2\text{ATP}$$
$$\searrow\ 2\text{NADH} + 2\text{ATP}$$

Of course in practice in fermentations such as the mixed acid fermentation carried out by enteric bacteria, acetate and ATP production are less due to the need to balance the fermentation by regenerating NAD through the reduction of acetyl-CoA to ethanol thus providing necessary substrate (NAD) required for further sugar degradation.



As well, actual yields of hydrogen are reduced by hydrogen recycling as an uptake hydrogenase consumes a portion of the hydrogen produced generating NADH (used to increase yields of ethanol) and potentially a proton gradient across the membrane. This can be shown using strains that are specifically defective in one of the hydrogenase isozymes (unpublished observations). Interestingly, organisms which possess PFL as the major enzyme for pyruvate degradation also contain the second pathway PFOR, discussed below) as a minor constituent needed for activation of PFL (formation of the active center glycyl-free radical) under anaerobic conditions. The hydrogen-evolving enzyme from this complex has yet to be studied in a purified state. Analysis of the *fhl* genes indicates that the hydrogenase component belongs to the NiFe hydrogenase family.

2. Pyruvate:ferredoxin (flavodoxin) oxidoreductase (PFOR)

Pyruvate + CoA + 2Fd (ox) $\longrightarrow$ acetyl-CoA +CO_2 + 2Fd (red)

The acetyl-CoA is usually further metabolized to produce ATP and the reduced ferredoxin (Fd (red)) is used to drive hydrogen production;

2Fd (red) + $2H^+$ $\longrightarrow$ 2Fd (ox) + H_2

The net effect of these coupled reactions is the same as that described for PFL; a theoretical maximum of $2H_2$ produced per molecule of glucose. Thus the degradation of glucose leads to the production of equimolar amounts of ATP and reduced ferredoxin, a ratio insufficient to efficiently drive nitrogenase. A variety of organisms contain PFOR, in fact it is the enzyme responsible for providing the reducing power for H_2 evolution in the vast majority of organisms carrying out this reaction coupled to sugar degradation including eucaryotes. The enzyme has different subunit structures depending upon the organism from which it has been isolated. Thus both homodimeric and hetero subunit enzymes are known. Sequence analysis of the various enzymes shows however that they

contain common motifs including a TPP (thiamin pyrophosphate) binding site and an unusual (unique) Cys motif that directs the chelation of a [4Fe-4S] cluster near the active site. The three-dimensional structure of a homodimeric PFOR has recently been solved (10) and gives insight into the mode of action of this enzyme. We can propose that TPP and the unique [4Fe-4S] cluster participate in the sequential abstraction of 2 electrons from pyruvate with the transfer of the first electron apparently generating a free-radical intermediate (11, 12). The other two clusters are organized such that they can serve as a "wire" conducting electrons to the surface to a docked ferredoxin or flavodoxin. This hypothesis, derived from structural considerations, is corroborated by stopped-flow kinetic data, which indicates that reduction of substrates requires that the enzyme act as a "wire" rather than a "battery" (i.e. accumulating electrons for later discharge to electron carriers (unpublished)). PFOR is capable of generating very low potential electrons (the midpoint of the proximal [4Fe-4s] cluster is –540 mV (13)) which are well below that required for hydrogen evolution. PFOR isolated from *Desulfovibrio africanus* has a turnover number of 290 s^{-1} (per 2 e^-) (13). It is perhaps noteworthy in this regard that under some conditions PFOR (from *Clostridium thermoaceticum*) itself evolves hydrogen, albeit at low rates, 0.54 s^{-1}, compared with its rate of ferredoxin (flavodoxin) reduction (14).

Attempts to increase hydrogen evolution by these systems could include: 1) genetic manipulation to increase expression of *fhl* which is usually induced merely to relieve acid stress, 2) development of conditions which permit high rates of metabolism without requiring growth and its attendant demands on metabolically derived reductant and energy, 3) elimination of the hydrogen uptake activity, or actually reversing the normal flow of electrons such that H_2 could be evolved from catabolically generated NADH, 4) schemes for abstracting the protons contained in the acetyl-CoA; for example generation of pyruvate through a co-fermentation process.

Reductant Generation in the Photosynthetic Bacteria

Photosynthetic bacteria have long been studied for their capacity to produce hydrogen through the action of their nitrogenase system. These bacteria display some of the highest rates of in vivo nitrogenase activity known, yet how reductant is supplied to nitrogenase has remained unclear. Rates are considerably higher in the light, yet their photosystem does not generate reducing power of a sufficiently low potential to directly drive nitrogenase. It has been suggested that in vivo nitrogenase is not limited by the amount of active nitrogenase protein present but rather by supply of reductant (1). *Rhodobacter capsulatus* has been shown to possess both a *nif* specific ferredoxin (15) and a *nifF* (16). In addition, *R. capsulatus* contains a set of *nif* specific genes, *rnf* (17, 18), which are required for in vivo nitrogenase activity, at least under photoheterotrophic conditions (*rnf* mutants can grow on N_2 microaerobically (unpublished)). These genes code for a putative membrane complex with proteins possessing motifs capable of binding [4Fe-4S] clusters and FMN, and potentially function in generating reductant for nitrogenase through reverse electron transport. The energy to drive this process is presumably obtained from an ion gradient. This complex has yet to be studied on a biochemical level, so nothing is known about its mode of action or relative catalytic

efficiency. In addition, *R. capsulatus* has been shown to possess a PFOR capable of reducing nitrogenase in vitro with either FdI or NifF (12). It is more effective at reducing NifF, which, taken together with the results of a comparative rapid kinetic study (9), suggests that PFOR and NifF form a high affinity, low capacity system for reducing nitrogenase whereas RNF and FdI form a low affinity, high capacity system.

Reductant Generation in Cyanobacteria

Hydrogen production by cyanobacteria, first demonstrated 25 years ago (19), has been extensively studied since. In this system, hydrogen evolution is by nitrogenase contained in the heterocyst, ~10% of the total cells, so in addition to the limitations to the nitrogenase system discussed above must be added the additional energy costs involved in heterocyst differentiation and maintenance. Despite the number of studies on this system, it is still unclear as to what the immediate electron donor to nitrogenase is and how it in turn is reduced. Heterocysts have been show to contain a heterocyst-specific [2Fe-2S] plant-type ferredoxin (FdH), however this ferredoxin is not essential for nitrogen fixation (21). Likely reductant generating mechanisms could include direct light driven reduction FdH. However, recent results, in which *zwf* (glucose-6-phosphate dehydrogenase) was inactivated demonstrated unequivocally that the major source of reductant to nitrogenase is derived by dark sugar metabolism in which the oxidative pentose pathway plays an essential role (22).

REFERENCES

1. Yakunin, A. F., Laurinavichene, T. V., Tsygankov, A. A., and Hallenbeck, P. C. (1999). *J. Bacteriol.* **181**, 1994-2000.
2. Volbeda, A., Charon, M.H., Piras, C., Hatchikan, E.C., Frey, M., and Fontecilla-Camps, J. C. (1995). *Nature* **373**, 580-587.
3. Higuchi, Y., Yagi, T., and Yasouka, N. (1997). *Structure* **5**, 1671-1680.
4. Pierik, A. J., Roseboom, W, Happe, R. P., Bagley, K. A., and Albracht, S. P. J. (1999) *J. Biol. Chem.* **274**, 3331-3337.
5. Rousset, M., Montet, Y., Guigliarelli, B., Forget, N., Asso, M., Bertrand, P., Fonteccilla-Camps, J. C., and Hatchikian, E. C. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 11625-11630.
6. Peters, J. W., Lanzilotta, W. N., Lemon, B. J., and Seefeldt, L. C. (1998). *Science* **282**, 1853-1858.
7. Pierik, A. J., Hulstein, M., Hagen, W. R., and Albracht, S. P. J. (1998). *Eur. J. biochem.* **258**, 572-578.
8. Moulis, J.-M., and Davasse, V. (1995) *Biochemistry*, **34**, 16781-16788.
9. Hallenbeck, P. C. and Gennaro, G. (1998) *Biochim. Biophys. Acta* **1365**, 433-442
10. Chabriere, E., Charon, M. H., Volbeda, A., Pieulle, L., Hatchikian, E. C., Fontecilla-Camps, J. C. (1999). *Nat. Struct. Biol.* **6**, 182-190.
11. Menon, S. and Ragsdale, S.W. (1997) *Biochemistry* **36**, 8484-8494.
12. Yakunin, A. F. and Hallenbeck, P. C. (1998). *Biochim. Biophys. Acta* **1409**, 39-49.

13. Pieulle, L., Guigliarelli, B., Asso, M., Dole, F., Bernadac, A., and Hatchikian, E. C. (1995). *Biochim. Biophys. Acta* **1250**, 49-59.
14. Menon, S. and Ragsdale, S. W. (1996). *Biochemistry* **35**, 15814-15821.
15. Hallenbeck, P. C., Jouanneau, Y., and Vignais, P. M. (1982). **1365**, 435-442.
16. Yakunin, A. F., Gennaro, G., and Hallenbeck, P. C. (1993). *J. Bacteriol.* **175**, 6775-6780.
17. Schmehl, M., Jahn, A., Meyer zu Vilsendorf, A., Hennecke, S., Masepohl, B., Schuppler, M., Marxer, M., Oelze, J., and Klipp, W. (1993) . *Mol. Gen. Genet.* **241**, 602-615.
18. Kumagai, H., Fujiwara, T., Matsubara, H., and Saeki, K. (1997). *Biochemistry* **36**, 5509-5521.
19. Benemann, J. R. and Weare, N. M. (1974). *Science* **184**, 174.
20. Benemann, J. R., Miyamoto, K., and Hallenbeck, P. C. (1980) .*Enzyme Microbiol. Technol.* **2**, 103.
21. Masepohl, B., Scholisch, K. Gorlitz, Kutzki, C., and Bohme, H. (1997). *Mol. Gen. Genet.* 253, 770-776.
22. Summers, M. L., Wallis, J. G., Campbell, E. L., and Meeks, J. C. (1995). *J. Bacteriol.* **177**, 6184-6194.

This Page Intentionally Left Blank

V. Genetic Engineering

This Page Intentionally Left Blank

SCREENING OF MARINE PHOTOSYNTHETIC MICROORGANISMS AND HYDROGEN PRODUCTION

T. Matsunaga and H. Takeyama

Department of Biotechnology,
Tokyo University of Agriculture and Technology
2-24-16 Naka-Cho, Koganei, Tokyo 184-8588, Japan

1. SUMMARY

In the diverse marine environment, photosynthetic microorganisms that are the largest primary producers of biomass are attracting attention as a new resource. To utilize those organisms, successful screening methods and its applications have to be developed. Here we describe a novel identification system for microorganisms with 16S rDNA-targeted hybridization using bacterial magnetic particles. Further discussed is the novel hydrogen producing photosynthetic bacterium *Rhodovulum sulfidophilum* NKPB160471R, and optimization of hydrogen production through transposon mutagenesis and use of a double phase photobioreactor under microaerobic conditions.

KEY WORDS:

Identification, magnetic capture hybridization, cyanobacteria, photosynthetic bacteria, hydrogen production, double-phase reactor, oxygen effect

2. INTRODUCTION

In the remarkably diverse marine environment, there are many unique species of organisms. One of the largest resources and biomass in the marine environment is the photosynthetic microorganism. We have found many useful chemicals and energy production from those marine photosynthetic microorganisms (Matsunaga and Takeyama, 1998). Successful isolation, manipulation, and optimized use of the novel organism are required for applications. The screening for novel organisms is the first step in a process. However, less than 1% of microbes

are available for study and most are unculturable under artificial growth conditions. Screening for microbes exclusively depends on primary culture followed by single cell isolation, and therefore many unculturable strains remain cryptic (Thornhill, et al., 1995, Ward, et al., 1990). In order to make the screening process complete and efficient, it is necessary to characterize target microbes in the collected samples before enrichment. Molecular approaches are useful to detect certain microbes in the natural environment where 16S rDNA and specific gene for target microbes are used to detection as molecular markers.

Biological production of hydrogen is of worldwide interest and is extensively investigated with photosynthetic bacteria. Effective screening of those bacteria and their characterization is important process. In this paper, we introduce a novel identification method of target microbes with magnetic capture hybridization using 16A rDNA. Analysis of the *nifH* gene is further discussed for application to the detection/identification of photosynthetic bacteria and cyanobacteria. Effective hydrogen production is also demonstrated using a double-phase reactor with addition of small amount of oxygen.

3. DETECTION OF PHOTOSYNTHETIC MICROORGANISMS FOR HYDROGEN PRODUCTION

3.1. 16S rDNA targeted identification of cyanobacteria using magnetic capture hybridization

Magnetic capture hybridization has attracted considerable attention owing to the ease with which specific target DNA may be isolated from a heterogenous sample. This simple method furthermore permits rapid concentration of target DNA from a bulk sample. It would therefore allow sensitive detection of target DNA from environmental samples, blood or other clinical samples.

Bacterial magnetic particles (BMPs) produced intracellularly by magnetic bacteria are ultrafine magnetite crystals (50-100 nm diameters) with regular morphology and covered with a stable lipid bilayer [Matsunaga, et. al., 1987]. Various functional biomaterials such as antibody, enzyme and DNA have demonstrated to conjugated onto the BMP via the appropriate chemical cross-linker reagents and these modified BMPs has been used in novel enzyme immunoassays and DNA recovery system [Matsunaga, et. al., 1996, Matsunaga, et. al., 1999, Tanaka, et. al., 2000, Takeyama, et. al., 2000,]. Here, we demonstrate the identification system with magnetic capture hybridization using bacterial magnetic particles (BMPs).

Genus-specific oligonucleotide probes for detection of *Anabaena* spp., *Microcystis* spp., *Nostoc* spp., *Oscillatoria* spp., and *Synechococcus* spp. were designed from the variable region of the cyanobacterial 16S rDNA of 148 strains. These oligonucleotide probes were immobilized on BMPs via streptavidin-biotin conjugation and employed for magnetic capture hybridization against digoxigenin-labeled cyanobacterial 16S rDNA. BMPs were magnetically concentrated, spotted in 100 μm-size micro-well on MAG-microarray, and the fluorescent detection was performed (Fig. 1).

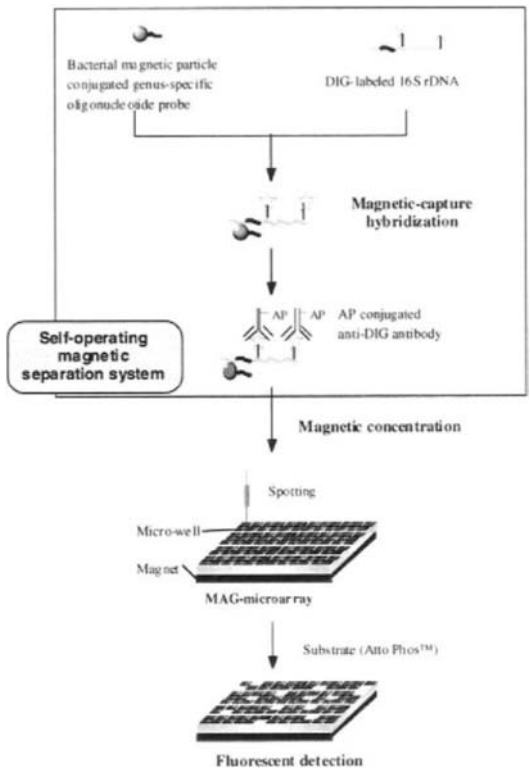


Figure 1: Procedure for detection of cyanobacterial DNA on MAG-microarray using genus-specific oligonucleotide probes conjugated to bacterial magnetic particles.

All procedure for hybridization was carried out by robot. Entire process of hybridization and detection was automatically performed using magnetic separation robot and all five cyanobacterial genera were successfully discriminated on MAG-microarray (Fig.2).

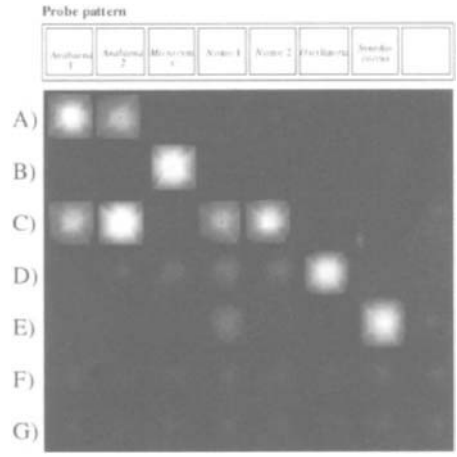


Figure 2: Detection of cyanobacterial DNA on MAG-microarray using BMPs conjugated genus-specific oligonucleotide probes. Oligonucleotide probes used for detection are given as probe pattern. Cyanobacterial strains used for analysis are follows. A: *Anabaena* sp. PCC7120, B: *Microcystis* sp. NIES-98, C: *Nostoc* sp. NKBG038601, D: *Oscillatoria* sp. NKBG091600, E: *Synechococcus* sp. PCC7942, F: *E.coli* K-12, G: *Pseudomonas cepacia* JCM5506

3.2. Identification of marine nitrogen fixing photosynthetic microorganisms capable of producing hydrogen using the nifH gene

The biocatalyst responsible for hydrogen production in these bacteria is the nitrogenase complex. The nitrogenase system is composed of two multiple subunit proteins (MoFe protein, composed of subunits encoded by *nifD* and *nifK* genes, and the Fe protein composed of subunits encoded by *nifH* gene). The amino acid sequence of the Fe protein is very similar amongst organisms, even those from distant taxonomic groups (Ben-Porath and Zehr, 1994). The *nifH* gene have been extensively investigated in various microorganisms such as cyanobacteria (Ben-Porath and Zehr, 1994, Colon-lopez, et al., 1999), archaea (Chien, 1996), gram positives (Normand and Bousquet, 1989), and proteobacteria (Hennecke, et al., 1985).

In order to make the screening process complete and efficient for diazotrophs capable of producing hydrogen, it is necessary to characterize them in the collected samples before enrichment. The PCR techniques such as nested PCR have developed for screening process of diazotrophs through the utilization of *nifH* gene, *vnfG* and *anfG* gene sequence (Loveless and Bishop, 1999).

We already demonstrated the identification of the *nifH* gene in cyanobacterial cells in a natural sample by direct PCR (Takeyama and Matsunaga, 1998). Direct PCR is advantageous because DNA does not have to be extracted from the cell and can be performed on natural samples. Using primers designed from the *nifH* sequence of *Anabaena* PCC7120, a few or single cells of *Anabaena* PCC7120 could be detected. When this technique was applied to 10^{-4} dilutions of an

algal mat and 20 times concentrated seawater samples, direct PCR products were obtained. However the *nifH* gene amplified from samples may have originated from other nitrogen fixing bacteria, diazotrophs. To overcome problems of diazotrophs *nif*H amplification, direct nested PCR combined with epifluoresence microscopy at the single cell level was performed. Using two primer sets, the *nifH* genes were amplified from all tested nitrogen fixing cyanobacteria. Furthermore, the nested PCR amplified the *nif*H gene from a single cell isolated by epifluorescence microscopy.

This procedure is directly applied toward the detection of other photosynthetic marine microorganisms. The same *nifH* gene sequence was used to characterize a novel marine purple non-sulfur photosynthetic bacterium *Rhodovulum sulfidophilum* NKPB 160471R, which possessed high hydrogen producing activity. Approximately 330 bp of the *nifH* genes from strain NKPB160471R and other representative members of the phototrophic purple bacteria were amplified by PCR and sequenced. These sequences were compared phylogenetically in order to determine this bacterial taxonomic position. The clustering pattern obtained from this analysis was similar to that obtained when the 16S rDNA sequences of the bacteria were compared. From this result, the *nifH* gene is useful marker for not only detection of diazotrophs but also identification of photosynthetic bacteria.

4. EFFICIENT HYDROGEN PRODUCTION BY MARINE PHOTOSYNTHETIC BACTERIA

4.1. Conversion efficiencies of light energy to hydrogen by an uptake hydrogenase mutant of Rhodovulum sulfidophilum

Transposon mutagenesis of the marine nonsulfur photosynthetic bacterium, *Rhodovulum sulfidophilum* NKPB160471R was carried out. Strains that could not grow with H_2 under photoautotrohpic conditions were isolated as uptake hydrogenase mutants. A Transposon mutant H-1 had 25% less uptake hydrogenase activity than the wild type strain under H_2 saturated conditions. At a light intensity of 1800 W/m^2, maximum hydrogen production rates of 8.8 and 11.2 μmol/mg dry weight/h were obtained by NKPB160471R and H-1 respectively. Under a light intensity of 13 W/m^2 maximum conversion efficiencies of 26 and 35% were obtained by NKPB160471R and H-1 respectively. In order to obtain the higher conversion efficiencies using marine photosynthetic bacteria, utilization of uptake hydrogenase mutants will be a great benefit.

4.2. Microaerobic hydrogen production by Rhodovulum sulfidophilum in a double phase photobioreactor

Bacterial hydrogen production depends on the activity of the highly oxygen-sensitive nitrogenase enzyme complex (Goldberg, et al., 1987). The nitrogenase activity is controlled by intracellular ATP. Some members of diazotrophic bacteria are able to fix nitrogen under the aerobic condition resulting in high cellular concentration of ATP (Bergersen and Turner, 1975, Cacciari and Lippi, 1979, Chan, et al., 1980, Haaker, et al., 1996, Linkerhäer and Oelze, 1997, Miller, et al., 1988). Furthermore, increase the partial pressure of oxygen stimulates respiration, thereby increasing intracellular ATP content and nitrogenase activity. Introduction of various microaerobic conditions and light and dark cycling in a double phase photobioreactor using NKPB 16471R was shown to increase hydrogen productivity.

The double-phase consisting light and dark compartments and conventional photobioreactor were shown in Figure 3.

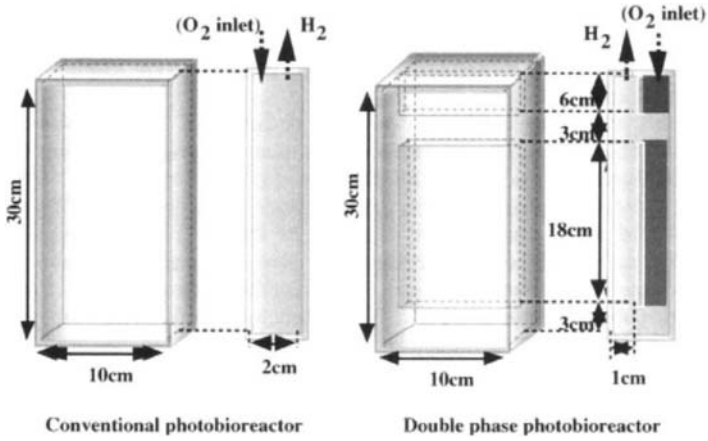


Figure 3: Schematic diagram of the conventional and double phase photobiorectors.

The strain NKPB16047R was shown to be reached a maximum hydrogen production rate of 9.4 μmol/mg dry weight/hr in conventional reactor. The effect of oxygen on hydrogen production in a conventional photobioreactor showed increased hydrogen production rates with increasing concentrations of O_2 up until 12 μmol under a light intensity of 18.5 W/m^2. The intracellular ATP content in this strain was also found to be higher under microaerobic rather than aerobic conditions (Table 1).

TABLE 1
EFFECT OF OXYGEN ON ATP CONTENTS IN *RHODOVULUM* SP. UNDER LIGHT AND DARK CONDITIONS

Condition	ATP content (nmol/mg dry weight)
Light illumination	
Anaerobic	**0.2± 0.1**
Micro-aerobic	**0.5± 0.1**
Dark condition	
Anaerobic	**0.6± 0.2**
Micro-aerobic	**2.6± 0.6**

*** Incubate for 2 h after addition of 20 μmol O_2.**

The ATP content increased to 2.6 nm/mg dry weight under microaerobic dark conditions. Therefore a photobioreactor consisting of both a light and dark compartment was constructed. Using the double-phase photobioreactor under that condition, hydrogen production exhibited enhanced production when compared with the conventional reactor (Table 2).

TABLE 2
HYDROGEN PRODUCTION RATES IN *RHODOVULUM* SP. USING THE DOUBLE PHASE AND CONVENTIONAL PHOTOBIOREACTORS

Condition	Hydrogen production rate (μmol/mg dry weight/h)
Anaerobic condition	
conventional photobioreactor	**0.09 ± 0.02**
double phase photobioreactor	**0.12 ± 0.01**
Micro-aerobic condition*	
conventional photobioreactor	**0.26 ± 0.05**
double phase photobioreactor	**0.38 ± 0.03**

*** 20 mmol O_2 was added to each reactor every 6 h.**

Furthermore, Hydrogen production was investigated under microaerobic conditions at a light intensity of 34.5 W/m^2 and 20 μmol of O_2 added every six hours. Under these conditions, strain NKPB16047R continuously produced hydrogen at a rate of 0.38 ± 0.03 μmol/mg dry weight/h for 150 h (Fig. 4).

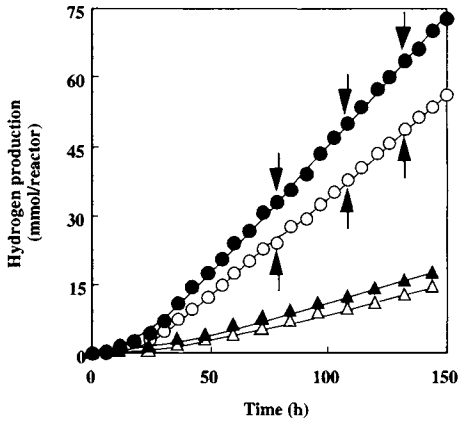


Figure 4: Hydrogen production by *Rhodovulum* sp. using the conventional and double phase photobioreactors. (○) Conventional photobioreactor under micro-aerobic condition. (addition of 20μmol O_2/ reactor every 6h) (●) Double phase photobioreactor under micro-aerobic condition. (addition of 20μmol O_2/ reactor every 6h) (△) Conventional photobioreactor under anaerobic condition. (▲) Double phase photobioreactor under anaerobic condition. Arrows indicate injection time point of 7.5 mmol of L-malate. Cell concentration was adjusted to 3.1 mg dry weight/ml. Light intensity was adjusted to 34.5W/m².

This research demonstrates that a double-phase photobioreactor under microaerobic condition showed to be better suited for hydrogen production using photosynthetic bacteria.

REFERENCES

Ben-Porath J and Zehr JP. (1994). Detection and characterization of cyanobacterial *nifH* genes. *Appl. Environ. Microbiol.* **60**, 880-887.

Bergersen BJ and Turner GL. (1975). Leghaemoglobin and the supply of O_2 to nitrogen-fixing root nodule bacteroids: presence of two oxidase system and ATP production at low free O_2 concentration. *J. Gen. Microbiol.* **91**, 345-354.

Cacciari I and Lippi D. (1979). Effect of oxygen on batch and continuous cultures of nitrogen-fixing *Arthrobactor* sp. *Can. J. Microbiol.* **25**, 746-751.

Chan YK, Nelson NM , Knowles R. (1980). Hydrogen metabolism of *Azospirillum brasilense* in nitrogen-free medium. *Can. J. Microbiol.* **26**, 1126-1131.

Chien T-Y, Zinder, S.H. (1996). Cloning functional organization, transcript studies and phylogenetic analysis of the complete nitrogenase structual genes (*nifHDK2*) and associated genes in the Archaeon *Methanosarcina barkeri*. *J. Bacteriol.* **178**, 143-148.

Colon-lopez MS, Tang H, Tucker DL , Sherman LA. (1999). Analysis of the *nifHDK* operon and structure of nifH protein from the unicellular, diazotrophic cyanobacterium, *Cyanothece* strain sp. ATCC 51142(1). *Biochem. Biophys. Acta* **1473**, 363-375.

Goldberg I, Nadler V , Hochman A. (1987). Mechanism of nitrogenase switch-off by oxygen. *J. Bacteriol.* **169**, 874-879.

Haaker H, Szafran M, Wassink H, Klerk H , Apples M. (1996). Respiratory control determines respiration and nitrogenase activity of *Rhizobium leguminosarum* bacteroids. *J. Bacteriol.* **178**, 4555-4562.

Hennecke H, Kaluza K, Thüy B, Fuhrmann M, Ludwig W , Stsckebrandt E. (1985). Concurrent evolution of nitrogenase genes and 16S rRNA *Rhizobium* species and other nitrogen fixing bacteria. *Arch. Microbiol.* **142**, 342-348.

Linkerhäer K and Oelze J. (1997). Nitrogenase activity and regeneration of the cellular ATP pool in *Azotobacto vinelandii* adapted to different oxygen concentrations. *J. Bacteriol.* **179**, 1362-1367.

Loveless TM and Bishop PE. (1999). Identification of genes unique to Mo-independent nitrogenase sysems in diverse diazotrophs. *Can. J. Microbiol.* **45**, 312-317.

Matsunaga T, Kamiya S. (1987). Use of magnetic particles isolated from magnetotactic bacteria for enzyme immobilization. *Appl. Microbial. Biotechnol.* **26**, 328-332.

Matsunaga T, Kawasaki M, Yu X, Tsujimura N, Nakamura N. (1996). Chemiluminescence enzyme immunoassay using bacterial magnetic particles. *Anal. Chem.* **68**, 3551-3554.

Matsunaga T and Takeyama H. (1998). Marine genome. Plenum Press, New York: 31-37.

Matsunaga T, Sato R., Kamiya S, Tanaka T, Takeyama H. (1999). Chemiluminescence enzyme immunoassay using Protein A-bacterial magnetite complex. *J. Magn. Magn. Mater.* **268**, 932-937.

Miller RW, McRae DG, Al-Jobore A , Berndt WB. (1988). Respiration supported nitrogenase activity of isolated *Rhizobium meliloti* bacteroids. *J. Cell. Biochem.* **38**, 35-49.

Normand P and Bousquet J. (1989). Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen-fixing microorganisms. *J. Mol. Evol.* **29**, 436-447.

Tanaka T, R. Sato R, Kamiya S, Obata K, Tamiya S, Matsunaga T. (2000). Fully-automated heterogeneous immuno assay of human insulin using protein A-bacterial magnetic particle complexes. *Anal. Chem.* **72**, 3518- 3522

Takeyama H and Matsunaga T. (1998). Detecton of marine nitrogen-fixing cyanobacteria capable of producing hydrogen by using direct nestid PCR on single cells. Plenum Press, New York: 197-202.

Takeyama H, Tsuzuki H, Chow S, Nakayama H, Matsunaga T. (2000). Discrimination between Atlantic and Pacific subspiecies of the northernbluefin tuna (*Thunnus thynnus*) by magnetic capture hybridization using bacterial magnetic particles. *Mar. Biotechnol.* **2**, 309.

Thornhill RH, Burgess JG , Matsunaga T. (1995). PCR for direct detection of indigenous uncultured magnetic cocci in sediment and phylogenetic analysis of amplified 16S ribosomal DNA. *Appl. Environ. Microbiol.* **61**, 495-500.

Ward DM, Wller R , Bateson MM. (1990). 16S rDNA sequence reveal numerous uncultured microorganisms in a natural community. *Nature* **345**,.

METABOLIC ENGINEERING APPROACHES FOR THE IMPROVEMENT OF BACTERIAL HYDROGEN PRODUCTION BASED ON *ESCHERICHIA COLI* MIXED ACID FERMENTATION

K. Sode[1], S. Yamamoto[1] and M. Tomiyama[2]

[1]Department of Biotechnology , Tokyo University of Agriculture and Technology,
Nakamachi 2-24-16, Koganei , Tokyo, 184-8588 Japan,
Tel. and Fax. +81 42 388 7027, E-mail sode@cc.tuat.ac.jp

[2]National Institute of Agrobiological Resources,
Kannondai 2-1-2, Tsukuba, Ibaraki, 305-8602 Japan
Tel. +81 298 38 7463, Fax. +81 298 38 7408, E-mail tomiyama@abr.affrc.go.jp

INTRODUCTION

Bacterial hydrogen production based on anaerobic metabolism has been studied as a means of efficiently using waste biomass, such as molasses from sugar manufacturing. Anaerobic processes can decompose saccharides, recovering molecular hydrogen gas with high efficiency. Considering that the anaerobic fermentation process can occur in a conventional bioreactor system with high volumetric yield, and does not require a large surface area such as for a photo-energy conversion system, the anaerobic fermentation will be a practical bioprocess for hydrogen production. Although the anaerobic fermentation cannot theoretically decompose saccharide completely into hydrogen and carbon dioxide, and some organic acids such as acetate and lactate remain, the combination with photosynthetic bacterial hydrogen production system enables the simultaneously utilization of organic acids for photo hydrogen production.

Among the various microorganisms which can produce hydrogen by an anaerobic fermentation pathway, *Escherichia coli* is an attractive target, considering the availability of genetic information for further improvement by genetic engineering.

Recent progress in molecular biology on the anaerobic metabolism of *Escherichia coli* has elucidated the enzymes and genes responsible for fermentative hydrogen production (1,2). *E.coli* produces hydrogen by mixed acid fermentation, mainly from glucose. In *E.coli*,

hydrogen evolution is by the formate hydrogenlyase system (FHL) containing formate dehydrogenase-H (FDH-H; hydrogenase linked), electron carrier intermediate(s), and hydrogenase 3 (3). Since hydrogen is not the only metabolite in the *E.coli* mixed acid fermentation process, the carbon and/or electron flux from glucose to FHL should be optimized and be improved.

In this review, we summarize the molecular biology of *E.coli* hydrogen production based on mixed acid fermentation, and our recent progress and approaches to enhance hydrogen production by metabolic engineering.

MIXED ACID FERMENTATION - BIOCHEMISTRY AND MOLECULAR BIOLOGY-

1. Biochemical Information

A brief introduction of the anaerobic mixed acid fermentation pathway of *E.coli*, remarking hydrogen production from glucose by pyruvate formate lyase (PFL)/FHL system is presented in Figure 1.

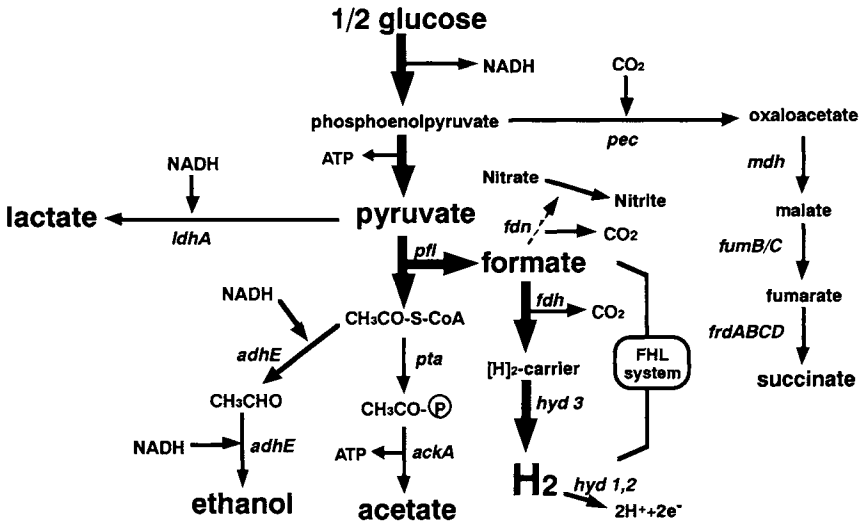


Figure 1: Fermentation pathways of Escherichia coli. Gene symbols: *ack*A, acetate kinase; *adh*E, alcohol dehydrogenase; *fdh*, formate dehydrogenase of FHL complex; *fhn*, nitrate-inducible formate dehydrogenase; *hyd*, hydrogenase 3 of FHL complex; *ldh*A, NAD+-dependent D-(-)-lactate dehydrogenase; *pfl*, pyruvate formate-lyase; *pta*, phosphotransacetylase. FHL, formate hydrogenlyase. Redrawn from reference 29

Under anaerobic conditions, in the absence of alternative and much more energetically favorable electron acceptors, such as nitrate or fumarate, *E.coli* utilizes the mixed acid fermentation pathway. Since the fermentation products are not only carbon dioxide and

hydrogen, but a mixture of ethanol and various organic acids, such as acetate, formate, lactate and succinic acid, the pathway is called a mixed acid fermentation. One molecule of glucose is taken up by the phosphoenolpyruvate phosphotransferase system (PTS), and oxidized by the Embden-Meyerhof-Parnas pathway to produce 2 molecules of pyruvate. Pyruvate is converted into acetyl-CoA and formate by pyruvate formate lyase (PFL) system, or reduced to lactate by fermentative lactate dehydrogenase, LDH-A. Formate is oxidized by formate dehydrogenase associated with FHL (FDH_H) and the released proton is reduced by hydrogenase 3, Hyd-3, to produce hydrogen.

2. Hydrogenase Isoenzymes

Table 1 summarizes the hydrogenase isoenzymes and genes relating to their expression in *E.coli*. Earlier molecular genetic approaches to elucidate the genes relating to hydrogenase(s) and to hydrogen production were based on isolation of mutants and their genetic complementation. However, the complex genetic organization of hydrogenase isoenzymes and the existence of genes responsible for their expression delayed the progress of their molecular biology. There are at least 3 hydrogenase isoenzymes in *E.coli*, designated as hydrogenase-1, Hyd-1, hydrogenase-2, Hyd-2 and Hyd-3. Hyd-3, as described above, is the component of the FHL system, and is responsible for hydrogen production from formate. Hyd-1 and Hyd-2 are, however, not involved in the mixed acid fermentation pathway but their function is to oxidize hydrogen, the so called uptake-hydrogenases. All of the hydrogenase isoenzymes are induced by formate. The structural gene of each hydrogenase is located independently of other hydrogenase isoenzymes, *hya*, *hyb* and on *hyc* operons. The *hyc* operon, encoding Hyd-3, is composed of 8 genes, *hyc*A to *hyc*H (4). The structural gene of the large subunit of Hyd-3 may be encoded in *hyc*E, according to homology analyses. *hyc*A encodes the repressor gene of the FHL system, including *hyc*. It has been reported that the deletion of *hyc*A results in an increase in hydrogenase activity (5).

TABLE 2
HYDROGEN PRODUCTION RATES FROM REDUCED MV AND GLUCOSE

Substrate	Hydrogen production rates ($mmol \cdot mg^{-1}protein \cdot hour^{-1}$)	
	MC4100	HD701(DhycA)
Reduced MV	0.8	1.5
glucose	1.8	1.8

5.6mM glucose and 16.5mM reduced MV were used in this study

Recently, the existence of a fourth hydrogenase isoenzyme and its operon was reported, Hyf (hydrogenase 4) and *hyf*, although the role of this isoenzyme is still unknown (6). Besides the genes responsible for each isoenzyme, several other genes are known whose products are responsible for the maturation or formation of active hydrogenase isoenzymes.

They are called *hyp* genes and encoded in the *hyp* operon and in *hyp*F former by known as *hyd*A gene. The mutants lacking some of these *hyp* genes do not produce all 3 hydrogenase isoenzymes in spite of the expression of their structural genes; therefore *hyp* genes are considered to be essential for the maturation of these enzymes. The function of *hyp* B has been vigorously studied and proposed to have a significant role in the incorporation of Ni ion into the active site of hydrogenase isoenzymes.

METABOLIC ENGINEERING FOR THE ENHANCEMENT OF HYDROGEN PRODUCTION

1. Over-expression of Hydrogenase 3

We tested the potential of hydrogen evolution of a mutant *E.coli* strain deficient in the *hyc*A gene, in order to evaluate whether the Hyd-3 expression level might be the rate-limiting step in an *E.coli* hydrogen production system(7).

Table 2 summarizes hydrogenase activity and hydrogen production from glucose of the *hyc*A mutant strain (HD701) (5) and its parent strain (MC4100). The HD701 strain showed 2 fold greater hydrogenase activity compared to MC4100. This was due to the overexpression of Hyd 3 by the deletion of *hyc*A. However, the hydrogen production rate from glucose was not affected by this mutation. This indicated that the enhancement of the terminal enzyme expression level will not result in improved hydrogen production efficiency.

TABLE 1
ESCHERICHIA COLI HYDROGENASE STRUCTUAL GENE AND OTHER GENES REGULATING HYDROGENASE ACTIVITY

operon	enzyme and gene	loci
hya	hydrogenase 1 (hydrogen cycling?) *hya*A small subunit *hya*B large subunit	22min
hyb	hydrogenase 2 (hydrogen uptake) *hyb*A small subunit *hyb*C large subunit	65min
hyc	hydrogenase 3 (formate hydrogen lyase system, dehydrogen evolution) *hyc*A repressor *hyc*E large subunit	58min
hyp	operon necessary for the activity of the 3 hydrogenase isozymes *hyp*A B (=*hyd*B), nickel transportation C D E F=*hyd*A *fhl*A transcriptional activator for *hyp* and *hyc* operon	58min

Figure 2 shows the correlation between formate concentration and the hydrogen production rate of each strain. At formate concentrations higher than 10 mM, the mutant strain showed a lower hydrogen production rate than its parent strain, even though it possessed higher hydrogenase activity. Assuming that this correlation was a Michaelis-Menten-type saturation curve, the apparent Km and Vmax were obtained for each strain. The apparent Vmax and Km values of HD701 strain were 18 μmol mg^{-1} protein h^{-1} and 24 mM, respectively, whereas those of the MC4100 strains were 36 μmol mg^{-1} protein h^{-1} and 67 mM, respectively. Therefore, by the overexpression of Hyd-3, a drastic decrease in Vmax value was observed. The FHL system is assumed to constitute a multiprotein complex located on the inner aspect of the cytoplasmic membrane (5). Therefore, the overexpression of Hyd-3 might cause the partial dissociation of the complex, resulting in the imbalance of formate utilization at a high substrate concentration.

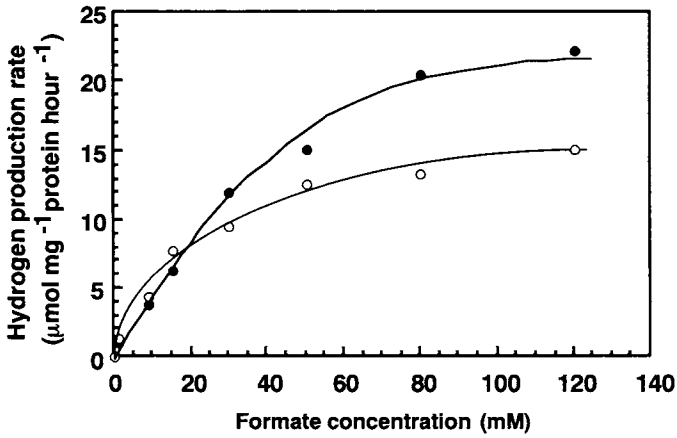


Figure 2: Correlation between formate concentration and hydrogen production rate by *E.coli* strains HD701 (open circles) and MC4100 (closed circles).

2. Construction of a mutant E.coli strain capable of producing hydrogen in the presence of nitrate

In the presence of nitrate, formate dehydrogenase-N (FDH-N) and nitrate reductase are induced (8). As a result, the formate is consumed preferentially by FDH-N due to its inherent high affinity for formate versus FDH-H. The Km value for formate of FDH-N is 0.12 mM, while that of FDH-H is 26 mM. The FHL system is induced by the presence of formate, however, formate consumption by an FDH-N - nitrate reductase system depresses the expression level of the FHL system. Consequently, both expression of FHL and hydrogen production are repressed by the presence of nitrate in the medium. The removal of nitrate from waste water is not a practical process; therefore a bacterial strain capable of hydrogen production even in the presence of nitrate is favorable. The subunits of nitrate reductase are encoded in the *fdn* operon. The a-subunit of nitrate reductase is encoded in the

*nar*G locus. Therefore, we tested the potential of a nitrate reductase mutant for hydrogen production in the presence of nitrate (7).

We used the *E.coli* strain, RK5265, in which *nar*G, a gene encoding for an a subunit of nitrate reductase was disrupted by a Tn10 insertion (9). Figures 3a and 3b show the time courses of hydrogen production from glucose in the absence or presence of nitrate by the strain deficient in the *nar*G locus (RK5265) and its parent strain (RK4353).

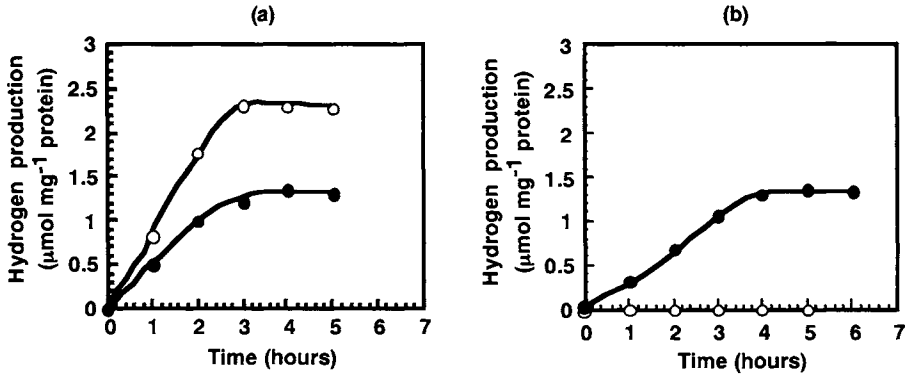


Figure 3: Time course of hydrogen production from 5.6mM glucose by *E.coli* strains RK5265 (closed circles) and RK4353 (open circles) in the (a) absence and (b) presence of 40mM nitrate.

In the presence of 40 mM nitrate, however, hydrogen production by the strain RK4353 was greatly repressed and no detectable hydrogen was produced. This indicated that the cultivation in a rich medium utilized for pre-cultivation resulted in the induction of a nitrate reductase system. Therefore, the strain with a nitrate reductase system cannot be applied to bacterial hydrogen production in the presence of nitrate. In contrast, the strain RK5365 produced hydrogen in the presence of 40 mM nitrate. Hydrogen production was the same as the condition without nitrate (1.3 μmol mg^{-1} protein). Considering that the nitrate reductase system of the parent strain, RK4353, was induced by pre-cultivation in a rich medium, the FDH-N in RK5265 would be induced. However, the absence of the nitrate reductase due to the state of the gene locus *nar*G, achieved hydrogen production even in the presence of the 40 mM nitrate.

These results clearly indicate that by disrupting the nitrate reductase system, an *E.coli* strain suitable for practical hydrogen production can be developed which will not be affected by the presence of nitrate in the utilizable substrate. The availability of a mutant strain enabled us to demonstrate the application of a mutant strain deficient in the *nar*G locus. However, the existence of a constitutively synthesized nitrate reductase was reported, a nitrate reductase Z (10). Indeed, by the cultivation of the strain RK5265 in the presence of 100 mM nitrate, no FHL activity was observed (results not shown). Therefore, the ideal *E.coli* strain will be constructed by introducing mutation in the structural gene for FDH-N.

3. Elimination of a branched pathway - construction of a LDH-A mutant E.coli

In order to improve the efficiency of bacterial hydrogen production, several metabolic branching points on carbon and/or electron flux from glucose to FHL should be precisely examined. One such metabolic branch exists in the utilization of pyruvate. In order to utilize pyruvate for the FHL system, it should be consumed by PFL. However, pyruvate can also be consumed by LDH-A. Recently the structural gene of LDH-A, *ldh*A, was cloned, sequenced and its biochemical regulation was also elucidated (11). Since the production of lactate by LDH-A is one of the major metabolites during the mixed acid fermentation of *E.coli*, the elimination of LDH-A activity will make possible the construction of an ideal *E.coli* strain which produces hydrogen with high efficiency.

We therefore attempted the construction and characterization of an *E.coli* strain lacking LDH-A activity, and demonstrated its potential for bacterial hydrogen production(12).

Mutant strains used in this study were constructed by P1 transduction (13). First, an *E.coli* strain KF1344 was used as the donor and FMJ39A as the recipient, and the allele containing the CAT gene of KF1344 was transferred. The resulted transductants were subjected to the LDH-A assay. Among the Cm^R colonies, a clone maintaining LDH-A mutation was selected, and designated as FM#13. Then using FM#13 as the donor, the second P1 transduction was carried out using MC4100 as the recipient, in order to transfer the allele containing both Cm^R and the LDH-A mutation. Among the colonies showing Cm^R, a clone lacking LDH-A activity was selected and designated as MC13-4.

Figure 4 shows the time courses of hydrogen production from 3 mM glucose (123 μmol glucose in a batch reaction), and also the time courses of other metabolites, by the strain MC4100 and its mutant, MC13-4, which lacks LDH-A.

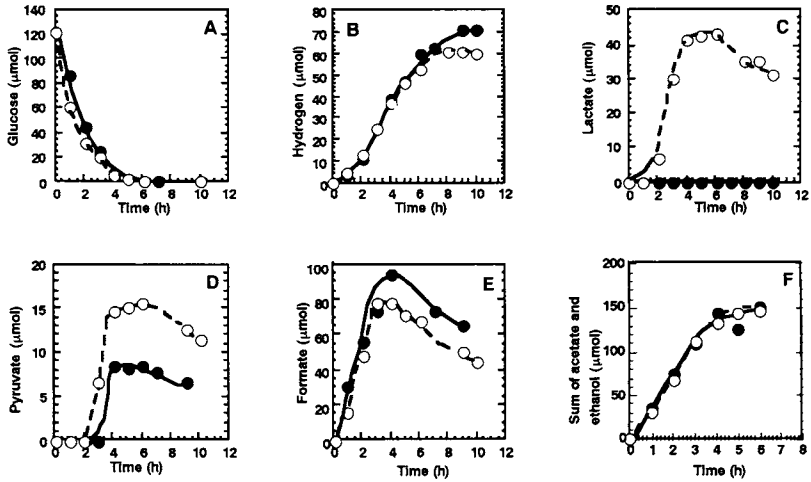


Figure 4: Time courses of glucose consumption and metabolite production during hydrogen production using the strain MC4100 (open circular) and its LDH-A mutant, MC13-4 (close circular). (A) glucose consumption, (B) hydrogen production, (C) lactate production, (D) pyruvate production, (E) formate production, and (F) sum of the produced ethanol and acetate.

Both strains consumed glucose within 6 hours. As MC13-4 lacks LDH-A, it did not produce lactate, whereas MC4100 accumulated about 40 μmol of lactate. The hydrogen production rate and the amount of hydrogen produced by MC13-4 were at almost the same levels as those achieved by MC4100. After 4 hours, the sum of formate and hydrogen reached a maximum, about 120 μmol for MC4100 and about 140 μmol for MC13-4, and maintained their levels for a further 4 hours. Therefore, the elimination of LDH-A resulted in an increase in the glucose flux which is utilized for formate and hydrogen production.

On the basis of the accumulated amount of ethanol and acetate, the amount of hydrogen consumed by the uptake hydrogenases, Hyd1 and Hyd2, can be calculated. Hydrogen was assumed to have been consumed by the uptake hydrogenases, 24 μmol hydrogen for MC4100 and 6 μmol hydrogen for MC13-4. Therefore, LDH-A mutation also repressed the effect of uptake hydrogenase.

Table 3 summarizes the yield and efficiency of hydrogen production by the engineered *E.coli* strain, MC13-4, and its parent strain, MC4100. The calculation was based on the fact that ideally 2 molecules of hydrogen can be produced from one molecule of glucose. The yields of hydrogen based on the total glucose consumed are 22 % and 24 % for the parent strain and MC13-4 respectively. On the basis of the calculation of the amount of glucose utilized at the downstream of pyruvate, PFL/LDH/FHL, this mutation yielded a 50% increase, from 26 % to 38 %. The ratios of the hydrogen consumed by the uptake hydrogenases were 17 % and 3 % for MC4100 and MC13-4 respectively.

TABLE 3
HYDROGEN PRODUCTION YEILD AND EFFICIENCY

strains	theoretical hydrogen yield(%)[1)]	hydrogen production efficiency[2)]	glucose consumption ratio[3)]	hydrogen uptake[4)]
MC4100	22 %	26 %	83%	17 %
MC13-4	24%	38 %	65%	3%

1) hydrogen yield = $\frac{\text{hydrogen produced}}{\text{total glucose consumed X 2}}$ X 100

2) hydrogen production efficiency = $\frac{\text{hydrogen produced}}{\text{glucose consumed in PFL-LDH}}$ X 100

3) glucose consumption ratio = $\frac{\text{glucose consumed in PFL-LDH}}{\text{total glucose consumed X 2}}$ X 100

4) hydrogen uptake = $\left(1 - \frac{\text{hydrogen and formate produced}}{\text{acetate and ethanol produced}}\right)$ X 100

This calculation is based on that 2 molecules of hydrogen are produced from 1 molecule of glucose. Amount of glucose consumed in PFL-LDH is estimated by the sum of the amount lactate production, pyruvate production,acetate production, and ethanol production; 204 mmol in MC4100 and 160 mmol in MC13-4.

These results suggest that by eliminating LDH-A, hydrogen production efficiency is in principle increased. The physiological roles of uptake hydrogenases are still unknown; however, these enzymes may have an impact on the redox status of anaerobic metabolism and/or the formate/hydrogen equilibrium. The LDH-A mutation should result in the accumulation of NADH. Considering that the uptake hydrogenase catalyses the oxidation of hydrogen, the decrease in uptake hydrogenase activity might complement the LDH-A mutation on the basis of the cellular redox balance of native cells. However, the complementation of LDH-A by the repression of uptake hydrogenase might not be sufficient, and subsequently affect the glucose flux utilized in the PFL system. The further elucidation of the role of uptake hydrogenases is essential in order to advance the improvement of the *E.coli* hydrogen production system.

CONCLUDING REMARKS AND FUTURE

Here we presented some of our approaches for the improvement of hydrogen production by *E.coli* via metabolic engineering. Throughout this investigation, we encountered *in vivo* complementation of metabolic imbalance caused by the genetic manipulation. Although the whole genomic information is now available, we still lack knowledge of the regulation of the enzymes responsible for mixed acid fermentation and their relations. This is not only true for bacterial hydrogen production the future progress of Bioinformatics will precisely elucidate the network of genetic information in this metabolic pathway. Particularly, the elucidation of the role of uptake-hydrogenases, Hyd1 and Hyd2, is expected.

Another feature to be improved in bacterial hydrogen production is the expansion of the biomass which can be used, At the moment, glucose or some organic acid are utilized for bacterial hydrogen production. Since the major lignolytic compound is xylose, the development of an alternative metabolic pathway or microorganisms which can produce hydrogen from xylose is expected.

REFERENCES

1. Clark, D.P. (1989). Fermentation pathways of *Escherichia coli*. *FEMS Microbiol.* **Rev. 63**, 223-234.
2. Sawers., B.A.a.G. (1996). Fermentation. In *Escherichia coli* and *Salmonella* 2nd Ed., Neidhardt, F. C. et al eds., ASM Press, Washington, D. C., 262-282.
3. Sawers, R.G., S.P.Ballantine, and D.H.Boxer. (1985). Differential expression of hydrogenase isoenzymes in *Escherichia coli* K-12: Evidence for a third isoenzyme. *J.Bacteriol.* **164**, 1324-1331.
4. Böhm, R., M.Sauter, and A.Böck. (1990). Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenlyase components. *Mol.Microbiol.* **4**, 231-243.
5. Sauter, M., R.Böhm, and A.Böck. (1992). Mutational analysis of the operon (*hyc*) determining hydrogenase 3 formation in *Escherichia coli*. *Mol.Microbiol.* **6**, 1523-1532.

6. Andrews, S.C., M.A.Quail, P.Golby, J.R.Guest, J.McClay, A.Amber, and B.C.Berks. (1997). A new-12 gene operon (*hyf*) encoding a fourth hydrogenase in *E.coli*. In Abst. of International Meeting of Anaerobic Metabolism, Electron Transfer Systems and Regulation, Marseille-Carry-Le-Rouet, Apr. 1997.
7. Sode, K., Watanabe, M., Makimoto, H. and Tomiyama, M. (1998). Effect of hydrogenase 3 over-expression and disruption of nitrate reductase on fermentative hydrogen production in *Escherichia coli*; A metabolic engineering approach. Biohydrogen, O.R.Zaborsky Ed., Plenum Press :73-79.
8. Stewart, V. (1994). Regulation of nitrate and nitrite reductase synthesis in enterobacteria. *Antonie van Leeuwenhoek* **66**, 37-45.
9. Stewart, V., and C.H. MacGregor. (1982). Nitrate reductase in *Escherichia coli* K-12: involvement of *chl*C, *chl*E and *chl*G loci. *J.Bacteriol.* **151**, 1320-1325.
10. Bonnefoy, V., and J.A.Demoss. (1994). Nitrate reductases in *Escherichia coli*. *Antonie van Leeuwenhoek* **66**, 47-56.
11. Bunch, P.K., Mat-Jan, F., Lee, N. and Clark D.P. (1997). The ldh A gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. *Microbiology* **143**, 187-195.
12. Sode, K., Watanabe, M., Makimoto, H. and Tomiyama, M. (1999). Construction and characterization of fermentative lactate dehydrogenase *Escherichia coli* mutant and its potential for bacterial hydrogen production. *Appl. Biochem. Biotechnol.* **77-79**, 317-323.
13. Tarmy, E.M., Kapran, K.O. (1968). Chemical characterization of D-lactate dehydrogenase from *Escherichia coli* B. *J. Biol. Chem.* **10**, 2579-2586.

MOLECULAR HANDLING OF HYDROGENASE

M. Miyake[1,2], J. Schnackenberg[1], C. Nakamura[2], Y. Asada[3] and J. Miyake[2]

[1] National Institute of Bioscience and Human-Technology,
1-1 higashi, Tsukuba, Ibaraki 305-8566,
[2] National Institute for Advanced Interdisciplinary Research
1-1-4 Higashi, Tsukuba; Ibaraki 305-8562,
[3] Industrial Technology Center of Okayama Prefecture,
5301 Haga Okayama 701-1296 Japan

SUMMARY

Hydrogenase is a responsible enzyme catalyzed the reversible or irreversible conversion from proton to molecular hydrogen. Many cyanobacteria and alga retaining hydrogenase have been intensively studied for biological hydrogen production from water by using light energy. Recent progress of molecular handling of hydrogenase allows to variable application of hydrogenase for hydrogen production. Here in this chapter, new molecular handling and application of hydrogenase are described.

1. HYDROGENASE ENGINEERING IN HISTORY

The first application of hydrogenase would be *in vitro* hydrogen production. Benemann *et al.*, [Benemann, 1973 #2] demonstrated a photoproduction of H_2 by *in vitro* coupling of photosynthetic system, hydrogenase and mediator in which the system, water splitting at PSII results in production of proton, electron and O_2. The electron was transferred to hydrogenase via mediator like ferredoxin, cytochrome c3 or NADH and used for reduction of the proton to H_2.

The system was extensively studied by many researchers to elongate its stability (reviewed by Adams *et al*, [Adams, 1980 #3]). Hydrogenase is very unstable in the presence of O_2. Rao *et al.*, [Rao, 1976 #8] succeeded elongate the hydrogenase-dependent H_2 production by combination of O_2 scavenging system. Use of NADH resulted in reducing autooxidizability of mediator ([Rao, 1976 #8]). Stabilization of each element would be a major breakthrough for continuous hydrogen production.

Recently, some new molecular handling techniques and discovery of extrastable hydrogenase widen the availability of hydrogenase and open a new research field on the application of hydrogenase. In the next, the new molecular handling techniques will be overviewed.

2. MOLECULAR HANDLING OF HYDROGENASE

2.1. -Pseudotransformation- for Construction of Intracellular Hydrogen Production System

2. 1. 1. Pseudotransformation

Hydrogenase molecules can be incorporated into any kind of living cells by using electroporation techniques (Miyake et al., 1997; Schnackenberg et al., 1999). For example, a considerable increase of the hydrogenase activity was detected after the electroporation of *Synechococcus elongatus* cells with the clostridial hydrogenase at 9 $kV \cdot cm^{-1}$ of electric field strengt (Fig. 1). The incorporation of the clostridial hydrogenase into the cells was confirmed by western blot analysis (Fig. 2). Clostridial hydrogenase outside the cells were completely digested by proteinase K in 2 min (Fig. 2, lane 5). There were no compounds crossreacting with an antibody against clostridial hydrogenase in *S. elongatus* (Fig. 2, lane 2, 3, 4, 5, 7, 8). The signals of undigested clostridial hydrogenase in lane 6 and 9 of Fig. 2 suggest the incorporation of the clostridial hydrogenase into the cyanobacteria. The cells retaining clostridial hydrogenase were designated as "pseudotransformants" to distinct from the wild-type cells.

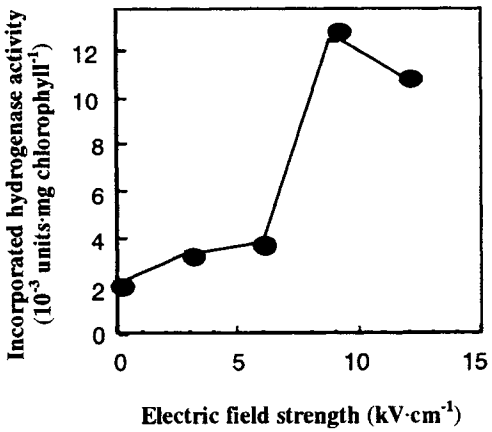


Figure 1: Introduction of clostridial hydrogenase into cells of *Synechococcus elongatus* by electroporation.

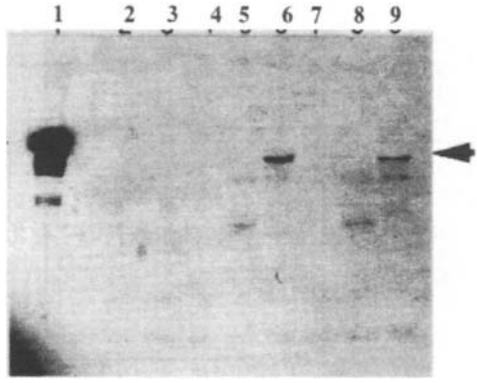


Figure 2: Western blot analysis of clostridial hydrogenase introduced into cells of *S. elongatus*. **Lane 1**, hydrogenase from *C. pasteurianum*; **lane 2&3**, total protein fraction from *S. elongatus* wildtype cells; **lane 4&7**, protein fraction from wildtype cells after electroporation in the absence of clostridial hydrogenase; **lane 5&8**, protein fraction from wildtype cells mixed with clostridial hydrogenase, no electroporation; **lane 6&9**, protein fraction from cyanobacterial cells containing clostridial hydrogenase. The samples separated in lane 7-9 have been treated with protease K for 10 min. The arrow indicates the position of the clostridial hydrogenase (64 kDa).

2.1.2. Measurement of stability of foreign hydrogenase

In general, hydrogenase is unstable in oxygenic photosynthetic organisms. However, measurement of intracellular stability of hydrogenase is difficult due to generation of the molecules. Pseudotransformation allows to measure the stability of enzyme molecules *in vivo* (Miyake et al., 1998).

Fig. 3 shows residual activity of hydrogenase in the pseudotransformants and the crude Extract from *C. pasteurianum* after transferred to aerobic conditions. Hydrogenase activity of the pseudotransformants under light conditions decreased more rapidly than under dark conditions, which was suspected to be due to O_2 photosynthetically evolved. The clostridial hydrogenase activity in the pseudotransformants was rather stable than that in the crude extract.

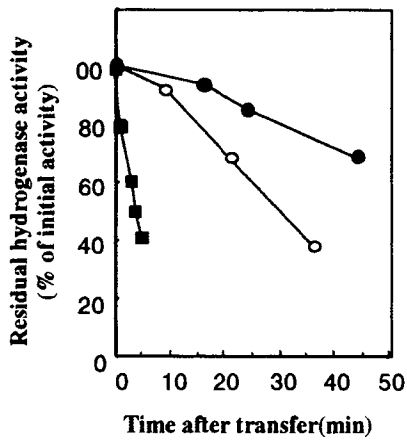


Figure 3: Stability of introduced hydrogenase under aerobic conditions. The open and the full circles indicate the hydrogenase activity of intact cells containing clostridial hydrogenase under illumination (14 $W \cdot m^{-2}$, fluorescent white light) and in the dark, respectively. The squares represent the hydrogenase activity in a crude extract from cells containing clostridial hydrogenase.

2.1.3. In vivo coupling between foreign hydrogenase and photosynthetic systems.

Pseudotransformation is applied to investigate in vivo coupling between foreign hydrogenase and photosynthetic systems for hydrogen production. Light-dependent H_2 and O_2 production were measured without any artificial electron carriers (Fig. 4. and Table 1). The rate of O_2 produced by the pseudotransformants was three-times lower as that by wild-type cells (Table 1) probably due to the damage for electroporation. The pseudotransformants produced H_2 for 5 min and the rate decreased (Fig. 4, b). Wild-type cells before and after the electroporation without clostridial hydrogenase produced no H_2 (Fig. 4, a and Table 1). These results suggest that clostridial hydrogenase could couple with photosynthetic system *in vivo* as well as in vitro system (Fry *et al.*, 1977).

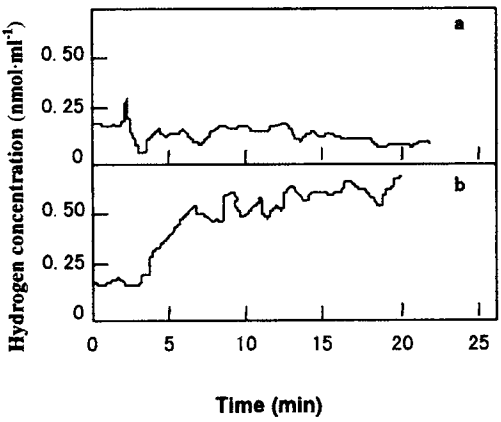


Figure 4: Photohydrogen evolution of wildtype cells and cells containing clostridial hydrogenase. The measurement was started with the onset of light.

TABLE 1
H_2 AND O_2 EVOLVING RATES UNDER LIGHT CONDITIONS

Cell	*evolution rate (nmol·mg chlorophyll^{-1}·min^{-1})	
	H_2	O_2
Wild-type cells	not detected	26.0
Wild-type cells electroporated, no hydrogenase	not detected	7.8
H_2ase-cells	0.23	8.3

* The rates were calculated from the shifts during the initial 5 min after the onset of light

2.1.4 Conclusion

Direct electroporation was useful to prepare the pseudotransformants. *In vivo* coupling of cyanobacterial photosynthesis and clostridial hydrogenase was demonstrated by the pseudotransformants. This method will be applicable to screen the appropriate hydrogenase for hydrogen production coupled with cyanobacterial photosynthesis.

2.2. Langmuir-Blodgett Film of Hydrogenase

The Langmuir-Blodgett (LB) method has a potential to make thin films of functional molecules on an electrode for efficient reactions. By using polyamino acids as counter ion, this method can be applied to make stable monolayer film of hydrogenase proteins (Noda *et al.*, 1998).

2.2.1. Formation of hydrogenase LB films

A hydrogenase isolated from a photosynthetic bacterium *Thiocapsa roseopersicina* strain BBS shows a profound stability against oxygen and heat (Gogotov *et al.*, 1978; Zorin, 1986; Zorin *et al.*, 1995). The hydrogenase is used to make a functional hydrogenase LB film since the hydrogenase (Noda *et al.*, 1998).

A KSV Minitrough (7.5 x 33 cm, 260 ml; KSV Instruments, Finland) was used for the preparation of the LB films. Poly-L-Lysine (PLL) (20-mer) as the counter ion was synthesized by the solid-phase system using a P9050 Plus PepSynthesizer (Millipore, Bed-fold, MA) and purified using an ODS column (C18 19 x 150 mm, Millipore).

The subphase contained 10 mM Tris-HCl buffer (pH 7.4). $CaCl_2$ (2 mM) or PLL (0.5 mM, concentration of amino acid residues) was added to the subphase. The temperature of the subphase was adjusted to 18°C. Hydrogenase solution of 20 µl (1.1 mg/ml) was spread on the air/water interface of the subphase (surface area of 248 cm^2) using the glass-rod method (Hirata *et al.*, 1992). After 10 min, the monolayer was compressed at a rate of 7.5 cm^2/min. The layer of hydrogenase was transferred to hydrophilic quartz plates and indium tin oxide (ITO) electrodes (40 x 5 x 1 mm). The vertical deposition onto the substrate was performed a surface pressure of 30 mN/m. The dip-ping and withdrawal rates were 50 and 5 mm/min, respectively. The density of hydrogenase molecules in an LB film was calculated based on the number of molecules spread on the subphase and the transfer ratio (TR).

2.2.2. Stability of the hydrogenase LB films

PLL is effective to make a stable hydrogenase film. The π-A isotherms of the hydrogenase from *T. roseopersicina* in the presence of PLL and Ca^{2+} are shown in Fig. 5. A typical two-step curve with the transition point at 20 mN/m was observed with using PLL. Over 25 mN/m of surface pressure, the two plots became steep and identical. The hydrogenase molecules were transferred onto quartz substrates at the upstroke. TRs were 1.3 at the first transfer and the averages for four-layer deposition were in the range of 0.8-1.0 for counter ions. TRs were same for the ITO electrode.

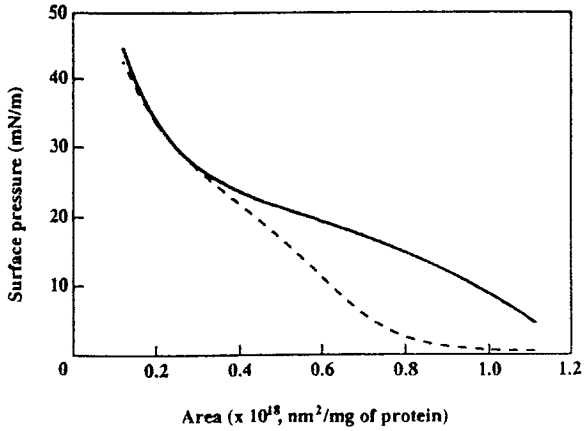


Figure 5: Pressure-area isotherms of the hydrogenase monolayer. The counter ions are PLL (————) and Ca^{2+} (·····).

Large differences were observed in the hydrogen evolution activities of the hydrogenase films made with each counter ion (Fig. 6). A high and linear increase of the activity was seen by the repeated transfer with PLL, whereas much low activity was observed with Ca^{2+}. PLL was also effective for transfer to ITO electrode. The activity for hydrogen uptake was also examined.

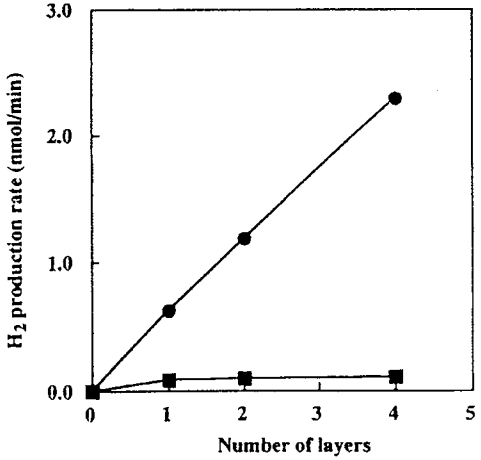


Figure 6: Hydrogenase activity in the films *versus* the number of layers. The counter ions are PLL (●) and Ca^{2+} (■).

The blue color from the MV reduced by the hydrogenases on the quartz substrate was observed immediately after soaking of the substrate in the reaction solution (Fig. 7). The reduction of the MV could be repeated more than twenty times by using the same substrate.

The thermal stability of the hydrogenase LB film was investigated to compare with the those in solution as reported previously (Zorin *et al.*, 1995). The hydrogen evolving activity of the hydrogenase LB film was retained the same from 30 to 70°C. The latter is the optimal temperature for the hydrogen evolution of the hydrogenase from *T. roseopersicina* (Zorin *et al.*, 1995). Complete denaturation was caused at 100°C. The hydrogenase LB film electrode was applicable even under such rigorous condition.

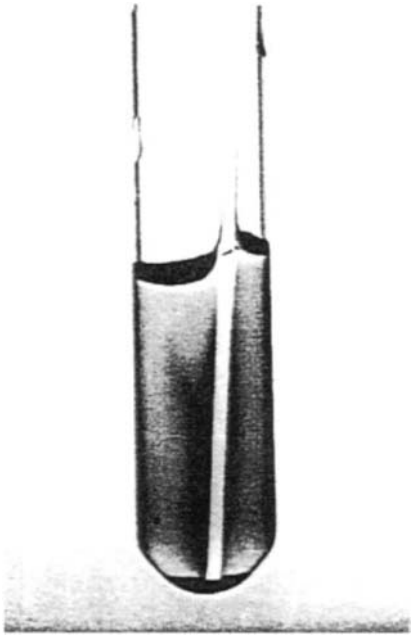


Figure 7: Reduction of methylviologen by a hydrogenase-LB film (four layers). A view parallel to the plane of the quartz plate. The dark area surrounding the surface of the hydrogenase-LB film shows the reduction of methylviologen.

2.2.3. Hydrogen evolution by hydrogenase LB films

Electrochemical hydrogen evolution by the hydrogenase LB film on an ITO electrode, measured with a three-electrodes system is shown in Fig. 8. In the presence of 1.6 mM oxidized MV, the hydrogen concentration increased up to 0.17 nmol/ml under 51.2 μA x 10 min

while the potential on the working electrode was maintained at -550 mV (vs. SHE). Initial rate of hydrogen production was 0.36 or 514 nmol/min per mg protein (Fig. 8a). The applied potential did not cause electrolysis of water, confirmed by using an ITO electrode without hydrogenase. In this case, only the reduction of MV was observed in the vicinity of the surface of the ITO electrode. Without MV, hydrogen evolution was not observed at the potential (Fig, 8b). Increasing the potential to –440 mV (vs. SHE) did not cause hydrogen evolution as the potential was too high to reduce MV. This was confirmed by a measurement of cyclic voltammetry of MV using the same electrode system as used in this experiment (data not shown). The result indicates that hydrogen was evolved by the electron transfer from ITO electrode MV then to the hydrogenase LB film for the reduction of protons. The saturation of hydrogen evolution (Fig. 8a) may be caused by the characteristics of the detection system as hydrogen consumption by electrode. Hydrogen evolution was not detected using the same amount of free hydrogenase in solution (Fig. 8c), The hydrogenase in the solution of the reaction chamber (0.35 pg/ml) could not interact effectively with reduced MV because the mediator was rapidly oxidized in the open-air reaction chamber. The redox potential of the buffer in the chamber was 375 mV, which is enough oxidative condition. In the case of LB film, the hydrogenases on top of the electrode could accept electrons from the reduced MV immediately after the electrode reaction. LB film of hydrogenase provided an effective electrochemical method for hydrogen production.

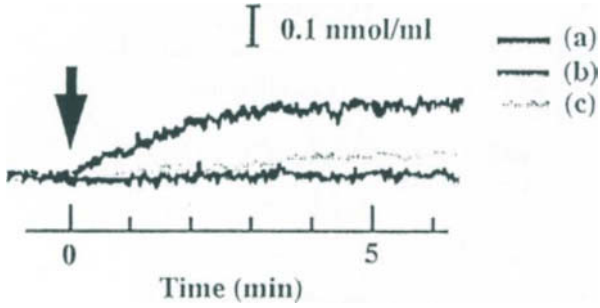


Figure 8: Electrochemical hydrogen evolution of the hydrogenase-LB film (four layers) in the presence (a) and in the absence (b) of methylviologen. The vertical axis represents the relative amounts of hydrogen evolved. The hydrogen evolution of hydrogenase in solution is shown in (c).

2.2.4. Conclusion

LB films of thermostable hydrogenase were successfully prepared on electrodes using poly-L-lysine. Activities of hydrogenase were maintained in the film. Hydrogen production was demonstrated by applying the bias to the no electrode covered with hydrogenase LB film using methyl viologen as a mediator. The electrode was efficient because all the electron transfer

from electrode, MV then to hydrogenase, occurs on the electrode. Much less bias (higher potential) was enough for hydrogen production than electrolysis of water. The results shows that the hydrogenase LB film has a potential for effective electrode system for hydrogen production.

2.3. Genetic Expression of Heterogeneous Hydrogenase

Genetic expression of heterogeneous hydrogenases is desired for application of hydrogenase to biological hydrogen production. The structural genes coding [Ni-Fe] hydrogenase from several organisms have been cloned and sequenced and recent studies revealed an increasing number of genes involved in the expression, processing and assembly of functional [Ni-Fe] hydrogenase (11-16). The expression and activation of [Ni-Fe] hydrogenases seems to be a highly specific and complex process suggesting the involvement of additional genes. In contrast to [Ni-Fe] hydrogenase only the genes coding for [Fe-only] hydrogenase *Clostridium pasteurianum* (18), *C. acetobutylicum* (19) and *D. vulgaris* (20) have been cloned and characterized. Yet, genes required for the regulation and expression of the genes could not be localized (20). So far the heterologous expression of [Ni-Fe] hydrogenase has only been reported for *Desulfovibrio*, where the [Ni-Fe] hydrogenase from *D. gigas* could be expressed in *D. fructosovorans* (17).

Recently, active clostridial hydrogenase was expressed in cyanobacteria. In this section, this first heterologous expression of active hydrogenase is described.

2.3.1. Construction of the plasmid for expression of clostridial hydrogenase

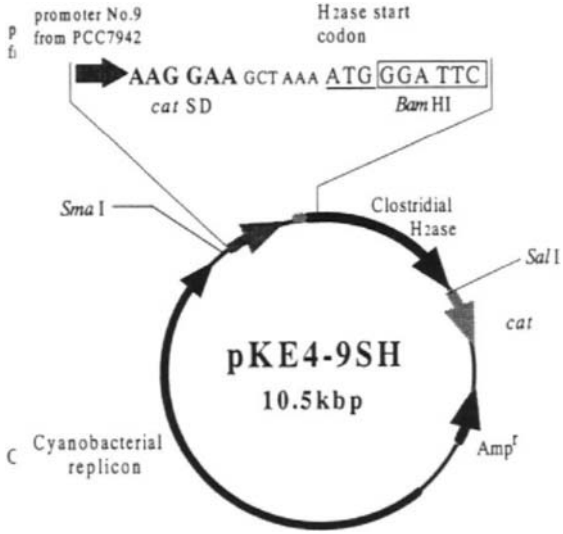


Figure 9: Construction of plasmid pKE4-9SH. The promoter region and the clostridial hydrogenase structure gene were amplified by PCR. Both fragments were ligated at their BamHI restriction sites and cloned into the shuttle vector pKE4.

Fig, 9 shows the structure of the plasmid pKE4-9SH for expression of the clostridial hydrogenase. The putative SD sequence of the gene coding for hydrogenase in *C. pasteurianum* was changed to that of the cat gene (32), which homologous to the SD sequence of the D1 from *Synechococcus* PCC7942 protein (33) and cloned into the shuttle vector pKE4-9 (27). In the plasmid pKE4-9SH the structural gene of the clostridial hydrogenase is flanked by a strong promoter (28), isolated from *Synechococcus* PCC7942 and the cat gene as well as an additional BamH1 site downstream of the initiation codon (Fig. 9). The strong promoter is active in both of *E. coli* and *Synechococcus* PCC7942 (27). Also, the plasmid pKE4-9SH has two replicating *ori* for *E. coli* and *Synechococcus* PCC7942 (27). Therefore, the plasmid is available to compare expression of clostridial hydrogenase between *E. coli* and *Synechococcus* PCC7942.

2.3.2. Expression of clostridial hydrogenase in E. coli and Synechococcus PCC7942

E. coli is not available organism for expression of active clostridial hydrogenase. Northern- and Western blot analyses of *E. coli* (strain JM109), harboring pKE4-9SH, revealed the presence of hydrogenase-specific mRNA as well as an expressed clostridial hydrogenase protein. Expressed clostridial hydrogenase protein was found in both the soluble and the membrane fraction. Even after extensive washing of the membrane fraction significant amounts of expressed hydrogenase remained in the insoluble fraction, suggesting of the partial expression of clostridial hydrogenase in form of inclusion bodies. This suggestion was confirmed by Western blot analysis, revealing strong signal with the solubilized and reconstituted protein fraction derived from isolated inclusion bodies. Both, the soluble fraction and the reconstituted hydrogenase faction from *E. coli* were thoroughly investigated for hydrogenase activity. Neither the soluble protein fraction nor the reconstituted hydrogenase protein expressed any hydrogenase activity.

Northern blot analyses of total mRNA from *Synechococcus* PCC7942, harboring pKE4-9SH, with the clostridial hydrogenase gene as a probe revealed a strong signal. The results obtained from Northern blot analysis are future substantiated by additional Western blot analysis of cell-free extracts from *Synechococcus* PCC7942 harboring pKE4-9SH or pKE4-9 (lacking the structual hydrogenase gene) revealing the presence of expressed clostridial hydrogenase only in cells containing pKE4-9SH.

The hydrogenase from *C. pasteurianum* is extremely oxygen-sensitive and strict anaerobic conditions are required for the induction and maintenance of the hydrogenase activity (34). To provide complete induction and activation of the enzyme, the cells of *Synechococcus* containing pKE4-9SH adapted to anaerobic conditions. *Synechococcus* cells, transformed with plasmid pKE4-9SH, demonstrated a significantly higher H_2-evolution activity than the wild-type cells. Upon anaerobic adaptation the transformant cells and the wild-type cells showed a continuous increase in H_2-production rates, reaching a maximum after approximately 40 hours (Fig.10).

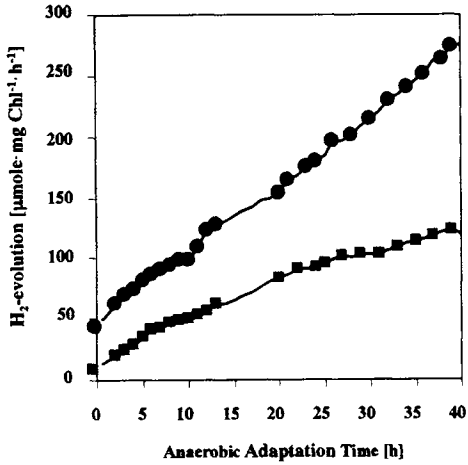


Figure 10: Expression of hydrogenase activity during the anaerobic adaptation of *Synechococcus* PCC 7942 harboring pKE4-9SH. Cells were harvested in their midlogarithmic growth phase and resuspended in fresh growth medium. The cell suspension was repeatedly evacuated and flushed with Argon for 1h. Further anaerobic adaptation was achieved at 30°C in the dark. Determination of hydrogen evolution was started before the dark anaerobic adaptation period and measured amperemetrically using a Clark-type electrode in the presence of 10 µm reduced methylviologen. ●*Synechococcus harboring* pKE4-9SH; ■*Synechococcus*, wildtype cells.

Similar results were obtained with cell-free extracts of anaerobically adapted transformant cells in comparison with extracts of *Synechococcus* wild-type cells. Cell-free extracts of transformant cells showed an H_2-production of 45 $\mu mol \cdot mg^{-1} \cdot h^{-1}$, whereas the cell-free extracts of *Synechococcus* wild-type cells produced only 14 µmol $H_2 \cdot mg^{-1} \cdot h^{-1}$. After the separation of soluble protein fractions from anaerobically adapted transformant cells and *Synechococcus* wild-type cells on a non-denaturing polyacrylamide gel, the subsequent activity staining revealed an additional positive band in the fraction derived from the transformant containing plasmid pKE4-9SH. The additional band is located at the same migration distance as the hydrogenase active protein band from the soluble protein fraction from *C. pasteurianum* (Fig. 11). No additional hydrogenase activity could be detected in the soluble protein fraction from wild-type cells at the corresponding migration distance. The hydrogenase active bands from *Synechococcus* PCC7942 wild-type cells at the top of the gel can be attributed to the indigenous [Ni-Fe] hydrogenase and its aggregative forms.

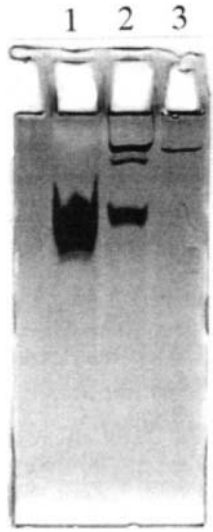


Figure 11: Activity staining of a cell-free extract from the *Synechococcus* transformant. The extracts were separated on a 8% homogeneous nondenaturing polyacrylamide gel. The gel was incubated with menthylviologen (0.125% w/v) and 2,3,5-triphenyltetrazolium chloride (0.125% w/v) in a hydrogen-saturated solution as described in material and methods. Lane 1, soluble protein fraction from *Chostridium pasteurianum*, 0.357 mg.; lane 2, total protein fraction from the transformant harboring pKE4-9SH, 0.383 mg.; lane 3, total protein fraction from *Synechococcus* wildtype cells, 0.429 mg.

At present, the successful heterologous expression of the clostridial [Fe-only] hydrogenase in *Synechococcus* can only be explained for by mechanism, although specific for the expression of the indigenous [Ni-Fe] hydrogenase, sufficiently variable to allow the synthesis, assembly and activation of the clostridial hydrogenase. The *Synechococcus* mutant introduced here demonstrates the possibility to introduce foreign hydrogenase into the cells of *Synechococcus*, resulting genetically engineered organism with a significantly increased hydrogen production capacity. It furthermore represents an ideal model for detailed studies on the expression and regulation of [Fe-only]- and [NiFe] hydrogenases from photosynthetic microorganisms.

3. FUTURE ASPECTS

Current molecular handling techniques of hydrogenase introduced here would be just the entrance of applied technology for hydrogenase: Heterologous expression of hydrogenase allow to modify hydrogenase proteins and produce hydrogenase in the large scale. The molecular handling like LB films allow to develop some fuel-cell-type molecular battery which would be available for medical micromachine. LB film technology would also be available for

development of sensing interface of hydrogen and electricity-chemical conversion device which is available for construction of electric signal transduction system. Pseudotransformation would be useful to build up suitable metabolic systems in the living cells for biological hydrogen production.

Development of the new technology for hydrogenase allow to create basis of clean world mediated by hydrogen.

REFERENCES

1. Arnon, DI, Mitsui, A Arnon and Paneque, A. (1961). Photoproduction of hydrogen gas coupled with photosynthetic phosphorylation. *Science* **134**, 1425.
2. Rao, KK, Gogotov, IN and Hall, DO. (1978). *Biochemie* **60**, 291-296.
3. Adams, MWW, Mortenson, LE and Chen, J-S. (1981). Hydrogenase. *Biochim. Biophys. Acta* **594**, 105-176.
4. Miyake, M and Asada, Y. (1997). Direct electroporation of clostridial hydrogenase into cyanobacterial cells. *Biotechnol. Tech.* **11**, 787-790.
5. Schnackenberg, J, Miyake, M, Miyake, J, Zorin, NA and Asada, Y. (1999). *In vitro* and *in vivo* coupling of *Thiocapsa* hydrogenase with cyanobacterial and algal electron mediators. J. *Biosci. Bioeng.* **88**, 30-34.
6. Gogotov, IN, Zorin, NA Serebriakova, LT and Kondratieva, EN. (1978). The properties of hydrogenase from the phototrophic bacterium *Thiocapsa roseopersicina. Biochim. Biophys. Acta* 523, 335-343.
7. Zorin, NA. (1986). Redox properties and active center of phototrophic bacteria hydrogenases. *Biochimie* **68**, 97-101.
8. Noda, K, Zorin, NA, Nakamura, C, Miyake, M, Gogotov, IN, Asada, Y, Akutsu, H and Miyake, J. (1998). Langmuir-Blodgett film of hydrogenase for electrochemical hydrogen production. *Thin Solid Films*, 327-329.
9. Hirata, Y, Nukanobu, K, Hara, M. (1992). Preparation of stable Langmuir-Blodgett film of photosynthetic bacterial reaction center from *Rhodopseudomonas viridis* using poly-L-lysine. *Chem. Lett.*, 227-230.
10. Greenbaum, E. (1988). Hydrogen production by algal water splitting. In: Lembi, CA Waaland, JR (eds.): Algae and Human Affairs. 238-304 Cambridge Univ. Press, Cambridge.
11. Boichenko, VA and Hoffmann, P. (1994). Photosynthetic hydrogen production in Prokaryotes and Eukaryotes: Occurrence, mechanism and functions. *Photosynthetica* **30**, 527-552.
12. Leclerc, M., Colbeau, A., Cauvin, B. and Vignais, P.M. (1988). Cloning and sequencing of the genes encoding the large and the small subunits of the H_2 uptake hydrogenase (hup) of *Rhodobacter capsulatus. Mol. Gen. Genet.* **214**, 97-104.
13. Cauvin, B., Colbeau, A. and Vignais, P.M. (1991). The hydrogenase structural operon in *Rhodobacter capsulatus* contains a third gene, hupM, necessary for the formation hydrogenase. *Mol. Microbiol.* **5**, 2519-2527.
14. Vignais, P.M. and Toussaint, B. (1994). Molecular biology of membrane bound H_2 uptake hydrogenases. *Arch. Microbiol.* **161**, 1-10.
15. Olson, J.W., Fu, C. and Maier, R.J. (1997). The HypB protein from *Bradyrhizobium japonicum* can store nickel and is required for the nickel-dependent transcriptional regulation of hydrogenase. *Mol. Microbiol.* **24**, 119-128.

16. Durmowicz, M.C. and Maier, R.J. (1997). Roles of HoxX and HoxA in biosynthesis of hydrogenase in *Bradyrhizobium japonicum*. *J. Bacteriol.* **179**, 3676-3682
17. Schwartz, E., Gerischer, U. and Friedrich, B. (1998. Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. *J. Bacteriol.* **180**, 3197-3204.
18. Rousset, M., Magro, V., Forget, N., Guigliarelli, B., Belaich, J.P. and Hatchikian, E.C. (1998). Heterologous expression of the *Desulfovibrio gigas* [Ni-Fe]hyrogenase in *Desulfovibrio fructosovorans* MR400. *J. Bacteriol.* **180**, 4982-2986.
19. Meyer, J. and Gagnon, J. (1991). Primary structure of hydrogenase I from *Clostridium pasteurianum*. *Biochemistry* **30**, 9697-9704.
20. Santangelo, J.D., Durre, P. and Woods, D.R. (1995). Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262. *Microbiology* **141**, 171-180.
21. van den Berg, W.A., Stokkermans, J.P. and van Dongen, W.M. (1993). The operon for the Fe-hydrogenase in *Desulfovibrio vulgaris* (Hildenborough): mapping of the transcript and regulation of expression. *FEMS Microbiol. Lett.* **110**, 85-90.
22. Brosius, J. (1984). Plasmid vectors for selection of promoters. *Gene* **27**, 151-160.
23. Bustos, S.A., Schaefer, M.R. and Golden, S.S. (1990). Different and rapid responses of four cyanobacterial *psbA* transcripts to changes in light intensity. *J. Bacteriol.* **172**, 1998-2004.
24. Miyake, M., Yamada, J., Aoyama, K., Uemura, I., Hoshino, T., Miyake, J. and Asada, Y. (1996). Strong expression of foreign protein in *Synechococcus* PCC7942. *J. Mar. Biotechnol.* **4**, 61-63.
25. Aoyama, K., Miyake, M., Yamada, J., Miyake, J., Uemura, I., Hoshino, T., and Asada, Y. (1996). Application of vector pKE4-9 carrying a strong promoter for the expression of foreign proteins in *Synechococcus* PCC7942. *J. Mar. Biotechnol.* **4**, 64-67.

This Page Intentionally Left Blank

VI. Photobioreactors

This Page Intentionally Left Blank

PRODUCTION OF HYDROGEN BY AN *ANABAENA VARIABILIS* MUTANT IN A PHOTOBIOREACTOR UNDER AEROBIC OUTDOOR CONDITIONS

A.S. Fedorov[1,2], A. A. Tsygankov[1,2], K. K. Rao[2], and D. O. Hall[2]

[1] Institute of Basic Biological Problems RAS,
Pushchino, Moscow Region, 142292, Russia;
[2] Division of Life Sciences, King's College London,
Campden Hill Rd., London W8 7AH, UK.

SUMMARY

This study demonstrates the principal possibility of hydrogen production in an outdoor photobioreactor (PhBR) incorporating a cyanobacterial mutant of *Anabaena variabilis* (PK84) under aerobic conditions. A computer-controlled helical tubular PhBR was operated over 4 summer months. A maximum rate of 80 mL H_2 per hr per reactor volume (4.35 L) was obtained on a bright day (400 $W \cdot m^{-2}$) from a batch culture. Also the culture was grown in chemostat mode at dilution rate D of 0.02 h^{-1}. The maximum efficiency of conversion of light to chemical energy of H_2 in the PhBR was 0.33% and 0.14% on a cloudy and a sunny day, respectively.

INTRODUCTION

An *Anabaena variabilis* mutant PK84 without uptake hydrogenase activity (Mikheeva *et al.*, 1995) has been shown to be able to evolve H_2 with high rates under aerobic (Sveshnikov *et al.*, 1997; Tsygankov *et al.*, 1998b) and simulated outdoor (Borodin *et al.*, 1999) conditions. In spite of the fact that extensive biochemical and physiological H_2 production studies have been performed there are no reports of biohydrogen production by cyanobacteria under outdoor, aerobic condition. Nevertheless, such process would be appealing as the cost of H_2 production would be less under such conditions. The aim of the present study was to demonstrate long-term, continuous and stable hydrogen production in a helical photobioreactor incorporating the cyanobacterium *A. variabilis* PK84 under aerobic outdoor conditions.

MATERIALS AND METHODS

The filamentous heterocystous cyanobacterium *Anabaena variabilis* PK 84, a mutant without uptake hydrogenase activity, was obtained from Prof. S. Shestakov (Moscow State University). The cyanobacterium was maintained and grown in inorganic, double strength Allen and Arnon (1955) medium in the absence of fixed nitrogen with 2 μM Na_3VO_4 instead of a molebdenium salt to synthesise the V-containing nitrogenase (Kentemich *et al.*, 1991; Tsygankov *et al.*, 1997). 20 $mg \cdot l^{-1}$ of actidion (Aldrich) were added to suppress a possible contamination.

Cultivation of the cyanobacterium was performed in a 4.35 L automated helical photobioreactor (PhBR) described by Tsygankov *et al.* (1998a). *A. variabilis* PK 84 was cultivated autotrophically in the PhBR under sterile air containing 2% CO_2 (500 $ml \cdot min^{-1}$). Growth conditions in the PhBR were monitored by a computer system connected to built-in pH, temperature, optical density, pO_2 and sunlight intensity sensors. When necessary 1 or 2 polyurethane foam balls were circulated along with the culture in the PhBR to clean the inner surface of the PVC tubing.

The rate of H_2 production by the culture under growth conditions (the actual rate) was calculated on the basis of the H_2 content in the effluent gas and the gas flow rate in the PhBR. The H_2 content in the effluent gas was measured with a gas chromatograph (Hewlett Packard 5890).

The chlorophyll *a* content of the cells was determined spectrophotometrically at 665 nm in 90% methanol extracts (Tandeau de Marsac and Houmard, 1988). Dry weight of biomass was determined as described by Tsygankov *et al.* (1999).

The efficiency of light energy conversion to H_2 energy by the PhBR incorporating cyanobacterium was calculated as follow:

$$\text{Conversion efficiency} = \frac{\text{Combustion energy of evolved } H_2,\ E_H}{\text{Energy of incident light, } E_I}$$

where $E_I=(I_s \times S_{ps}+I_d \times S_{pd}) \times t$, where S_{ps} - the surface of the PhBR receiving direct light, and S_{pd} - the surface of the PhBR exposed by diffused light, t - time;

$S_{ps}=D \times h \times \cos\alpha + D \times \sin\alpha \times D \times tg\alpha$, where D - the PhBR diameter (55 cm), h - the PhBR height (32 cm), and α - the angle of the sun (0 at sunrise and sunset and 51.5 grad at midday on 21st March; the value was assumed to be approx. 55 grad in July when the measurements were made);

$S_{pd}=3.14 \times D \times h$;

unit for light energy - 1W per hour is 3.3 kJ.

On the figures there are results presented typical operations of the PhBR. The whole data includes a period of over 4 months (not shown).

RESULTS AND DISCUSSION

In spite of a recent report of *A. variabilis* PK84 cultivation under simulated outdoor conditions (Borodin *et al.*, 1999), the behaviour of the organism under natural day-night periods has not been reported. Firstly, the cells were inoculated in the PhBR and grew in batch culture mode (Fig. 1a, 1b).

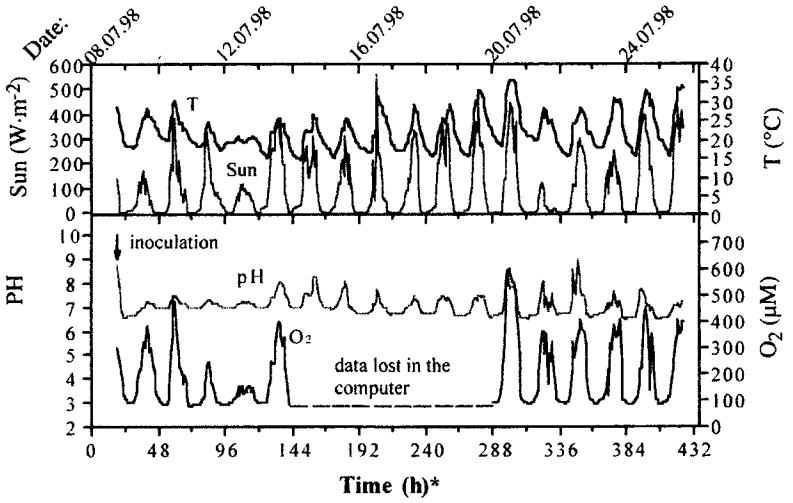


Figure 1a: Physico-chemical parameters tn the outdoor PhBR during the batch cultivation of *Anabaena variabillis* PK84. *18 days from July, 8(18:30) to July, 25(16:50)1998. All sensors connected to the computer and recorder automatically.

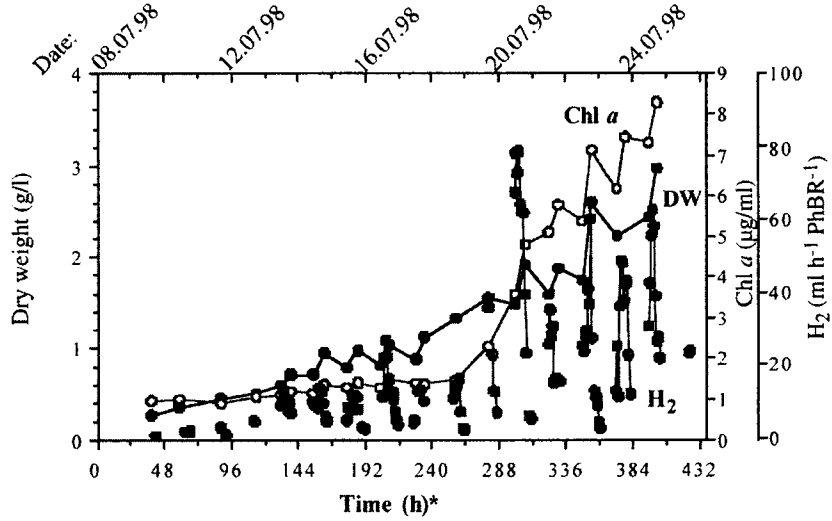


Figure 1b: H_2 production, dry weight and chlorophyll concentration in the outdoor PhBR during the batch cultivation of *Anabaena variabillis* PK84. *18 days from July, 8(18:30) to July, 25(16:50)1998. All parameter measured manually from gas and liquid samples with drawn from the PhBR.

The average irradiance was 300 $W \cdot m^{-2}$ (on one or two sunny days - up to 400 $W \cdot m^{-2}$) and declined to 100 $W \cdot m^{-2}$ on cloudy days. The maximum rate of H_2 production, 80 ml h^{-1} $PhBR^{-1}$, occurred after 12 days of growth when the Chl *a* concentration was 7 mg ml^{-1} and irradiance about 400 $W \cdot m^{-2}$. On that day the PhBR was producing 652.3 ml H_2 (per daytime per PhBR); total light energy incident on the PhBR was 4669 kJ and the efficiency of light energy conversion was 0.14%. On the next (cloudy) day the PhBR was producing 254 ml of H_2 (per daytime per PhBR); total light energy incident on the PhBR surface was 863.5 kJ and the efficiency of light energy conversion was 0.33%. The light conversion efficiency has recently been shown to be ten times higher for a similar H_2 production system. However, a very low light intensity (3 $W \cdot m^{-2}$) was used (Markov *et al.*, 1997). Thus, *A. variabilis* PK84 is able to be grown under natural outdoor conditions and evolve H_2 with high rates aerobically.

Continuous H_2 production by *A. variabilis* PK84 under outdoor, aerobic conditions was shown by the next experiment. The cells growing in batch culture in the PhBR were shifted to the chemostat mode of cultivation at dilution rate D=0.02 h^{-1} (Fig. 2a). The H_2 production rate of 25 to 35 ml h^{-1} $PhBR^{-1}$ was sustainable through out the experimental period of 36 days excepting 2 or 3 dull days. During these days it could be noticed a correlation between the Chl *a* concentration and density of cells and H_2 production (Fig. 2b).

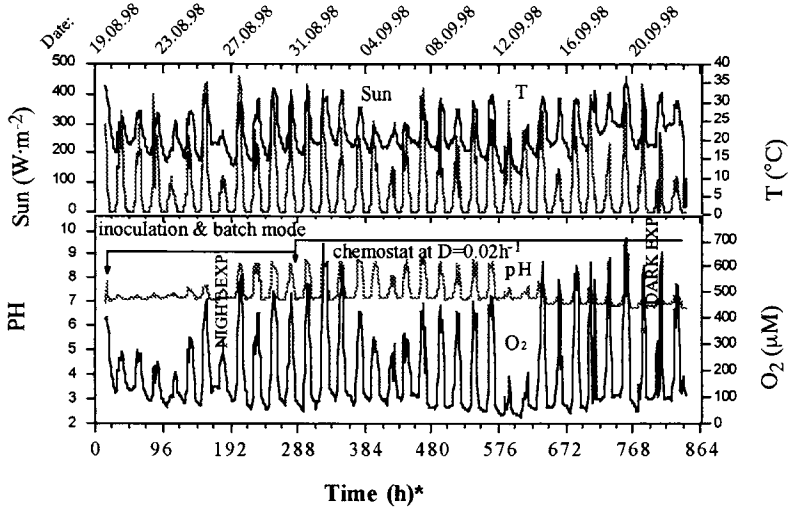


Figure 2a: Physico-chemical parameters tn the outdoor PhBR during the batch cultivation of *Anabaena variabillis* PK84. *36 days from August, 19(16:00) to September, 23(04:00)1998. All sensors connected to the computer and recorder automatically.

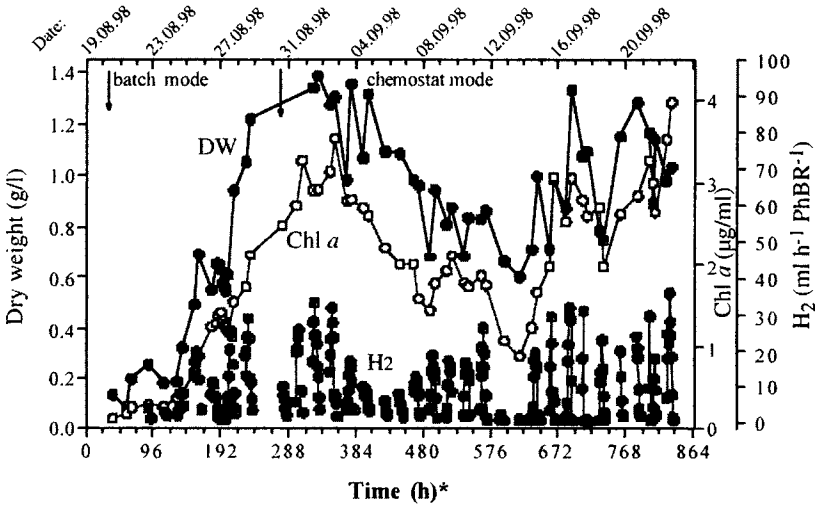


Figure 2b: H_2 production, dry weight and chlorophyll concentration in the outdoor PhBR during the batch cultivation of *Anabaena variabillis* PK84. *36 days from August, 19(16:00) to September, 23(04:00)1998. All parameter measured manually from gas and liquid samples with drawn from the PhBR.

Also, there was no drop in the volumetric rate of H_2 production when the cells were shifted to dilution. Although, some decrease of H_2 evolution occurred because of the weather hydrogen was being produced continuously and stable over a month and even longer (data not shown).

To summarize: we have shown a principal possibility of H_2 production in an outdoor PhBR incorporating *A. variabilis* PK84 under aerobic conditions. This process might be continuous, stable and long-term. Further investigations should be directed to research of factors limiting of efficiency of light energy conversion to chemical H-H bond energy.

ACKNOWLEDGEMENTS

The authors thank Prof. S. Shestakov and Dr. L. Mikheeva of Moscow State University for the kind gift of the PK84 mutant. This work was supported by RITE (Tokyo) whom we gratefully acknowledge.

REFERENCES

1. Allen, M. B., Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. 1. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol.* **30,** 366-372.

2. Borodin, V. B., Tsygankov, A. A., Rao K. K., Hall D. O. (1999). Hydrogen production by *Anabaena variabilis* PK84 under simulated outdoor conditions. *Submitted to Biotechnol. Bioeng.*
3. Kentemich, T., Haverkamp, G., Bothe, H. (1991). The expression of a third nitrogenase in the cyanobacterium *Anabaena variabilis*. *Z. Naturforsch.* **46c**, 217-222.
4. Markov, S. A., Weaver, P. F., Seibert, M. (1997). Spiral tubular bioreactors for hydrogen production by photosynthetic microorganisms. Design and operation. *Appl. Biochem. Biotechnol.* **63-65,** 577- 584.
5. Mikheeva L. E., Schmitz O., Shestakov S. V., Bothe H. (1995). Mutants of the cyanobacterium *Anabaena variabilis* altered in hydrogenase activities. *Z. Naturforsch.* **50c**, 505-510.
6. Sveshnikov, D.A., Sveshnikova, N.V., Rao, K.K., Hall, D.O. (1997). Hydrogen metabolism of mutant forms of *Anabaena variabilis* in continuous cultures and under nutritional stress. *FEMS Microbiol. Lett.* **147**, 297-301.
7. Tandeau de Marsac, N., Houmard, J (1988). Complementary chromatic adaptation: physiological conditions and action spectra. *Methods Enzymol.* **167**, 318-328.
8. Tsygankov, A. A., Serebriakova, L. T., Sveshnikov, D. A., Rao, K. K., Gogotov, I. N., Hall, D. O. (1997). Hydrogen photoproduction by three different nitrogenases in whole cells of *Anabaena variabilis* and the dependence on pH. *Int. J. Hydrogen Energy*, **22**, 859-867.
9. Tsygankov, A.A., Hall, D.O., Liu, J.-G., Rao, K. K. (1998a). An automated helical photobioreactor incorporating cyanobacteria for continuous hydrogen production. In: Biohydrogen 97, (O.R. Zaborsky ed.), Plenum Pub., N.Y. pp. 431-440.
10. Tsygankov, A. A., Serebryakova, L. T., Rao, K. K., Hall, D. O. (1998b). Acetylene reduction and hydrogen photoproduction by wild-type and mutant strains of *Anabaena* at different CO_2 and O_2 concentrations. *FEMS Microbiol. Lett.* **167**, 13-17.
11. Tsygankov, A.A., Borodin, V.B., Rao, K.K., Hall, D.O. (1999). H_2 photoproduction by batch culture of *Anabaena variabilis* ATCC 29413 and its mutant PK84 in a photobioreactor. *Biotechnol. Bioeng.* (in press - Production number: **BB-2884**).

HYDROGEN PHOTOPRODUCTION BY PURPLE BACTERIA: IMMOBILIZED VS. SUSPENSION CULTURES

A. A. Tsygankov

Institute of Basic Biological Problems RAS,
Pushchino, Moscow Region, 142292, Russia

SUMMARY

Factors affecting H_2 photoproduction by purple bacteria are described in this minireview. Based on comparison of maximum H_2 photoproduction rates by suspension and immobilized cultures, it is concluded that immobilized culture is more attractive for application. Possibility for scaling up of photobioreactors with immobilized purple bacteria is discussed.

INTRODUCTION

Development of biotechnological systems for non-polluting fuels as H_2 attracts the attention of researchers. Phototrophic anoxygenic bacteria are able to produce H_2 catalyzed by nitrogenase under diazotrophic or nitrogen-limiting conditions. Although H_2 photoevolution was observed among members of purple sulfur and green sulfur bacteria, intensive work has been done on the purple nonsulfur bacteria where higher rates of nitrogen fixation (hydrogen evolution) were measured.

The potential of purple nonsulfur bacteria for application as biological element in systems of light energy conversion into H_2 energy is discussed.

SPECIFIC RATE OF H_2 PHOTOPRODUCTION BY SUSPENSIONS OF PURPLE BACTERIA AND INFLUENCE OF ENVIRONMENTAL FACTORS

Purple bacteria contain two enzymatic systems involved in hydrogen metabolism: nitrogenase and hydrogenase(s). Nitrogenase produces H_2 under phototrophic conditions. Hydrogenase(s) is responsible mainly for H_2 utilization. Enormous progress in our understanding of peculiarities of these systems in purple bacteria was shown in the last two decades. Genes participating in

nitrogenase (Dean and Jacobson, 1991) and hydrogenase (Vignais and Toussant, 1994) synthesis, maturation and translocation were characterized. Molecular structure and active center composition were described for nitrogenase (Peters et al., 1995) and hydrogenase (Volbeda et al., 1995; 1996). Mechanisms of substrate activation and electron translocation by nitrogenase (Mortenson et al., 1993) and hydrogenase (Thauer et al., 1996) were studied in detail. Control mechanisms that permit purple bacteria to turn their nitrogenase system on and off by covalent modification of their dinitrogenase reductases were found (Ludden and Burris, 1979).

Together with basic investigations, intensive attempts were undertaken to apply the potential of purple bacteria for commercial H_2 photoproduction (for review see Sasikala et al., 1993). The most important parameter for industrial H_2 production by the system is the rate of H_2 evolution per unit of illuminated surface. It depends on the specific H_2 evolving activity of purple bacteria (fundamental property), on the concentration of an active biomass (technological parameter) and on the surface-to-volume ratio (construction parameter).

From an evaluation of the highest specific rates of H_2 photoproduction by different purple bacteria using various organic substrates published by different authors, one notes little success in acceleration of this important parameter during the last two decades (Table 1).

It is difficult to compare the data published by different authors due to differences of strains, physiological conditions, organic substrates, durability of the process, and size and peculiarities of photobioreactors. However, an evaluation of the main factors affecting the specific rate of hydrogen photoproduction can be attempted.

TABLE 1

THE SPECIFIC RATES OF H_2 PHOTOPRODUCTION BY DIFFERENT PURPLE BACTERIA DATA LISTED CHRONOLOGICALLY

Strain	Organic compound for H_2 production	Rate of H_2 production, $\mu l\ h^{-1}\ mg^{-1}$ dw	Author and year of publication
Rhodobacter capsulatus	Pyruvate	130	Hillmer, Gest, 1977
Rb. capsulatus	Malate	176	Weaver et al., 1980
Rhodospirillum rubrum	Lactate	160	Zurrer, Bachofen, 1982
Rb. capsulatus	Lactate	110	Tsygankov et al., 1982
Rhodobacter sphaeroides	Lactate	262	Miyake and Kawamura, 1987
R. rubrum, hup⁻ mutant	Lactate	41	Kern et al., 1994
Rb. sphaeroides	Lactate	75	Fascetti, Todini, 1995
Rhodobacter capsulatus	Lactate	102	Zorin et al., 1996
Rhodobacter capsulatus, hup⁻ mutant	Lactate	135	Zorin et al., 1996
Rhodopseudomonas palustris	Acetate	44.8	Kohring et al., 1996
Rb. capsulatus	Lactate	100	Tsygankov et al., 1998

Environmental factors affecting the specific rate of H_2 production

The specific rate of H_2 photoproduction by purple bacteria depends on the total activity of nitrogenase in the cells and on the environmental factors:

- light intensity, spatial and spectral distribution;
- nature and concentration of nitrogen compounds;
- concentration and nature of reduced compounds for anoxygenic photosynthesis;
- pH, temperature, and salinity of the medium;
- oxygen presence; and
- presence of different metals (Mo, Fe, V, W) and other factors.

Activity of the nitrogenase complex is determined genetically. Taking into account that the content of nitrogenase proteins in *Rb. capsulatus* can reach 40% of cell protein (Jouanneau et al., 1985), we can conclude that an increase of nitrogenase content in cells by superexpression is not a way for acceleration of specific rate of H_2 production by purple bacteria.

Light

Light intensity is an important factor for H_2 production by purple bacteria. Under limiting light intensity, the efficiency of light energy conversion is higher but the rate is lower (Miyake, 1994). The rate of H_2 production by *Rb. sphaeroides* was highest under supersaturating light intensities (Miyake, Kawamura, 1987). However, under supersaturating light intensities H_2 production by this bacterium was not stable and decreased after several hours (Miyake, 1994). In a majority of publications an incident light intensity (I_0) is described. Nevertheless, irradiation of a culture includes additional parameters: average light intensity (I_a) and spatial light distribution (this parameter can be described as the ratio of minimal irradiation (I_{min}) to incident irradiation, I_{min}/I_0). Saturating light intensity in terms of I_0 depends on culture properties, on the cell concentration, and on the thickness of the culture layer. That is why it is difficult to summarize data of different authors on the light saturation as experiments with different strains were done using different cell concentrations and various photobioreactors. In general, light saturation in terms of I_0 for different purple bacteria corresponds to light saturation for growth (Tsygankov and Gogotov, 1990) and lies in the range of 40-80 W m^{-2} (Miyake et al., 1982; Zurrer and Bachofen, 1982; Laurinavichene et al., 1989; Tsygankov et al., 1996). However, in terms of I_a the saturation for H_2 production was shown to be lower (app. 25 W m^{-2}; Tsygankov et al., 1996). Furthermore, it was shown that the light distribution inside the culture influences the H_2 production activity. The culture of *Rb. capsulatus* grown at I_0/I_{min} =3 produced 108 µl h^{-1} mg^{-1} dw at $I_0 \geq 80$ W m^{-2} whereas the culture grown at I_0/I_{min} =260 produced 58 µl h^{-1} mg^{-1} dw and the rate of H_2 evolution did not saturate at I_0=220 W m^{-2} (Tsygankov et al., 1996). Spatial distribution of light inside the suspension of purple bacteria also influenced the efficiency of light energy conversion. For example, cultures of *Rb. capsulatus* grown under homogeneous irradiation used light energy for biomass synthesis more efficient than cultures grown under irradiation with a high gradient (Tsygankov, Laurinavichene, 1996). From these basic observations we can draw a technological conclusion. For efficient light - H_2 energy conversion it is important to apply moderate (40-100 W m^{-2}) light intensities and distribute it with maximum homogeneity.

Nitrogen

An excess of ammonium and other easy utilizable nitrogen compounds repressed the synthesis of nitrogenase in purple bacteria. Even N_2 supplied in non-limiting concentrations decreased the nitrogenase activity and, as a result, the H_2 production rate (Tsygankov and Gogotov, 1982). However, purple bacteria require nitrogen for their metabolism. Under nitrogen starvation the rate of H_2 production decreased (Miyake et al., 1982). For this reason, continuous chemostat cultures with N-limitation are useful (Zurrer and Bachofen, 1982,; Tsygankov et al., 1982, Arik et al., 1996). Using these cultures the degree of N-limitation can be changed simply by variation of the dilution rate. It was found that for *Rb. capsulatus* an optimum of N-limitation exists independently on the source of nitrogen (Yakunin et al., 1987; Tsygankov et al., 1998). However *R. rubrum* has no such optimum: the highest rate of H_2 production was found at minimum rate of dilution (Zurrer and Bachofen, 1982). Summarizing the data obtained with synthetic media and with waste water treatment with simultaneous H_2 photoproduction (for review see Sasikala et al., 1993) it is possible to suggest that for maximum rate of H_2 production purple bacteria should be supplied wit nitrogen in any form available for the culture but with a rate of app. 1-10% of the rate of carbon supplement. However, more experiments are necessary to prove this suggestion.

Electron donors

For anoxygenic photosynthesis, purple bacteria need reduced compounds for their metabolism. Different purple bacteria consume for growth and H_2 production many simple organic acids, some sugars, and ethanol (Hillmer and Gest, 1977), some aromatic compounds (Kohring et al., 1996) and other reduced compounds such as H_2S, sulfur, thiosulfate (van Gemerden, 1984). However, it is important to note that for commercial H_2 photoproduction the use of synthetic media is impossible: the price of H_2 will be extremely high. That is why intensive work has been done to evaluate the purple bacteria's potential in H_2 production with simultaneous waste water treatment (for review see Sasikala et al., 1993; Sasikala and Ramana, 1995).

Waste waters contain many different organic compounds, part of which are not utilized by purple bacteria. If a pure culture of purple bacteria applied to such waste waters, the water treatment will not be complete and H_2 production will not use all the available reduced compounds. For this case a mixed culture of different bacteria should be very useful. In this mixture chemotrophic bacteria should convert the main part of the poorly utilizable compounds to simple organic acids and, possibly, even to H_2. Purple bacteria convert simple organic acids with light energy to the H_2. Several reports have been published using photosynthetic bacteria with *Klebsiella pneumoniae* (Weetal et al., 1981) and *Clostridium butiricum* (Miyake et al., 1984). Future investigations should focus on clarification of the optimal chemotrophic strain(s) and the quantity and composition of purple bacteria. Evidently, different waste waters should be processed by different mixtures of culture. Optimization of the waster water – cultural mixture relation is of prime importance and offers the possibility for entire waste water treatment and elevated H_2 production.

Other possible electron donors from wastes for H_2 production by purple bacteria were studied less intensively. As purple bacteria can utilize reduced sulfur compounds, it is very attractive to use them for treatment of gas mixtures containing high levels of H_2S such as flue or oil gases. The maximum rate of hydrogen production by purple sulfur bacterium *Chromatium* Miami1071 using H_2S was app. 0.03 ml h^{-1} g^{-1} of gel (Ikemoto and Mitsui, 1984). However purple nonsulfur bacteria also can transform H_2S (van Gemerden, 1984). According to our measurements

succinate-limited diazotrophic culture of *Rb. sphaeroides* ATCC 17023 using Na_2S evolved H_2 with the rate 0.5 $\mu l\ h^{-1}\ mg^{-1}$ dw (Tsygankov and Miyake, unpublished data). This is not a very high rate of H_2 production, but taking into account that this culture produced H_2 using succinate with the rate 6.7 $\mu l\ h^{-1}\ mg^{-1}$ dw there is a chance for improvement of the process.

The most important peculiarity of some purple nonsulfur bacteria is that elemental sulfur is deposited extracellularly and does not damage the cell when accumulated in great excess. In this case elemental sulfur is the end product. It is a neutral (in contrast to sulfuric acid produced by purple sulfur bacteria or by green sulfur bacteria) and non-toxic product for bacteria up to high concentration. Thus, this approach to combine light energy conversion into H_2 with simultaneous waste (or oil) gases treatment is very attractive but poorly explored.

pH and Temperature

pH of the cultural suspension producing H_2 is an important parameter as it influences the growth of purple bacteria. Optimum pH for H_2 production by these bacteria corresponds to the optimum pH for growth and biomass accumulation (Tsygankov and Gogotov, 1982; Zurrer and Bachofen, 1982). This is true for the cultures grown in the presence of Mo. When *Rb. capsulatus* grew without Mo in diazotrophic conditions the optimum pH for growth was wider (7-8 and 6.2-8.7 correspondingly). Furthermore, the H_2 production was highest at pH 8.5-9.0 (Tsygankov and Laurinavichene, 1996a).

Temperature is another important factor for hydrogen production. The optimum temperature for long-term H_2 production by purple bacteria (Zurrer and Bachofen, 1982) as well as for nitrogenase activity (Tsygankov et al., 1982) corresponds to the optimum for growth and lies in the 27-35°C region.

Oxygen and Other Factors

Oxygen is a strong repressor of nitrogenase synthesis and activity in purple bacteria. Another negative influence of oxygen on H_2 production is that the efficiency of organic compounds utilization during respiration is lower than during anoxygenic photosynthesis. This was shown for *Rb. capsulatus* (Tsygankov et al., 1983) but it follows for other purple bacteria from the comparison of the stoichiometry of anoxygenic photosynthesis and respiration. That is why the development of biotechnological systems for H_2 production has to be done taking into account the absence of oxygen.

Influence of other factors on H_2 production by purple bacteria was not well studied. The presence of chelating agents like EDTA activated H_2 photoproduction by *R. rubrum* (Kern et al., 1992). It was concluded that EDTA enhanced H_2 photoproduction by inhibition of biosynthesis of Hup hydrogenase and mobilization of iron, thereby activating the biosynthesis of the nitrogenase complex. Ni deficiency enhanced H_2 photoproduction by different purple bacteria due to decreased synthesis of active uptake hydrogenase. Deficiency of Mo initiated synthesis of alternative Fe-nitrogenase by *Rb. capsulatus* but not *Rb. sphaeroides* (Yakunin et al., 1991). However, cells with alternative nitrogenase showed lower rates of C_2H_2 reduction and H_2 production activities (Tsygankov and Laurinavichene, 1993).

In any case it is important to note that H_2 evolving culture must be nitrogen-limited. Deficiency of another element(s) will transfer culture to the double-limited state which is not favorable for H_2 production (Tsygankov et al., 1996). However, a requirement for excess of macro- and micro-elements is not considerative for the biotechnological systems since many

waste waters contain enough of these elements; for example milk factory wastes (Tsygankov et al., 1998), sugar refinery wastes (Vincensini et al., 1981), distillery waste waters (Sasikala et al., 1993), clarified slurry of biogas (Vrati and Verma, 1983) and others.

Summarizing the data on an influence of environmental factors on H_2 production by purple bacteria, and analyzing the experiments with the highest specific rates of H_2 production, it is possible to conclude that most studies used an optimal combination of environmental factors. Thus, optimization of H_2 production by variation of environmental factors is close to a final evaluation.

The Actual H_2 Photoproduction in a Photobioreactor

The rate of H_2 production by one unit of illuminated surface (F_s) depends on the specific activity of cells, on biomass concentration and on the surface-to-volume ratio of photobioreactor:

$$F_s = R_{sp} X_b \frac{V}{S}$$

where R_{sp} is a specific rate of hydrogen production by 1 mg of biomass, X_b is a biomass concentration, S is an illuminated surface, and V is a volume of photobioreactor.

By increasing X_b we can expect an increase of F_s. Experiments with *Rb. capsulatus* showed a complicated dependence of F_s on X_b (Table 2). Specific H_2 production rate (measured in small vials) depended on X_b and was lower at high concentrations of biomass. The potential H_2 production (as recalculated from data measured in small vials) increased at higher biomass concentrations. However, the actual H_2 production by photobioreactor (with surface-to-volume ratio 69 $m^2 \cdot m^{-3}$) increased up to 1.05 g l^{-1} of biomass. The actual H_2 production in the photobioreactor was lower than the potential activity of this culture at any concentration of biomass.

At any biomass concentration the culture was ammonium-limited (data not shown). The increase in incident light intensity (Table 2) affected neither the hydrogen production by the photobioreactor nor the specific activity of the cells. Thus, the culture was not limited by light even at high biomass concentrations. Measurements of residual lactate concentration showed that only at the highest biomass content it was quite low (100 μM), possibly limiting the culture.

Analysis of differences between H_2 production in the photobioreactor and in the small vials revealed three possibilities.

1. Both the potential and the actual rates of H_2 production were measured at saturating light but with a different light path: 13 mm in the photobioreactor and 6 mm in the vials. Possibly the higher light gradient in the photobioreactor (compared with the vials) may account for the decreased level of actual H_2 production. It is known that under high gradient the efficiency of light utilization by this bacterium decreased (Tsygankov and Laurinavichene, 1996).

2. H_2 production by the photobioreactor took place under constant addition of the medium containing ammonium salt and the residual concentration of NH_4^+ was low but not zero, whereas in the vials the ammonium ions were all assimilated. It is known that low concentrations of ammonium affect the nitrogenase activity (Zumft, Castillo, 1978). So, H_2 production in the photobioreactor might be partially inhibited by residual ammonium ions.

3. During H_2 measurements in the vials the gas phase was 100% argon. In the photobioreactor the gas phase was 64-70% H_2 + 36-30% CO_2. Analysis of H_2 influence on the rate of H_2 production in the vials showed that H_2 inhibited H_2 production (data not shown).

TABLE 2

THE SPECIFIC RATE OF HYDROGEN EVOLUTION AND THE VOLUMETRIC RATE OF HYDROGEN EVOLUTION BY AMMONIUM-LIMITED CHEMOSTAT CULTURE OF *RB. CAPSULATUS* AS A FUNCTION OF BIOMASS CONCENTRATION (TSYGANKOV ET AL., 1998)

Biomass, g l^{-1} (X_b)	Specific rate of H_2 production, ml g^{-1} h^{-1} (R_{sp})	Volumetric rate of H_2 production, ml l^{-1} h^{-1} (F_s)	Potential volumetric rate of H_2 production, ml l^{-1} h^{-1}	Incident light intensity, W m^{-2}
0.40	52.0	26.1	28.1	250
0.61	42.6	46.5	35.1	250
1.05	42.7	53.4	60.5	250
1.67	29.7	43.3	67.0	250
1.67	29.7	43.3	67.0	400
2.65	21.9	0	78.3	400
2.65	21.9	0	78.3	1260

Other conditions of cultivation. D=0.08 h^{-1}; the steady state biomass concentration was varied by different concentrations of ammonium salt in the input medium; lactate concentration in the input medium 42 mM; pH 7.0. There was no argon flow through the photobioreactor.

Two Conclusions Follow from This Study.

1. The specific H_2 production activity of purple bacteria decreased with increase of biomass concentration. The most probable reason of that decrease lies in an increased gradient of irradiation in dense culture. It was shown that increased light gradient decreased specific nitrogenase activity of *Rb. capsulatus* (Tsygankov et al., 1996). Two solutions for this problem exist. The first is creation of novel photobioreactors with much higher surface-to-volume ratio. Calculations show that photobioreactors with 10-fold higher surface-to-volume ratio will have the same light gradient at 10-fold higher biomass concentration. So, the creation of novel photobioreactors with surface-to-volume ratio higher than 500 m^2/m^3 (with a possibility for scaling up) is an important technical problem. Another solution is creation of novel strains with decreased pigment content but with the same (or higher) specific nitrogenase activity. Strains with 10-fold lower concentration of pigments will have 10-fold lower light gradient.

2. The actual rate of H_2 production decreased with increase of biomass concentration. All these factors are implicated, namely, light gradient, residual ammonium ions content and high H_2 percentage in gas phase. Possibilities for light regime improvement were discussed above. Inhibition of H_2 production by residual ammonium ions can be avoided by application of two-stage chemostat culture (Fascetti, Todini, 1995). However it complicates the system greatly. Another approach is application of mutants with derepressed nitrogenase synthesis. Inhibition of H_2 production by H_2 can be avoided technologically using argon sparging (Tsygankov et al., 1998) or partial vacuum (Rao and Hall, 1996).

At present, we do not have novel photobioreactors and their creation is questionable at present. So, application of alternative systems incorporating purple bacteria for the light energy conversion is problem of great importance.

H_2 PHOTOPRODUCTION BY IMMOBILIZED PURPLE BACTERIA

Immobilized cultures of microorganisms have some advantages over suspension cultures. A photobioreactor with an immobilized culture is simpler in operation. Effluent from the photobioreactor with immobilized cells is cell-free. This is important for H_2 production with simultaneous waste water treatment. Due to immobilization the biomass concentration can be higher than in suspension. These systems have also some disadvantages. Due to the density of the culture the control of environmental factors (for example, pH and H_2 content) is impossible. This disadvantage can be minimized using a continuous flow regime. Immobilized cells are inhomogeneous in definition, which is why some parts of photobioreactor might be limited by substrate (or inhibited by product). This drawback can be eliminated by special organization of the matrix space.

Purple bacteria have been immobilized on various kinds of gels (Zurrer and Bachofen, 1979; Vincenzini et al., 1982; Francou and Vignais, 1984). In some cases gels limit the rate of H_2 evolution due to a diffusion barrier (Vincenzini et al., 1982b). Immobilization on a porous transparent matrix eliminates this problem. In addition, the transparency of a matrix allows light to be delivered at any point due to multiple refraction of light beams inside the matrix. Glass has the best transparency. Porous glass provides a high surface-to-volume ratio for effective medium exchange. One problem with this material is the difficulty of immobilizing bacteria: negative charge on the glass surface decreases bacterial adsorption.

A method the positive charge modification of the glass surface was proposed (Tsygankov et al., 1993). The ability of various species and strains of phototrophic microorganisms was shown to bind to the activated glass surface including green microalgae, cyanobacteria and anoxygenic photosynthetic bacteria (Tsygankov et al., 1998a).

Photobioreactors with photosynthetic bacteria immobilized on porous glass for H_2 production were fabricated (Tsygankov et al., 1994; Tsygankov et al., 1998a). Photobioreactors were made as a rectangular cuvette with porous glass (thickness, 0.5 mm; pore diameter, 9.8 μm) sheets and tested in continuous flow regime. The influence of medium feed, light intensity, N_2 and O_2 presence on the rate of H_2 production, as well as the conversion of organic substrates, was studied. Photobioreactors based on porous glass produced hydrogen for 37-40 days without major limitations of the process duration. Hydrogen production by photobioreactor occurred using waste water of milk factory (Tsygankov et al., 1998a).

Maximum volumetric rates of H_2 production by purple bacteria immobilized on porous glass were much higher in comparison with suspension cultures of purple bacteria (Table 3, compare with Table 1). However, the highest rate calculated per unit of surface was lower than the 7900 ml h^{-1} m^{-2} recorded for suspension culture (Miyake, Kawamura, 1987) due to very high surface-to-volume ratio (2000 $m^2 \cdot m^{-3}$).

Porous glass is stable, inert to microorganisms, does not have diffusion barriers for substrates and products (as gels have), and is transparent. This is why it is useful in biological light energy conversion systems. Using a surface modification procedure the immobilization of bacteria on glass surfaces is rapid. Unfortunately, porous glass is rather expensive.

Polyurethane (PU) foam has many characteristics similar to porous glass (light penetration, stability, surface-to-volume ratio) and has been used for the immobilization of cyanobacteria (Hall and Rao, 1996). In addition, it is much cheaper than porous glass.

H_2 production by *Rb. sphaeroides* GL-1 immobilized on PU foam in continuous flow photobioreactor was shown to occur for a prolonged period (Fedorov et al., 1998). The rate of H_2 production was not as high as in a photobioreactor with porous glass (Table 3). However, this

rate increased with the time of operation. After 34 days of operation it was evident that the bacteria attached to the PU foam, but even this time was not sufficient for the complete colonization of the matrix (Fedorov et al., 1998). So, relatively low rate of H_2 production is a result of slow colonization of PU foam by bacteria. Evidently, a special method for acceleration of bacterial attachment to the PU surface should accelerate the process of immobilization and improve the rate of H_2 production.

So, purple bacteria immobilized on porous transparent matrix are very perspective for application in biotechnological systems for light energy conversion whereas suspension cultures are very useful for preliminary search of optimum conditions. A relatively low rate of H_2 production based on the illuminated surface does not mean that photobioreactor will occupy a large land area: during the scale-up procedure the configuration of the photobioreactor could be changed. For example, in the case of direct exposure to sunlight the plates of porous matrix with purple bacteria should be fixed as shown in Fig. 1. The angle of inclination can be calculated based light intensity saturation and maximum intensity of sunlight.

TABLE 3
MAXIMUM VOLUMETRIC RATES OF H_2 PRODUCTION BY PURPLE BACTERIA IMMOBILIZED ON POROUS MATRIX

Strain	Matrix	Conditions	H_2 prod., ml h^{-1} ml^{-1} of matrix	H_2 prod., ml h^{-1} m^{-2} surface	Conversion efficiency of organic compound (%)	Reference
Rb. sphaeroides RV	Porous glass	Continuous flow, 10 mM succinate, 1 mM glutamate, 300 W m^{-2}	1.3	660	55	Tsygankov et al., 1994
Rb. sphaeroides GL1	Porous glass	Continuous flow, 10 mM lactate, 1 mM glutamate, 300 W m^{-2}	3.6	1800	75	Tsygankov et al., 1998
Rb. sphaeroides GL1	PU foam	Continuous flow, 10 mM lactate, 1 mM glutamate, 300 W m^{-2}	0.21	1680	86	Fedorov et al., 1998

In the case of application of photobioreactor based on porous matrix together with light concentrators, the sheets with purple bacteria should be fixed as shown in Fig. 2. The distance between sheets as well as the depth of the sheets should be determined experimentally. In both cases an illuminated surface is much lower than an occupied surface.

CONCLUSION

This minireview does not cover all aspects of H_2 photoproduction by purple bacteria. However, based on data reviewed here, we can formulate some requirements for bacteria as possible elements of a system for photobiological H_2 production as well as for photobioreactors incorporating purple bacteria.

1. Decreased quantity of pigments. Suspensions and immobilized cells of purple bacteria for H_2 photoproduction with decreased quantity of pigments (but with very effective photosynthetic apparatus) allow for use of higher concentrations of biomass and, consequently, higher rates of H_2 photoproduction per unit of volume.
2. Insensitivity to combined nitrogen. Strains without switch-off regulation of nitrogenase by ammonium and with derepressed synthesis should produce H_2 with high rate using wastes with high nitrogen content.
3. Absence of uptake hydrogenase and decreased accumulation of storage material. This requirement was not discussed in this minireview but, evidently, all photosynthetic energy coupled to electron donors should be directed to H_2 production.
4. Possibility for assimilation of wide range of organic compounds (or compatibility with chemotrophic bacteria which can convert unconvertible compounds into simple organic compounds).

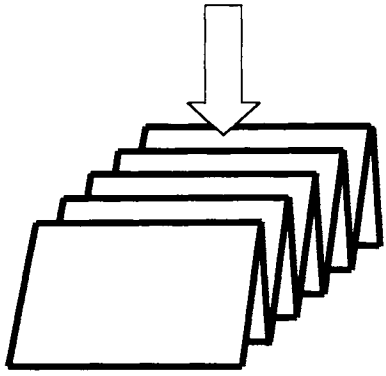


Figure 1: An example of photobioreactor with many porous glass sheets exposed to sunlight.

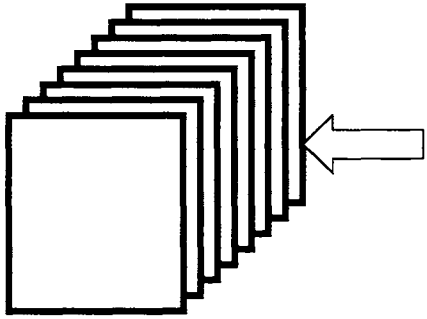


Figure 2: An example of photobioreactor with many porous glass sheets with illumination by intensive light

Evidently, much research is necessary for creation of an "ideal" strain.

At present, photobioreactors with immobilized cells are more attractive than photobioreactors with suspension cultures. It follows from the next:

- immobilized cultures allow use of more dense cultures;
- H_2 production by purple bacteria should be coupled to waste treatment. In this case a bacteria-free effluent from photobioreactor is much more useful. Photobioreactors with immobilized bacteria provide this type of effluent;
- light delivery through a transparent porous matrix is simpler than through a culture suspension.

However, for the creation of a practical, scalable photobioreactor with immobilized culture(s) efforts must consider these questions:

- screening of different transparent porous materials for possible application in photobioreactors;
- development of a method for quick and cheap attachment of bacteria to the matrix;
- special organization of medium flow and gas accumulation to avoid limitations by substrates and inhibition by products;
- development of optimal configuration of photobioreactor for effective light capture; and
- theoretical and experimental search for optimal size of one sheet of matrix as the scalable unit for photobioreactor.

ACKNOWLEDGEMENTS

This work was supported by Japanese-Russian International Project (Biotechnoloical potential of photosynthetic bacteria), INTAS 97-862, the Royal Swedish Academy of Sciences (Cyanobacterial hydrogen and nitrogen metabolism), and CF Lundstrom Foundation (Hydrogen as a future energy-carrier).

REFERENCES

Arik, T., Gunduz, U., Yucel, M., Turker, L., Sediroglu, V., Eroglu, I. (1996). Photoproduction of hydrogen by *Rhodobacter sphaeroides* OU001. In: Hydrogen'96 Designing the energy link. Proc. 11th Hydr. Conf., Stuttgart, Germany. 2417-2426.

Dean, D.R., Jacobson, M.R. (1992). Biochemical genetics of nitrogenase. In: Biological Nitrogen Fixation. (Stacey G. et al. eds). Chapman and Hall, New York. 763-834.

Fascetti, E., Todini, O. (1995). *Rhodobacter* RV cultivation and hydrogen production in one- and two-stage chemostat. *Appl. Microbiol. Biotechnol.* **22**, 300-305.

Fedorov, A.S., Tsygankov, A.A., Rao, K.K., Hall, D.O. (1998). Hydrogen photoproduction by *Rhodobacter sphaeroides* immobilised on polyurethane foam. *Biotechnol. Lett.* **20**, 1007-1009.

Francou, N., Vignais, P.M. (1984) Hydrogen production by *Rhodopseudomonas capsulata* cells entrapped in carrageenan beads. *Biotechnol. Lett.* **6**, 639-644.

Van Gemerden, G. (1984). The sulfide affinity of phototrophic bacteria in relation to the location of elemental sulfur. *Arch. Microbiol.* **139**, 289-294.

Hillmer, P., Gest, H. (1977). H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. *J. Bacteriol.* **129**, 724-731.

Ikemoto, H., Mitsui, A. (1984). Hydrogen photoproduction by marine purple sulfur bacterium *Chromatium*. In: Adv. Photosynth. Res. (Sybesma ed.). Hague. 789-792.

Jouanneau, Y., Wong, B., Vignais, P.M. (1985) Stimulation by light of nitrogenase synthesis in cells of *Rhodopseudomonas capsulata* growing in N-limited continuous cultures. *BBA.* **808**, 149-155.

Kern, M., Klipp, W., Klemme, J.-H. (1994). Increased nitrogenase-dependent H_2 photoproduction by hup mutants of *Rhodospirillum rubrum*. *Appl. Env. Microbiol.* **60**, 1768-1774.

Kohring, G.-W., Fissler, J., Schirra, C., Giffhorn, F. (1996). Hydrogen production from aromatic acids by liquid cultures and immobilized cells of *Rhodopseudomonas palustris*. In: Hydrogen'96 Designing the energy link. Proc. 11th Hydr. Conf., Stuttgart, Germany. 2743-2748.

Laurinavichene, T.V., Vasilieva, L.G., Tsygankov, A.A., Gogotov, I.N. (1989). The purple nonsulfur bacterium *Rhodobacter sphaeroides* isolated from the rice field of South Vietnam. *Mikrobiologiya (Russ.)* **57**, 810-815.

Ludden, P.W., Burris, R.H. (1979). Removal of an adenine-like molecule during activation of dinitrogenase reductase from *Rhodospirillum rubrum*. *Proc. Natl. Acad. Sci. USA.* **76**, 6201-6205.

Miyake, J., Tomizuka, N., Kawamura, S. (1982). Effect of glutamate on the production of hydrogen by *Rhodospirillum rubrum*. *Rep. Ferm. Res. Inst.* **58**, 25-31.

Miyake, J., Mao, X.Y., Kawamura, S. (1984). Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butiricum*. *J. Ferment. Technol.* **62**, 531-535.

Miyake, J., Kawamura, S. (1987). Efficiency of light energy conversion to hydrogen by the photosynthetic bacterium *Rhodobacter sphaeroides*. *Int. J. Hydr. Energy* **12**, 147-149.

Miyake, J. (1994). Photosynthetic bacteria for solar energy conversion. In: Proc. 6th Eur. Cong. Biotechnol. (Alberghina et al. eds.) 1019-1025.

Mortenson, L.E., Seefeldt, L.C., Morgan, T.V., Bolin, J.T. (1993). The role of metal-clusters and MgATP in nitrogenase catalysis. *Adv. Enzymol.* **67**, 299-373.

Peters, J.W., Fisher, K., Dean, D.R. (1995). Nitrogenease structure and function - a biochemical-genetic perspective. *Ann. Rev. Microbiol.* **49**, 335-366.

Rao, K.K., Hall, D.O. (1996). Hydrogen production by cyanobacteria: potential, problems and prospects. *J. Mar. Biotechnol.* **4**, 10-15.

Sasikala, K., Ramana, Ch.V., Rao, P.R., Kovacs, K.L. (1993). Anoxygenic phototrophic bacteria: physiology and advances in hydrogen production technology. *Adv. Appl. Microbiol.* **38**, 211-295.

Sasikala, K., Ramana, Ch.V. (1995). Biotechnological potentials of anoxygenic phototrophic bacteria. II. Biopolyesters, biopesticide, biofuel, and biofertilizer. *Adv. Appl. Microbiol.* **41**, 227-278.

Thauer, R.K., Klein, A.R., Hartmann, G.C. (1996). Reactions with molecular hydrogen in microorganisms. Evidence for a purely organic hydrogenation catalyst. *Chem. Rev.* **96**, 3031-3042.

Tsygankov, A.A., Gogotov, I.N. (1982). Influence of temperature and pH of the medium on the nitrogenase and hydrogenase activity of nitrogen-fixing *Rhodopseudomonas capsulata. Mikrobiologiya (Russ.)* **51**, 396-401.

Tsygankov, A.A., Pavlova, E.A., Gogotov, I.N. (1982). Activity of certain enzymes participating in the metabolism of H_2 in *Rhodopseudomonas capsulata* depending on the conditions of growth of the cultures. *Mikrobiologiya (Russ)* **51**, 188-192.

Tsygankov, A.A., Yakunin, A.F., Gogotov, I.N. (1983). Hydrogenase activity of Rhodopseudomonas capsulata growing on organic media. *Mikrobiologiya (Russ.)* **52**, 543-547.

Tsygankov, A.A., Gogotov, I.N. (1990). Biomass production of purple bacteria. *Prikl. Biokhim. Mikrobiol. (Russ)* **26**, 819-824.

Tsygankov, A.A., Hirata, Y., Asada, Y., Miyake, J. (1993). Immobilization of the purple nonsulfur bacterium *Rhodobacter sphaeroides* on glass surfaces. *Biotechnol. Techn.* **7**, 283-286.

Tsygankov, A.A., Laurinavichene, T.V. (1993). *Rhodobacter capsulatus - growth and nitrogen fixation in the presence and absence of molybdenum. Mikrobiologiya (Russ.)* **65**, 499-504.

Tsygankov, A.A., Hirata, Y., Miyake, M., Asada, Y., Miyake, J. (1994). Photobioreactor with photosynthetic bacteria immobilized on porous- glass for hydrogen photoproduction. *J. Ferm. Bioeng.* **5**, 575-578.

Tsygankov, A.A., Laurinavichene, T.V. (1996). Influence of the degree and mode of light limitation on growth characteristics of the *Rhodobacter capsulatus* continuous cultures. *Biotechnol. Bioeng.* **51**, 605-612.

Tsygankov, A.A., Laurinavichene, T.V. (1996a). Dependence of growth and nitrogenase activity on the illumination and pH in *Rhodobacter capsulatus* with and without Mo. *Microbiology.* **65**, 436-441.

Tsygankov, A.A., Laurinavichene, T.V., Gogotov, I.N., Asada, Y., Miyake, J. (1996). Switching from light limitation to ammonium limitation in chemostat cultures of *Rhodobacter capsulatus* grown in different types of photobioreactor. *J. Mar. Biotechnol.* **4**, 43-46.

Tsygankov, A.A., Fedorov, A.S., Laurinavichene, T.V., Gogotov, I.N., Rao, K.K., Hall, D.O. (1998). Actual and potential rates of hydrogen photoproduction by continuous culture of purple non-sulphur bacterium *Rhodobacter capsulatus*. *Appl. Microbiol. Biotechnol.* **49**, 102-107.

Tsygankov, A.A., Fedorov, A.S., Talipova, I.V., Laurinavichene, T.V., Miyake, J., Gogotov, I.N. (1998a). Application of immobilised phototrophic bacteria for waste water purification and hydrogen production. *Appl. Biochem. Microbiol.* **34**, 362-366.

Vignais, P.M., Toussaint, B. (1994). Molecular-biology of membrane-bound H_2 uptake hydrogenases. *Arch. Microbiol.* **161**, 1-10.

Vincenzini, M., Balloni, W., Manneli, D., Florenzano, G. (1981). A bioreactor for continuous treatment of waste-waters with immobilized cells of photosynthetic bacterua. *Experimentia.* **37**, 710-711.

Vincenzini, M., Materassi, R., Tredici, M.R., Florenzano, G. (1982). Hydrogen production by immobilized cells. I. Light-dependent dissimilation of organic substances by *Rhodopseudomonas palustris*. *Int. J. Hydr. En.* **7**, 231-236.

Vincenzini, M., Materassi, R., Tredici, M.R., Florenzano, G. (1982b). Hydrogen production by immobilized cells. II. H_2-photoevolution and wastewater treatment by agar-entrapped cells of *Rhodopseudomonas palustris* and *Rhodospirillum molishianum*. *Int. J. Hydr. En.* **7**, 725-728.

Volbeda, A., Charon, M.H., Piras, C., Hatchikian, E.C., Frey, M., Fontecillacamps, J.C. (1995). Crystal-structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. *Nature* **373**, 6515, 580-587.

Volbeda, A., Garcia, E., Piras, C., deLacey, A.L., Fernandez, V.M., Hatchikian, E.C., Frey, M., Fontecillacamps, J.C. (1996). Structure of the [NiFe] hydrogenase active site: Evidence for biologically uncommon Fe ligands. *J. Amer. Chem. Soc.* **118**, 12989-12996.

Vrati, S., Verma, J. (1983). Production of molecular hydrogen and single cell protein by *Rhodopseudomonas capsulata* from caw dung. *J. Ferment. Technol.* **19**, 199-202.

Weetall, H.H., Sharma, B.P., Detar, C.C. (1981). Photo-metabolic production of hydrogen from organic substrates by free and immobilized mixed cultures of *Rhodospirillum rubrum* and *Klebsiella pneumoniae*. *Biotechnol. Bioeng.* **23**, 605-614.

Weaver, P.F., Lien, S., Seibert, M. (1980). Photobiological production of hydrogen *Sol. Energy* **24**, 3-45.

Yakunin, A.F., Tsygankov, A.A., Gogotov, I.N. (1987). Regulation of two forms of nitrogenase in a continuous culture of *Rhodobacter capsulatus*. *Mikrobiologiya (Russ.)* **56**, 916-921.

Yakunin, A.F., Tsygankov, A.A., Troshina, O.Yu., Gogotov, I.N. (1991). Growth and nitrogenase activity of continuous cultures of the purple bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* depending on the presence of Mo, V, and W in the medium. *Mikrobiologiya (Russ.)* **60**, 41-46.

Zorin, N.A., Lissolo, T., Colbeau, A., Vignais, P.M. (1996). Increased hydrogen photoproduction by *Rhodobacter capsulatus* strains deficient in uptake hydrogenase. *J. Mar. Biotechnol.* **4**, 28-33.

Zumft, W.F., Castillo, F. (1978). Regulatory properties of the nitrogenase from *Rhodopseudomonas palustris*. *Arch. Microbiol.* **117**, 53-57.

Zurrer, H., Bachofen, R. (1979). Hydrogen production by the photosynthetic bacterium *Rhodospirillum rubrum*. *Appl. Environ. Microbiol.* **37**, 789-793.

Zurrer, H., Bachofen, R. (1982). Aspects of growth and hydrogen production of the photosynthetic bacterium *Rhodospirillum rubrum* in continuous culture. *Biomass.* **2**, 165-174.

This Page Intentionally Left Blank

PHOTOBIOREACTOR DESIGN FOR PHOTOBIOLOGICAL PRODUCTION OF HYDROGEN

J. C. Ogbonna and H. Tanaka

Institute of Applied Biochemistry,
University of Tsukuba, Tsukuba, Japan.

SUMMARY

Biohydrogen production by a biophotolysis process is limited by many problems which include low light intensity at which biohydrogen production saturates, low light conversion efficiency, photoinhibition of cell growth and hydrogen production at high solar light intensities, hydrogen uptake/oxidation under dark condition and sensitivity of the key enzymes (hydrogenase and nitrogenase) to oxygen. Although numerous efforts are underway to develop cell strains which can overcome these problems, some of the problems can be reduced by development of efficient photobioreactors. For example, the problems of photoinhibition and dark hydrogen uptake can be reduced by using a photobioreactor which is uniformly illuminated at the optimum light intensity. Also the problem of sensitivity of the enzymes to oxygen can be minimized by using a photobioreactor with high mass transfer capacity so that the generated oxygen can be simultaneously and efficiently removed from the reactor. Thus efficient biohydrogen production can be achieved by using specifically designed photobioreactors. It is technically possible to design such photobioreactors by internally illuminating tank type photobioreactors which can be aerated and agitated just as ordinary stirred tank bioreactors. However, the construction and operation costs for such systems are expected to be comparatively high.

Environmental, rather than commercial, issues should be the driving force in biohydrogen R&D. Thus, development of innovative technologies for efficient biohydrogen production should be encouraged no matter the present costs of such systems. The technologies used for such systems may become cheaper while environmental problems and future circumstances may favor the use of such systems instead of the present seemingly cheap systems. Furthermore, the aims of research on biohydrogen production should not be limited to developing just commercially

competitive production systems. Efficient personal or community scale production systems may be more practical even though such small scale systems may be more expensive in terms of unit production than large scale systems.

INTRODUCTION

Presently, the world economy depends almost entirely on the use of fossil fuel but the present world oil reserve will eventually be exhausted, given the present increase in the rate of consumption (due to both increase in the world population and industrialization). Moreover, the environmental problems associated with the use of fossil fuel are more immediate than the energy problem per se. People are now very much concerned about the increasing pollution of the atmosphere. There has been a 25% increase in the atmospheric carbon dioxide within the past 150 years (Barnola et al., 1987) and a probable link between the atmospheric carbon dioxide and the global temperature has been suggested by several authors (Genthon et al., 1987). Higher plants play a major role (through photosynthesis) in maintaining the balance between the atmospheric oxygen and carbon dioxide concentrations. However, increase in the world population and industrialization have led to considerable deforestation with consequent decrease in the sink for carbon dioxide. Thus, much research is now focussed on development of sustainable and clean energy sources. Biohydrogen is one of the most promising candidates as a clean and renewable energy source (Miura, 1995).

Many systems for biological production of hydrogen are being investigated but biophotolysis is an ideal system for production of a sustainable and clean energy source. With this system, hydrogen is produced from abundant natural resources (water and solar light energy) and it is coupled with CO_2 mitigation. Although research on hydrogen production by biophotolysis process has been going on for decades, only limited progress has been made due to many scientific and technological problems which include:

1) The low light intensity at which biohydrogen production saturates;
2) Low light conversion efficiency;
3) Photoinhibition of cell growth and hydrogen production at high solar light intensities;
4) Hydrogen uptake/oxidation under dark condition;
5) Sensitivity of the key enzymes (hydrogenase and nitrogenase) to oxygen; and
6) Technical problems of designing and constructing efficient photobioreactors.

These problems have been the focus of many investigations. For example, production of cells with reduced chlorophyll contents (antenna size) is being investigated as a means of solving the first two problems (Melis et al., 1998; Vasilyeva et al., 1998). Also screening for, and genetic engineering research for nitrogenase and hydrogenase, enzymes which are not sensitive to oxygen is being done by many workers (Ghirardi et al., 1997), while cells without or with reduced hydrogen uptake hydrogenase activity has been reported (Mikheeva et al., 1995). Although, at least in principle, the first five problems can be solved by genetic/molecular biology research, some of these problems can be minimized by designing and constructing appropriate

photobioreactors. For example, designing a photobioreactor where optimum light intensity is uniforming distributed within the reactor can reduce problems 1)~ 4) while a photobioreactor with high mass transfer capacity can be used for efficient removal of the generated O_2 and thus minimize problem 5). Thus, efficient biohydrogen production can be achieved by using photobioreactors which are specifically designed to overcome the above problems.

DESIGNING PHOTOBIOREACTORS WITH GOOD LIGHT SUPPLY COEFFICIENT

Perhaps, this is the most difficult problem in photobioreactor design. Because of rapid light attenuation in a culture, a very high illuminated surface to volume ratio is required. Consequently, shallow open cultivation ponds have been successfully used for cultivation of some strains of photosynthetic cells. However, these open systems are not efficient and, due to serious contamination problems, they have been used only for the strains that can grow under conditions very unfavorable for the growth of other strains. Furthermore, open systems cannot be applied for direct biohydrogen production without major modifications for collection of the produced hydrogen. They can be used for the starch production stage of indirect two stage or multistage processes but they are not suitable for the direct biophotolysis or the light-driven hydrogen production stage of the indirect process because light distribution in such reactors is very heterogeneous.

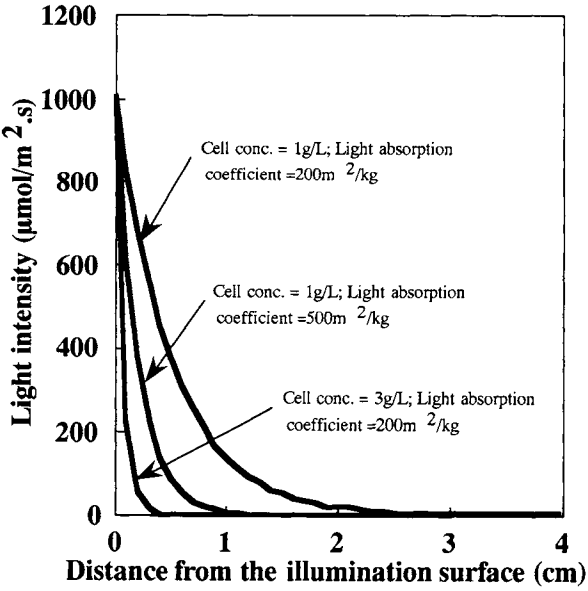


Figure 1: Effects of cell concentration and light absorption coefficient on light extinction inside photobioreactor.

A lot of research is now focused on reducing the antenna size of the photosynthetic cells (Melis et al., 1998). Aside from the physiological effects of antenna size reduction, it also helps to reduce mutual shading and thus improves light penetration into the culture (Fig. 1). However, when the incident light intensity is 1000 μmol/m^2, less than 5 mm of the culture broth containing 3 g/L cells would be illuminated even if the light absorption coefficient is reduced from 500 m^2/kg to 200 m^2/kg (achieved by reducing the chlorophyll contents of the cells). There is a limit to which the light absorption coefficient can be reduced. With cells devoid of chlorophyll, the light penetration zone is still very narrow especially at high cell concentrations. Thus even for very thin photobioreactors with optical paths of 1 cm, light inhibition, light saturation, light limitation and complete dark zones can exist simultaneously within the same reactor (Fig. 2).

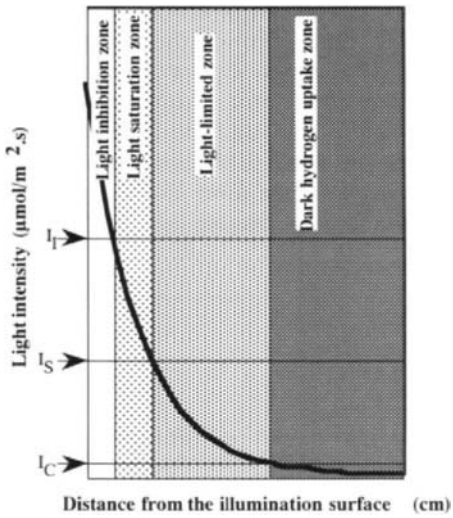


Figure 2: Light distribution pattern inside photobioreactors.

Ordered pattern of bidirectional mixing can be used to move the cells between these zones and thus reduce the problems of photoinhibition and dark hydrogen uptake but it is technically very difficult to operate such a mixing system in large scale photobioreactors. In most photobioreactors, mixing is random (Ogbonna et al., 1995a) and probability analyses have shown that cells in a reactor under turbulent flow would experience different durations of light and dark periods (Powell et al., 1965). In the case of tubular photobioreactors, broth flow and thus cell movement through the tube approaches a plug flow whether air or pumps are used for broth circulation.

Another possible method of ensuring that the whole reactor is illuminated would be to increase the incident light intensity but this has only limited effects in increasing the light penetration

zones while such a measure will increase the problem of photoinhibition. Using a very narrow tubes or thin panels may be effective in reducing the dark zone but constructing large scale production systems with tubes less than 5 mm in diameter will be difficult and mixing would become a problem as will be discussed later. Also under high light intensity, the proportion of the light inhibition zone in very narrow tubes or thin panels will be very high with consequent decrease in the productivity of the system.

The above discussion points out that the most effective method of avoiding the problems of the simultaneous existence of light inhibition, light saturation, light limitation and dark zones in the same photobioreactor is to use an internal illumination method to distribute an optimum light intensity uniformly inside the photobioreactor. Many people have designed such photobioreactors using very fine optical fibers (Mori, 1985; Matsunaga et al., 1991). In such systems, optical fibers were inserted directly inside the photobioreactor so that even for very small diameter photobioreactors, several hundreds of optical fibers would be required. In terms of light distribution, these photobioreactors are very efficient. However, they are structurally complex and thus difficult to maintain and scale up. Furthermore, only bubble aeration can be used for mixing and, as will be discussed later, broth mixing is poor with consequent cell sedimentation and adhesion on the fiber surfaces.

In order to overcome such problems, we designed a photobioreactor where fluorescent lamps, or light radiators to which optical fibers are connected, are used for internal illumination. Good light distribution within the reactor can be achieved by determining the optimum spacing between the lamps. Reactors of various diameters are equipped with a centrally placed lamp and the optimum diameter of the reactor which can be efficiently illuminated by the single light source is determined experimentally (Fig. 3). The optimum diameter will depend on the light intensity, the cell concentration, and the critical light intensity at which cell growth stops or dark hydrogen uptake starts. The optimum diameter can also be calculated when we have all the required parameter values but the most accurate method is to determine it experimentally so as to avoid making many assumptions - some of which may not be valid under the actual production conditions.

In order to construct a large scale photobioreactor, the optimum diameter with the single light source is considered as a single unit and the desired photobioreactor size can be obtained by increasing the number of units in three dimensions as shown in Figure 3 (Ogbonna and Tanaka, 1997; Ogbonna et al., 1996, 1998). Depending on the size of the reactor (number of units), different unit arrangements can be used to achieve this, but it is easiest if 4^n units (n = integer) are used as the base, and the desired reactor size is then obtained by increasing the height (increasing the number of units in a vertical direction). For example, to construct a reactor comprising of 48 units, 16 units (n=2) can be used as the base and the height increased 3 times. By this method, the light condition inside the large photobioreactor will be the same as that in the single unit. This "unit concept" can be used for construction of large scale internally or externally illuminated photobioreactors of any geometry and it is believed that by using this method, a very efficient photobioreactor for biohydrogen production can be constructed. In conclusion, internal illumination seems to be the most effective and practical method of avoiding light intensity heterogeneity inside large scale photobioreactors. By use of the "unit concept", it is technically easy to design and construct efficient large scale internally illuminated photobioreactors.

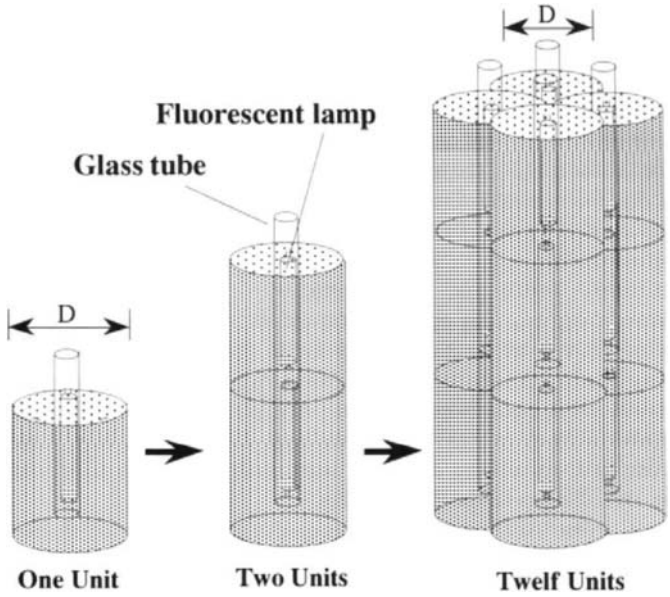


Figure 3: A unit concept" method for construction of a large scale photobioreactor with the same light condition as a small photobioreactor.

DESIGNING PHOTOBIOREACTORS WITH HIGH MASS TRANSFER CAPACITY

In bioreactors, aeration with or without agitation is used to supply the substrate gas to the cells, remove the gas products, keep the cells in suspension, and distribute both the nutrients and the heat generated within the bioreactor. An additional function of mixing in photobioreactors is that of moving the cells in and out of the illuminated part of the photobioreactor and thus decreasing the problem of light shading and lowering the probability of photoinhibition.

In a biophotolysis process, production of hydrogen is coupled to oxygen generation. Unfortunately, the key enzymes in hydrogen production (nitrogenase and hydrogenase) are both sensitive to oxygen. Thus oxygen inhibition of the enzymes is a big problem in biohydrogen production especially in a direct biophotolysis process. The problem is reduced in indirect processes since the oxygen evolution stage is separated in time or space. However, even in the indirect biophotolysis where algae are used in the light-driven hydrogen production phase, photosynthesis is easily activated during the light phase so that oxygen evolution remains a problem.

By designing a photobioreactor with good mass transfer capacity, the generated oxygen can be efficiently removed from the reactor, thus improving the productivity and stability of the system. Furthermore, in systems where the photosynthetic phase is separated from the hydrogen

evolution phase during which a carrier gas is used to sweep out the oxygen, the efficiency of replacing all the dissolved gasses (CO_2 and O_2) with the carrier gas depends on the mass transfer capacity of the photobioreactor. The level of oxygen which can be tolerated depends on the enzyme but it has been demonstrated that with green algae, a very high light conversion efficiency can be achieved only at low oxygen partial pressure (Greenbaun, 1988).

Aside from the light supply problems discussed earlier, the mass transfer capacity of the currently used open cultivation ponds is not enough for hydrogen production. The aeration/mixing systems (for example, using the blade wheel mixing system shown in Fig. 4) may be enough to supply the inorganic carbon, either in the form of carbon dioxide or bicarbonate, but not enough to remove the photosynthetically generated oxygen.

Direct bubbling with a mixture of air and CO_2 is used in many closed photobioreactors. The mass transfer capacity (as measured by the volumetric oxygen transfer coefficient, k_La) increases with decrease in the bubble diameters (achieved by using spargers with very small diameter pores) and with increase in the aeration rate. In vertical columns (Fig. 4), the gas transfer from the gas bubbles to the medium is high since the gas bubbles remain submerged in the broth until they exit the reactor. Thus bubble column bioreactors have been used for many biological processes.

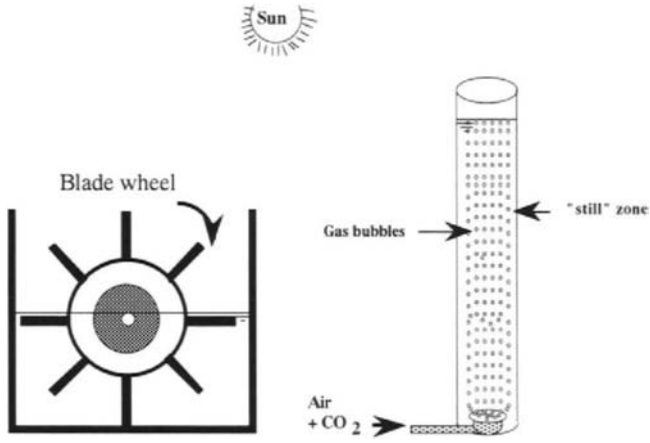


Figure 4: Conventional methods of aeration and mixing photobioreactors by blade wheel or direct gas bubbling.

However, in the case of very shallow photobioreactors (e.g., horizontal thin panels), the mass transfer from the bubbles to the medium is low because of the very short contact time between the bubbles and the medium before they escape from the reactors. Furthermore, air bubbling alone is often not sufficient to achieve good broth mixing which is necessary for degassing the

produced gasses. As shown in Figure 4, "still" (unmixed) zones often exist very close to the reactor wall, leading to cell adhesion on the reactor walls. This problem may be solved by using a coiled sparger that covers the entire base area of the photobioreactor. However, with such a system, a very high pressure (high air flow rate) is needed to distribute the bubbles along the entire sparger. In ordinary bioreactors, the problem of liquid mixing has been solved by the use of air lift systems equipped with external loop or internal draft tubes (Fig. 5).

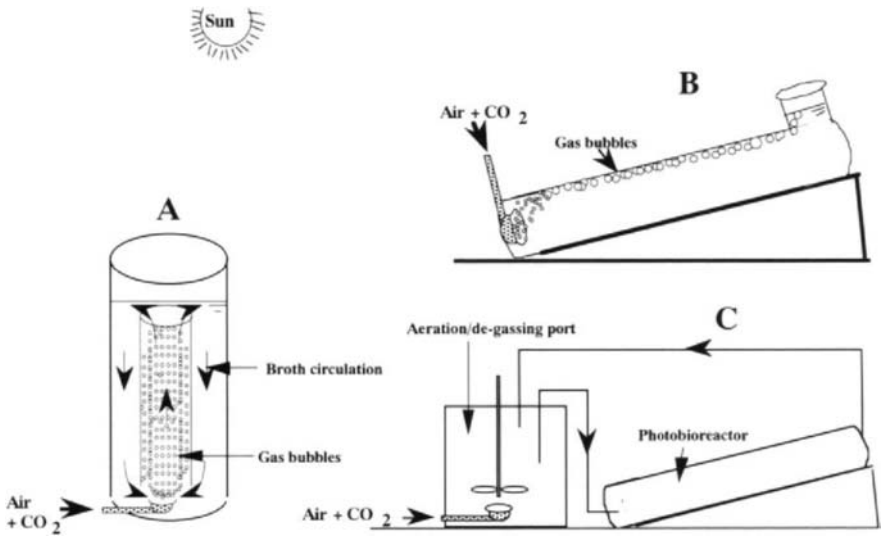


Figure 5: Various types of mixing systems. A: direct gas bubbling in a column equipped with an internal draft tube, B: direct gas bubbling in an inclined column and C: Broth circulation through an aeration/degassing port.

However, such systems will be difficult to apply to large scale photobioreactors because of the need to keep the illumination surface area to volume ratio high. Again, direct air bubbling is efficient for gas transfer to the medium only in vertically oriented systems. Unfortunately, the efficiency of solar light interception per unit reactor volume is low when a photobioreactor is placed vertically. Thus most tubular photobioreactors as well as thin panels are usually placed at an inclined angle. The optimum angle of inclination depends on the location (average insolation for the area) and the process (the optimum light intensity). When inclined orientation is applied, the gas supply capacity decreases sharply. This is because the gas bubbles coalesce into big bubbles and form an interface between the reactor wall and the liquid broth (Fig. 5). Thus the contact area between the culture broth and the gas bubbles is greatly reduced.

An effective method which has been used for gas supply and degassing of evolved gasses is to circulate the broth between the photobioreactor and an aeration/degassing port. The conventional method of aeration and agitation in bioreactors can be adapted so that by optimizing the aeration and agitation speed, very high mass transfer can be achieved inside the port. However, even with very high mass transfer in the port, CO_2 limitation and O_2 inhibition may occur inside the photobioreactor. This problem can be solved by keeping the residence time of the culture in the photobioreactor very short. In the case of tubular photobioreactors, this is achieved by reducing the length of the tubes. The optimum length of the tube depends on the rate of gas exchange in the port, the rate of photosynthesis, the maximum non-inhibiting O_2 concentration, the minimum non-limiting CO_2 concentration, and the broth circulation rate. When pumps are used for circulation, very high circulation rates can be achieved by increasing the pumping speed. However, depending on the type of pump and the type of photosynthetic cells, hydrodynamic stress due to pumping can be a serious problem. In order to save energy and avoid stress due to pumping, airlift systems can be used but the circulation rate is usually low.

It would be better to do the aeration and degassing directly inside the photobioreactor. Aerated stirred tank photobioreactors can be optimized in terms of aeration (aeration gas composition, gas flow rates and types of spargers) and agitation (types of impellers and agitation rates) for efficient gas supply into, and removal of the generated gas from, the photobioreactor in the same way as is currently done for ordinary bioreactors. As discussed earlier, by using an internal illumination systems, a homogeneously illuminated aerated stirred tank photobioreactor can be constructed. In ordinary bioreactors, various types of turbine impellers (Figs. 6A, B and C) are used.

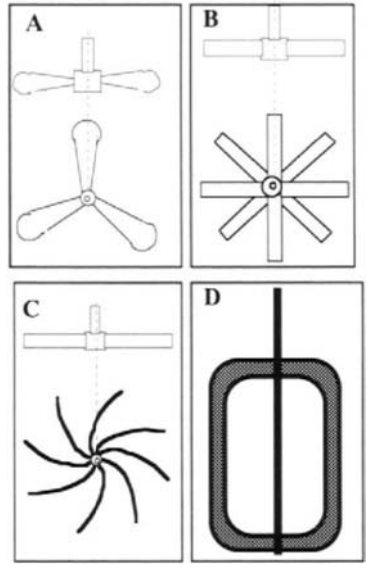


Figure 6: Various types of turbine (A, B and C) and paddle (D) impellers.

However, the hydrodynamic stress generated by the turbine impellers may be too high (depending on the agitation speed) for some photosynthetic cells without cell walls such as *Euglena* (Ogbonna et al., 1998a), some mobile cells such as *Haematococcus* as well as filamentous ones such as *Spirulina*. Moreover, with internal illumination, it will be difficult to include turbine impellers. To avoid the impeller hitting the internal lights, a core of unilluminated zone has to be maintained (Fig. 7).

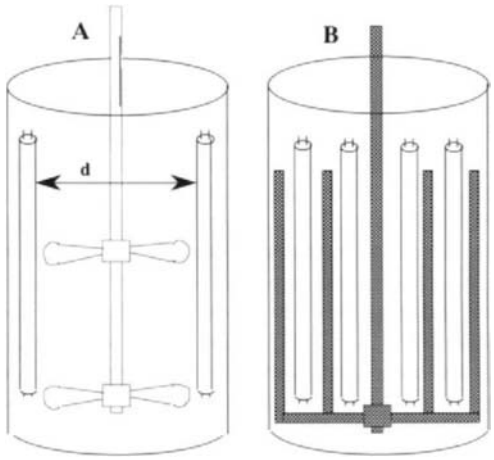


Figure 7: Internally illuminated stirred tank photobioreactors equipped with a turbine impeller (A) or a modified paddle impeller (B).

The required area of the unilluminated zone (represented by diameter d in Fig. 7A) depends on the diameter of the impeller. However, for efficient mass transfer, an optimum ratio of the impeller diameter to the reactor diameter has to be maintained. Thus, the larger the diameter of the reactor the larger would be the required space for the impeller and thus the lower the light supply coefficient. On the other hand, paddle type impellers (Fig. 6D) are known to have very good mixing capacity but low hydrodynamic stress (Ogbonna et al.,1991; Yokoi et al., 1993). Furthermore, paddle impellers can be modified so that they can be used for mixing in an internally illuminated photobioreactor without the problem of the impeller hitting the lights during rotation. Depending on the number and spacing of the lights, the paddle impeller can be modified as shown in Figure 7A. Each light is surrounded by two blades of the impeller, thus creating good mixing around the lamps and helping to avoid cell adhesion to the lights. The lights also serve as baffles to break the aeration gas into fine bubbles and thus improving the mass transfer of the system.

Depending on the inhibitory concentration of O_2 and the rate of O_2 evolution, aeration gas composition, aeration rate and agitation can be optimized to keep the dissolved oxygen concentration at the desired level. If need be, various types of O_2 scavengers such as sodium

dithionite and glucose oxidase (Pow and Krasna, 1979) or adsorbents such as deoxyhemoglobin and deoxymyoglobin (Rosenkrans et al., 1983) can be added to the broth to keep O_2 very low. However, even in such systems, good mass transfer capacity is still required to keep the adsorbents in suspension and thus facilitate their O_2 uptake.

AN INTERNALLY ILLUMINATED AERATED STIRRED TANK PHOTOBIOREACTOR

As discussed above, an ideal photobioreactor for hydrogen production by a biophotolysis process must be uniformly illuminated (no photoinhibition and dark zones) and must have high mass transfer capacity for efficient CO_2 supply and, most importantly, for O_2 removal. Good mass transfer will also facilitate pH and temperature control when necessary. Also operation under sterile conditions is desirable for long term contamination-free operation.

We have been working on development of photobioreactors which meet the above requirements. In order to design a photobioreactor with good light supply, a method for quantitative evaluation of light conditions inside the photobioreactors is necessary. We have reported the limitations of the incident light intensity, mean light intensity and light energy supplied per unit volume as parameters for the light supply in photobioreactors and proposed the use of light supply coefficient as a reliable parameter useful for quantitative assessment of light conditions inside photobioreactors (Ogbonna et al., 1995b).

Since each cell and process has its own optimum light condition, it is necessary that the target cell/process be defined and the optimum light condition (in case of the "unit concept", the optimum size of a unit) be determined experimentally before photobioreactor design is attempted. In the case of hydrogen production, the effects of unit size on hydrogen evolution rate, light conversion efficiency, process stability and overall performance of the system should be determined experimentally. If cell growth is considered an important parameter, the linear growth rate is preferred to specific growth rate as the parameter for assessing cell growth performance (Ogbonna et al., 1995c). In determining the optimum unit size, trade offs between the various parameters must be made since the optimum unit size for one parameter may be different from that for other parameters. For example, the optimum unit size for hydrogen evolution rate is, in most cases, different from that for maximum light conversion efficiency. In designing a photobioreactor for CO_2 fixation by *Chlorella sorokiniana*, a unit size giving the maximum yield from the supplied light energy was used (Ogbonna et al., 1996) but at such a unit size, the productivity was low.

A schematic diagram of a 4-unit photobioreactor (3.5 L) with 4 watts lamps is shown in Figure 8. A modified paddle impeller is used for mixing while aeration is through a ring sparger which produces very fine bubbles. The glass tube housings for the lamps serve as baffle plates for dispersing the air bubbles. Thus by increasing the aeration and agitation rates, very high CO_2 transfer and O_2 removal rates can be achieved. Also, since the lamps are not mechanically fixed to the reactors, the lamps are removed during heat sterilization and inserted into the housing glass tubes during cultivation. Thus cultivation under strict sterile conditions is possible. By using the "unit concept", the CO_2 fixation rates by *Chlorella sorokiniana* and alfa-tocopherol production

by *Euglena gracilis* in an 8-unit (20.0L) photobioreactor were the same as those obtained in a single unit photobioreactor (Ogbonna et al., 1998b).

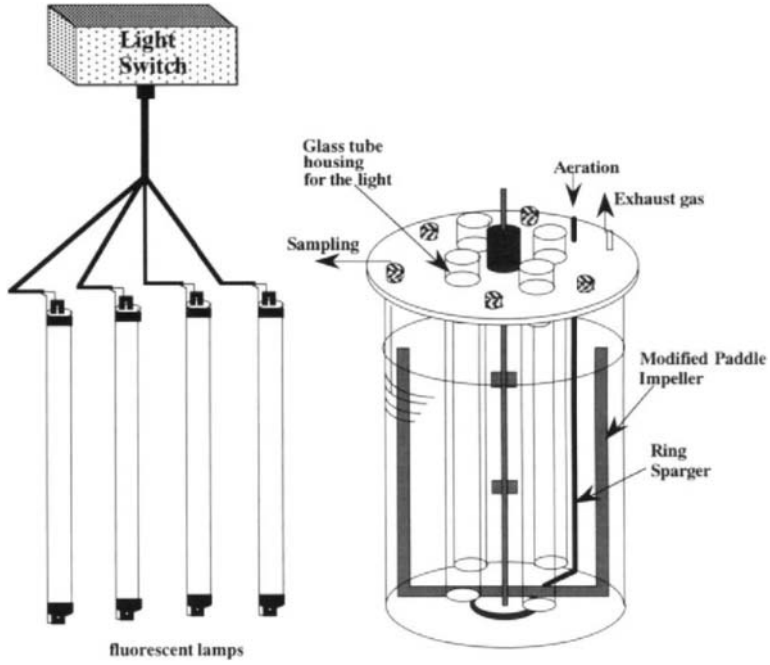


Figure 8: A schematic diagram of a four unit internally illuminated photobioreactor. The photobioreactor is illuminated by inserting the lamps into the glass tube housings.

Also, the photobioreactor can be illuminated by solar light energy. A solar light collecting device was installed on the roof and the collected light is transmitted to the photobioreactor inside the laboratory through optical fibers (Fig. 9). The optical fibers are divided into bundles according to the number of units and each bundle was connected to a light radiator which is used in place of fluorescent lamps. The light radiators are made of quart or glass rods which are vertically etched for efficient light radiation through the entire radiator surface (Ogbonna et al, 1999). When solar light is used for illumination, accurate determination of the optimum unit size is difficult since the solar light intensity is not constant. However, it can be estimated from the average solar light intensity for the experimental location.

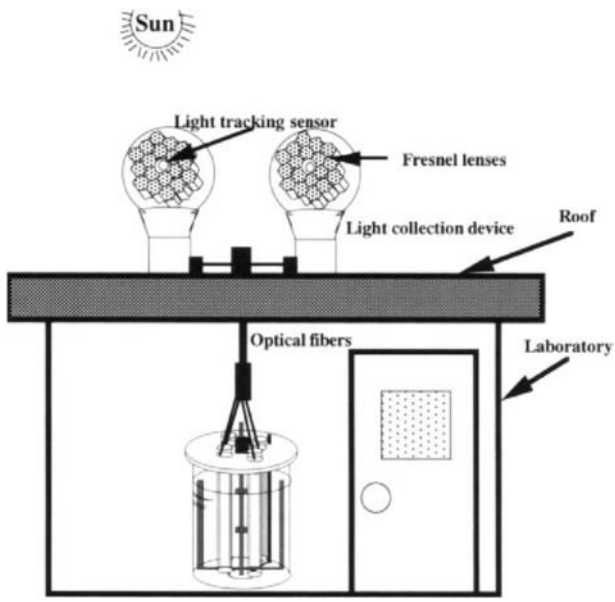


Figure 9: A schematic diagram of the system for solar light collection and transmission into the photobioreactor.

Furthermore, when solar light is used as the sole energy source, the photosynthetic process is inactive at night while prolonged bad weather (rainy and cloudy days) leads to reduced productivity or even total process failure. Thus an integrated solar and artificial light illumination system was constructed for continuous light supply to the photobioreactor (Fig. 10). Solar light is used during the day but when the intensity decreases below a pre-set value, the artificial light is switched on automatically, thus ensuring that the culture is continuously illuminated at a light intensity above the pre-set value (Ogbonna et al., 1999).

The photobioreactor shown in Figure 8 was developed for cultivation of *Chlorella* but the same concept can be used to design a photobioreactor for efficient production of biohydrogen. In the case of hydrogen production, it is expected that smaller unit size will be required while higher aeration and agitation rates will be employed. Furthermore, the aeration gas should be modified so that the O_2 concentration in the inlet gas is minimized. Again the exact values will depend on the cells to be employed i.e. the light inhibition levels, the degree of hydrogen uptake in the dark zone, the minimum non-limiting CO_2 concentration as well as the maximum non-inhibitory O_2 concentration.

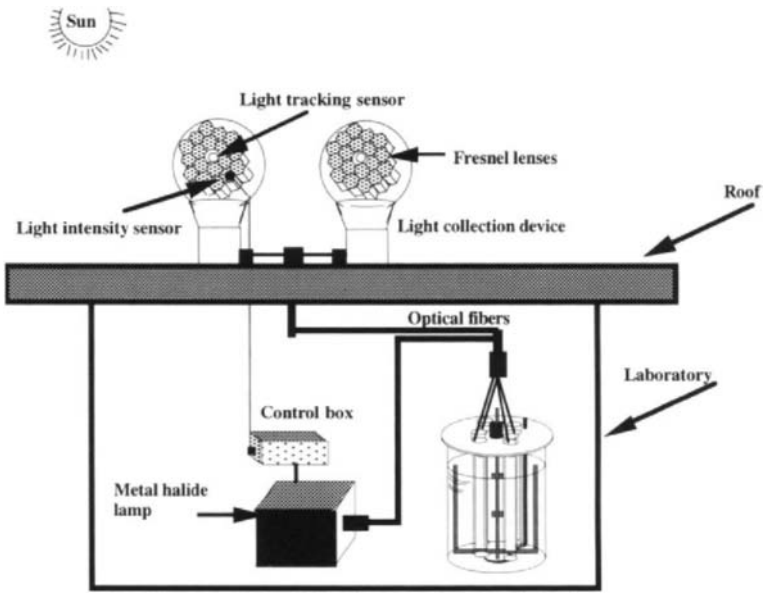


Figure 10: A schematic diagram of integrated solar and artificial light for internal illumination of a stirred tank photobioreactor.

STERILITY OF THE SYSTEM

Another issue which is worth considering while designing a photobioreactor for biological hydrogen production is the sterility of the culture. The level of sterility required depends on the nature of cells employed. For photosynthetic cells growing under normal (mild) condition, cultivation under sterile condition is necessary for long term stability of the process. As in other bioprocesses, continuous or long term operation will increase the productivity significantly. The current open or non-sterile photobioreactors can only be used for cultivation of those strains which grow under extreme/selective conditions (such as very high pH or salinity) not suitable for the growth of most other strains. Once a system is contaminated by fast growing species, it is often very difficult to get rid of the contaminants especially where heat sterilization cannot be employed. Chemical sterilization may be used but in large scale systems, soaking the whole system in chemicals and rinsing them with sterilized distilled water can be difficult, expensive and yet may not be very effective. Thus it may be necessary to use a photobioreactor which can be operated under strict sterile condition.

CONCLUSIONS

Although most economic analyses of photobiological hydrogen production are based on large scale systems, either "feasible productivities" or at best productivities obtained with very efficient laboratory scale systems are used. It is obvious that such productivities cannot be attained with current large scale photobioreactors. As discussed above, the heterogeneity in light intensity is so high that photoinhibition, light saturation, light limitation and dark zones exist simultaneously inside the same photobioreactor. Furthermore, the problem of oxygen sensitivities of the key enzymes involved in hydrogen production, as well as the dark hydrogen uptake activities of the cells, means that efficient hydrogen production can only be achieved in photobioreactors where light is uniformly distributed and where the mass transfer is high enough to supply CO_2 to the culture and remove the photosynthetically generated O_2. Thus, there is a need for photobioreactors specifically designed for biohydrogen production. Unfortunately, there seem to be very few photobioreactors designed specifically for hydrogen production, which take the above problems into consideration. The "unit concept" described here can be used to design a homogeneously illuminated aerated stirred tank photobioreactor with good mass transfer characteristics. Construction and operation of such photobioreactors will be more expensive than the conventional systems. However, environmental and sustainability rather than commercial issues should be the driving force in biohydrogen research and development. Thus development of innovative technologies for efficient hydrogen production should be encouraged no matter the present costs of such systems. The first step should be to develop very efficient systems and the second step should then be aimed at reducing the construction/operation costs of those systems that are proven to be efficient. It seems that biohydrogen production requires a long-term process development. Material and technological costs may change significantly before biohydrogen is ready for commercialization. Furthermore, environmental problems and future circumstances may favor the use of such systems instead of the present seemingly cheap systems. Again, the aims of R&D on biohydrogen should not be limited to developing only large scale commercially competitive production systems. Efficient personal/community scale systems may be more practical even if their unit production costs are higher than those of the large scale systems.

REFERENCES

Barnola, J. M.; Raynaud, D.; Korotkevich, Y.S.; Lorius, C. (1987). Vostok ice core provides 160,000-year record of atmospheric CO_2. *Nature* **329**, 408-414.

Genthon, C., Barnola, J. M., Raynaud, D., Lorius, C., Jouzel, J., Barkov, N. I., Korotkevich, Y. S., Kotlyakov, V. M. (1987). Vostok ice core: climatic response to CO_2 and orbital forcing changes over the last climatic cycle. *Nature* **329**, 414-418.

Ghirardi, M. L., Togasaki, R. K., Seibert, M. (1997). Oxygen sensitivity of algal hydrogen production. *Appl. Biochem. Biotech.* **63**, 141-151.

Greenbaun, E. (1988). Energic efficiency of hydrogen photoevolution by algal water splitting. *Biophys. J.* **54**, 365-368.

Matsunaga, T., Takeyama, H., Suso, H., Oyama, N., Ariura, S., Takano, H., Hirano, M., Burgess, J. G., Sode, K. and Nakamura, N., (1991), Glutamate production from CO_2 by marine cyanobacterium *Synechococcus* sp. using a novel biosolar reactor employing light diffusing optical fibers. *Appl. Biochem. Biotechnol.* **28/29**, 157-167.

Melis, A., Neidhardt, J., Baroli, I., Benemann, J. R. (1998). Maximizing photosynthetic productivity and light utilization in microalgae by minimizing the light-harvesting chlorophyll antenna size of the photosystems. In BioHydrogen Zaborsky O.R (Ed.) Plenum Press, pp. 41-52.

Mikheeva, L. E., Schmitz, O., Shestakov, S. V., Bothe, H. (1995). Mutants of the cyanobacterium *Anabena variabilis* altered in hydrogenase activities. *Zeitschrift für Naturforschung* **50**, 505 - 510.

Miura, Y. (1995). Hydrogen production by biophotolysis based on microalgal photosynthesis. *Process Biochem.* **30**, 1-7.

Mori K. (1985). Photoautotrophic bioreactor using solar rays condensed by fresnel lenses. *Biotechnol. Bioeng. Symp.* **15**, 331-345.

Ogbonna, J. C., Matsumura, M. ,and Kataoka, H. (1991) Production of glutamine by micro-gel bead-immobilized *Corynebacterium glutamicum* 9703-T cells in stirred tank reactor. *Bioprocess Eng.* **7**, 11-18.

Ogbonna, J. C., Yada, H. and Tanaka, H. (1995a). Effect of cell movement by random mixing between the surface and bottom of photobioreactors on algal productivity. *J. Ferment. Bioeng.* **79**, 152-157.

Ogbonna, J. C., Yada, H. and Tanaka, H. (1995b). Light supply coefficient - a new engineering parameter for photobioreactor design. *J. Ferment. Bioeng.* **80**, 369-376.

Ogbonna, J. C., Yada, H. and Tanaka, H. (1995c). Kinetic study on light-limited batch cultivation of photosynthetic cells. *J. Ferment. Bioeng.* **80**, 259-264.

Ogbonna, J. C.; Yada, H.; Masui, H; Tanaka, H. (1996). A novel internally illuminated stirred tank photobioreactor for large scale cultivation of photosynthetic cells. *J. Ferment. Bioeng.* **82**, 61-67.

Ogbonna, J. C. and Tanaka, H. (1997). Industrial-size photobioreactors. *CHEMTECH* **27**, 43-49.

Ogbonna, J. C., Tomiyama, S. and Tanaka, H. (1998a). Heterotrophic cultivation of *Euglena gracilis* Z for efficient production of alfa-tocopherol. *J. Appl. Phycol.* **10**, 67-74.

Ogbonna J. C., Soejima T. and Tanaka, H. (1998b). Development of efficient large scale photobioreactors; a key factor for practical production of biohydrogen. In BioHydrogen Zaborsky O.R (Ed.) Plenum Press, pp. 329-343.

Ogbonna J. C., Soejima T. and Tanaka, H. (1999). An integrated solar and artificial light system for internal illumination of photobioreactors. *J. Biotechnol.* **70**, 289-297.

Pow, T. and Krasna, A. I. (1979). Photoproduction of hydrogen from water in hydrogenase-containing algae. *Arch. Biochem. Biophys.* **194**, 412-421.

Powell, C. K., Chaddock, J. B., Dixon, J. R. (1965). The motion of algae in turbulent flow. *Biotechnol. Bioeng.* **7**, 295-308.

Rosenkrans, A. M., Rosen, M. M., Krasna, A. I. (1983). Effect of oxygen removal on hydrogen photoproduction in algae. *Biotechnol, Bioeng.* **25**, 1897-1904.

Vasilyeva, L., Miyake, M., Hara, M., Nakada, E., Nishikata, S., Asada, Y., Miyake, J. (1998). Characterization of a novel light-harvesting mutant of *Rhodobacter sphaeriodes* with relation to photohydrogen production. In BioHydrogen Zaborsky O.R (Ed.) Plenum Press, pp. 123-131.

Yokoi, H., Koga, J., Yamamura, K., Seike, Y., and Tanaka, H. (1993). High density cultivation of plant cells in a new aeration-agitation type fermentor, Maxblend Fermentor. *J. Ferment. Bioeng.* **75**, 48-52.

This Page Intentionally Left Blank

HYDROGEN PRODUCTION FROM FOOD PROCESSING WASTEWATER AND SEWAGE SLUDGE BY ANAEROBIC DARK FERMENTATION COMBINED WITH PHOTO-FERMENTATION

Mi-Sun Kim, T. J. Lee, Y. S. Yoon, I. G. Lee and K. W. Moon

Biomass Research Team, Korea Institute of Energy Research
71-2 Jang-Dong, Yusung-Gu, Daejon, Korea 305-343
Fax : 82 42 860 3739, E-mail: bmmskim@kier.re.kr

ABSTRACT

Anaerobic fermentation using *Clostridium butyricum* NCIB 9576, subsequently followed by photo-fermentation using immobilized *Rhodopseudomonas sphaeroides* E15-1 in hollow fibers, was performed for the production of H_2 from Makkoli (raw rice wine) and Tofu (soybean curd) wastewaters. From the Makkoli wastewater, which contained 0.54-1.2 $g \cdot l^{-1}$ sugars and 2.5-3.8 $g \cdot l^{-1}$ soluble starch, approximately 1 l $H_2 \cdot l^{-1}$ wastewater was produced during the initial 18 h of the anaerobic fermentation. Several organic acids such as butyrate, acetate, propionate and ethanol were also produced. The broth remaining after the anaerobic fermentation of the Makkoli wastewater, which contained 30-40 mg organic acids, 0.2-1.0 $g \cdot l^{-1}$ soluble starch, 0.1-0.3 $g \cdot l^{-1}$ sugars and 0.084-0.523 $g \cdot l^{-1}$ NH_3-N, was used as a feed for the photo-fermentation. About 0.44 l $H_2 g \cdot l^{-1}$ broth$\cdot day^{-1}$ was continuously evolved during the 10 days of photo-fermentation. Tofu waste water, pH 6.5, containing 5.0-8.0 $g \cdot l^{-1}$ soluble starch and 0.3-0.4 $g \cdot l^{-1}$ sugars, generated about 0.9 l $H_2 \cdot l^{-1}$ wastewater, along with some organic acids, during the initial 26 h of anaerobic fermentation. The remaining broth was also used as a feed for photo-fermentation, and about 0.2 l $H_2 \cdot l^{-1}$ broth$\cdot day^{-1}$ was produced for 10 days. When the sewage sludge was used as a substrate for the photo-fermentation, it was heated for 1 h at 150°C under 10 atm after alkali treatment. The pre-treated sewage sludge, which contained 25-40 mM organic acids, 0.04-0.05 $g \cdot l^{-1}$ NH_3-N, and a little amount of sugars, continuously produced 0.17-0.28 l $H_2 \cdot l^{-1}$ broth$\cdot day^{-1}$ during the 30 days of photo-fermentation under the average of 154 μM photon$\cdot m^{-2} \cdot sec^{-1}$ irradiance at the surface of the reactor using tungsten halogen lamps.

INTRODUCTION

Biological H_2 production is one of the most attractive research fields being considered for alternative energy production. The feasibility for creating renewable H_2 production

systems has been demonstrated through the anaerobic dark fermentation and/or photo-fermentation of organic wastes and wastewater by many researchers (1-6). Organic substances such as sugars, starch and cellulose, which are present in the waste effluents, are decomposed to organic acids and simultaneously H_2 is evolved by anaerobic/dark fermentation (5, 7, 8-10). It is well-known that purple non-sulfur photosynthetic bacteria produce H_2 from various organic acids more efficiently than from sugars under certain conditions (2-4, 11).

Disposal of organic biomass resources such as sewage sludge, food wastes and agricultural waste is one of the major environmental problems in Korea because the area for the landfill is limited and the average increase of organic wastes increases approximately 8% annually. Therefore serious considerations must be given to disposal of the organic wastes and effluents and lessening the burden of organic substances in the environment. Biological H_2 research is one of the most promising possibilities as a leading technology because biological H_2 research can accomplish two goals simultaneously; namely organic waste treatment and clean energy production without the greenhouse effect caused by fossil fuel.

This work is part of the project titled "H_2 production using biological and supercritical water technology from the biomass resources" for which funding has been provided since 1996 to the Biomass Research Team, Korea Institute of Energy Research as an alternative energy production project by the Ministry of Commerce, Industry, and Energy of Korea. The purpose of this H_2 project is to develop a biological process using anaerobic and/or photosynthetic microorganisms and to establish a basic H_2 production technology using organic wastes as raw materials. In conjunction with the biological process, the supercritical water technique will be used for substances which are not degradable or hard to be degraded by the biological process.

In this paper, we describe the biological H_2 production from anaerobic dark fermentation by *Clostridium butyricum* NCIB 9576 in combination with photo-fermentation by *Rhodopseudomonas sphaeroides* E15-1 using various organic wastewaters.

MATERIALS AND METHODS

Wastewater

Wastes and wastewater Makkoli and Tofu wastewaters were collected from the local manufacturing factories and sewage sludge from the municipal wastewater disposal utility in the area of Daejon.

Analysis

BOD, COD, total solids, suspended solids, and volatile solids were determined by standard methods (12). Sugar contents were measured by the dinitrosalicylic acid (DNS) method (13). Soluble starch contents were determined by the DNS method after acid hydrolysis using 5.5 N HCl. The concentration of organic acids was measured using Beckman gold model 420 high performance liquid chromatography fitted with a Aminex HPX-87H (Bio-Rad) organic acid analysis column and using Shimadzu 14-B gas chromatography equipped with a flame ionization detector after the sample was pre-treated with HCl. The column was packed with Porapak QS and nitrogen was used as a carrier gas. NH_3-N contents were estimated using an ammonia electrode (Corning) equipped with an ion/pH meter.

Immobilization of Bacteria

R. sphaeroides E15-1 was obtained from Bae et al(14). It was cultured with 12g of hydrophilic cuprammonium rayon hollow fibers (15) (diameter x length, 180 um x 3cm) in the modified Ormerod media (16) for 2 months at 30-32°C under 154 μM photon·m^{-2}·sec^{-1} irradiance using tungsten halogen lamps. Culture media was changed every week and flushed with oxygen-free argon.

Pretreatment of Sewage Sludge

Sewage sludge was pre-treated for 1 h at 150°C under 10 atm (17) after the pH was adjusted to 14 with NaOH. Ammonia in the gas phase was vented at 70-80°C after heat-treatment and 3-4 l of sewage sludge was processed in a 7 l reactor.

Hydrogen Production

C. butyricum NCIB 9576 was anaerobically precultured in PYG synthetic media containing 1% glucose for 24 h at 37°C after flushing the flask with oxygen-free argon for the dark anaerobic fermentation. Pre-cultures of 1 l were used as inocula for 10 l Makkoli and Tofu wastewaters, respectively, in a 15 l stainless steel fermentor to produce H_2 and various organic acids. The immobilized *R. sphaeroides* E15-1, was evenly spread in the rectangular photo-fermentor (width x length x depth, 50 cm x 50 cm x 4 cm). After the anaerobic fermentation of Makkoli wastewater, 3-5 l of broth was photo-fermented in the reactor under 154 μM photon·m^{-2}·sec^{-1} irradiance using tungsten halogen lamps. The pre-treated sewage sludge was also used for the photo-fermentation under similar experimental conditions as above except the volume was reduced (3 l), because the sewage sludge was dark brown in color and the light transparency in the sewage sludge was lower than in other samples.

RESULTS AND DISCUSSION

Dark Anaerobic Fermentation

C. butyricum NCIB 9576 produced approximately 1.1 l H_2·l^{-1} broth·day^{-1} when the synthetic PYG media containing 1% glucose was used as a feed (Fig. 1). The initial pH 6.8 decreased to 4.2–4.5 during the first 12–16 h of fermentation when pH was not controlled, resulting in the arrest of the H_2 evolution and cell growth with only 80% degradation of added glucose. However, when pH was controlled to 5.5–6.0, glucose was completely consumed, increasing H_2 production to 1.8 l H_2·l^{-1} broth·day^{-1}. Organic acids and solvents were also produced during the anaerobic fermentation by *C. butyricum* NCIB 9576, which were mainly butyrate, acetate, propionate and ethanol. When pH of the broth was controlled to 5.5–6.0, acetate production was increased to a maximum of 16mM at 20–24 h fermentation and then decreased to 10mM during 48 h fermentation. Butyric acid did not accumulate until 20 h of fermentation but was slowly produced up to 30 mM during 48 h fermentation. Approximately 1–2 mM and 2.5 mM of propionate and ethanol were produced during the anaerobic fermentation, respectively. The amounts of H_2, 1.1 l and 1.8 l, which were produced from 1 l synthetic PYG containing 1% glucose without and with pH control, are equivalent to about 0.89 and 1.46 mole of H_2·mole of $glucose^{-1}$, respectively. As compared to the result of Heyndrickx et al (18,19), we produced about 8.7 times more H_2 but about 3.2 times less acetate under similar experimental condition but using different strains of *C. butyricum*. However, 1.46 mole of H_2/ mole of glucose is still lower than the yield by Wood (20), which was 2.35 mole of H_2/ mole of glucose in a test tube. These differences in the H_2 and organic

acids production might be caused by the strain properties and the fermentation conditions.

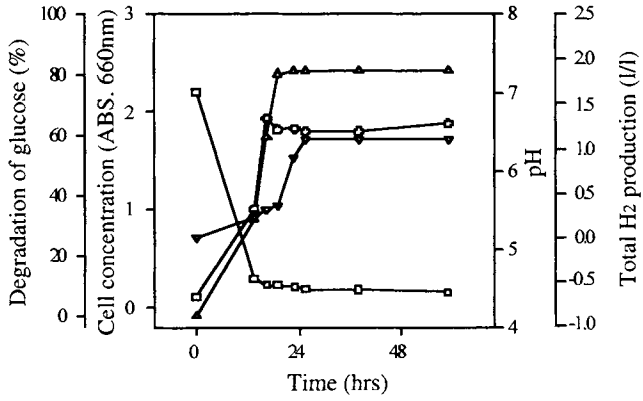


Figure 1: H_2 and organic acids production from PYG media containing 1% glucose by *C. butyricum* NCIB 9576. □ pH; ○ Cell concentration (Abs. 660nm); ▽ H_2 production; △ degradation of glucose

Makkoli wastewater has a large variation in the contents of sugars, starch, and ethanol, which are in the range of 0.54–1.2 $g \cdot l^{-1}$, 2.5–3.8 $g \cdot l^{-1}$ and 5–15 $g \cdot l^{-1}$, respectively (Table 1).

TABLE 1

COMPOSITION OF WASTEWATERS AND SEWAGE SLUDGE

	Wastewaters		Sewage sludge	
			Heat-treatment	
	Makkoli	Tofu	Before	after
BOD(mg/l)	20,580	9,100	3,120	ND
COD(mg/l)	13,330	8,100	2,040	ND
TS(%, w/w)	1.65	1.70	1.0-2.3	0.6-1.0
SS(mg/l)	12,900	1,400	2,200	7,120
VS(mg/l)	13,900	12,000	1,840	3,400
NH_3-N(g/l)	ND	ND	37.2	45.0
PH	5.6	5.7	6.7	12
Starch(g/l)	2.5-3.8	6.91	0	0
Reducing sugar(g/l)	0.5-1.2	0.74	0.08	0

TS, total solid; SS, suspended solid; VS, volatile solid; NH_3-N, ammonia nitrogen
ND, not determined

These variations were caused by the amount of water used for cleaning the fermentation tank, the period of sampling time and so on. In Korea, wastewater treatment utility is mandatory for Makkoli factory that has a capacity higher than 30,000 l/day Makkoli production. Unfortunately, more than 70% of Makkoli was produced by small scale local manufacturers and the wastewater is disposed to the drain, causing a serious contamination to sewage.

C. butyricum NCIB 9576 produced an average of 1 l H_2 from 1 l Makkoli wastewater during the initial 18 h of the anaerobic fermentation by *C. butyricum* NCIB 9576. pH was initially adjusted to 7.0 and the low limit of pH was 6.0 at 37°C during the fermentation of 10 l wastewater in a 15 l fermentor (Fig. 2). It is approximately equivalent to 1.70 mole of H_2 /mole of glucose for Makkoli wastewater by assuming that the conversion factor of soluble starch is 1.2. These results show that the Makkoli wastewater exhibits a better yield of H_2 production than the synthetic PYG media containing 1% glucose in which 1.46 mole of H_2 / mole of glucose was produced by *C. butyricum* NCIB 9576. When the cell concentration reached the maximum (dry cell wt., 1.2 $g \cdot l^{-1}$) and pH dropped to 6.0 after 20 h of batch fermentation, continuous fermentation *of C. butyricum* NCIB 9576 was performed. When the fresh Makkoli wastewater was added to the fermentor at the dilution rate of 0.15 h^{-1} for 2 h, approximately 400 ml H_2/l was additionally evolved for the next 15 h and then H_2 production ceased. The cell concentration dropped to 0.8 $g \cdot l^{-1}$ with the supply of the fresh wastewater after 35-40 h of fermentation. The result indicates that the pH 6.0 of the fermentation broth is not sufficient for the growth of *C. butyricum* NCIB 9576.

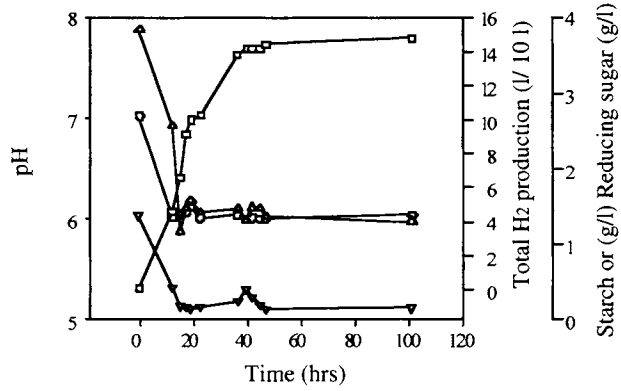


Figure 2: H_2 production by anaerobic fermentation of Makkoli wastewater by *C. butyricum* NCIB 9576. ○ pH; □ hydrogen production; △ starch; ▽ reducing sugar *, 1.5 l media exchange

Tofu wastewater, containing 5.0-8.0 $g \cdot l^{-1}$ soluble starch and 0.3-0.4 $g \cdot l^{-1}$ sugars, (Table 1) generated 0.9 l H_2/l wastewater, along with some organic acid, during 26 h of fermentation (Fig. 3). Due to the higher concentration of soluble starch in Tofu wastewater compared to the Makkoli wastewater, it took an additional 6-8 h to reach the maximum H_2 production

during the anaerobic fermentation. The rate of hydrolysis of soluble starch to produce sugars was faster than that of consumption of sugars by *C. butyricum* NCIB 9576 in Tofu wastewater, resulting in the slight increase of sugars after 8-10 h as shown in Figure 4. The H_2 production from Tofu wastewater using *C. butyricum* NCIB 9576 reached 1.05 l H_2 /l broth, which is equivalent to 0.97 mole of H_2 /mole of glucose, assuming that the conversion factor of soluble starch to sugar is 1.2. This result indicates that Tofu wastewater is less efficient for the H_2 production than Makkoli wastewater which produced 1.70 mole of H_2 /mole of glucose. The large amount of salts, such as Mg^{2+} and Ca^{2+} in Tofu wastewater which are used for the coagulation of soy protein curd, might cause the low efficiency of H_2 production by inhibiting the growth and other metabolism of *C. butyricum* NCIB 9576.

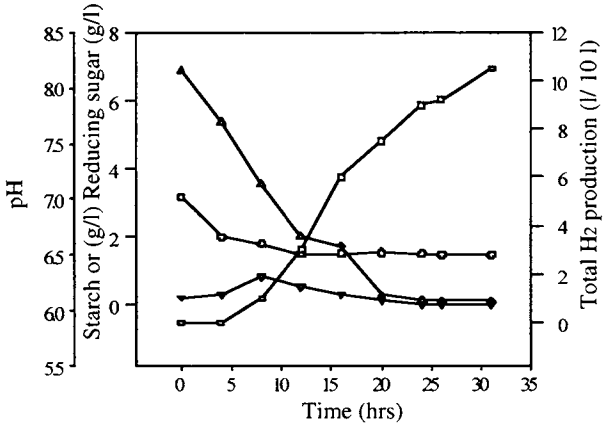


Figure 3: Anaerobic fermentation patterns from Tofu wastewater by *C. butyricum* NCI B 9576. ○ pH; □ hydrogen production; △ starch; ▽ reducing sugar

Photo-fermentation by R. sphaeroides E15-1

The broth remaining after the anaerobic fermentation of the PYG synthetic media containing 1% glucose, which had approximately 35 mM total organic acids, produced about 0.5 l H_2/l-broth for 3 days of fermentation (Fig. 4). The maximum rate of H_2 production was 0.38 l H_2/l-broth/day and 80% of total organic acids were degraded during the first 24 h of photo-fermentation by *R. sphaeroides*. The broth remaining after the anaerobic fermentation of Makkoli wastewater, which comprised 30-40 mM organic acids, 0.1-0.2 g/l soluble starch, and 0.084-0.523 g/l NH_3-N, was used as a feed for the photo-fermentation by *R. sphaeroides* E15-1. The organic acids and solvent in the broth were mainly butyrate (29-37 mM), acetate (1-3 mM), ethanol (70-150 mM), and very little lactate and propionate.

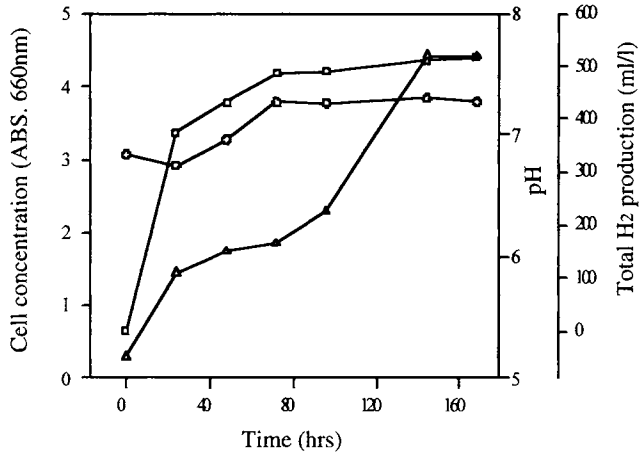


Figure 4: H_2 production by *R. sphaeroides* E15-1 using the residues of the anaerobic fermentation of PYG media by *C. butyricum.* ○ pH; △ Cell concentration (Abs. 660nm); □ hydrogen production

After photo-fermentation for 1 day, fresh broth was added to the fermentor to replace the old broth at the dilution rate of 0.5 h^{-1} for 1 h, every 24 h. About 0.44 l H_2 was continuously evolved from 1 l broth for the 10 days of photo-fermentation (Fig. 5).

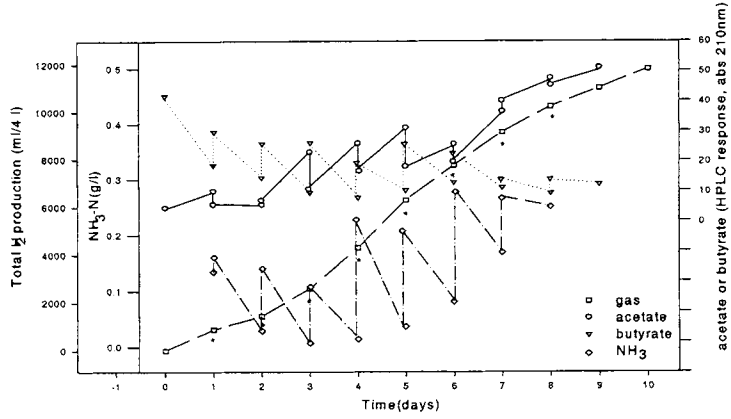


Figure 5: H_2 production by photo-fermentation of the immobilized *R. sphaeroides* E15-1 from remaining broth after anaerobic fermentation of Makkoli wastewater by *C. butyricum.*
★ 2L of broth exchanged

Butyrate, which was most abundant in the broth remaining after the anaerobic fermentation of Makkoli wastewater, was degraded 50-70% during the fermentation. Acetate was not metabolized by *R. sphaeroides* E15-1 and accumulated up to 16.9 mM for the 10 days of photo-fermentation. Accumulation of acetic acid during the photo-fermentation seems to be caused by two factors: the inability to metabolize acetic acid and the accumulation of acetic acid during the fermentation of sugars by *R. sphaeroides* E15-1. Ethanol in the broth was easily degraded 20-35% a day after the fresh media was supplied. NH_3-N contents of the broth remaining after anaerobic fermentation of Makkoli wastewater were in the range of 0.084- 0.523 $g \cdot l^{-1}$. NH_3-N at a concentration below 0.3 $g \cdot l^{-1}$ was metabolized about 80-96% by *R. sphaeroides* E15-1 in this experiment and did not affect H_2 production. However, NH_3-N was not degraded at a concentration above 0.3 $g \cdot l^{-1}$ in the broth and the rates of butyrate and ethanol hydrolysis and H_2 production declined. In a preliminary experiment, H_2 production by *R. sphaeroides* E15-1 was inhibited by 0.5 $g \cdot l^{-1}$ of NH_3-N. Sugars and starch were degraded well during hydrogen production and pH of broth dropped to 5.0-5.8.

Due to the high contents and variations of the soluble starch in Tofu wastewater, anaerobic fermentation by *C. butyricum* NCIB 9576 required longer fermentation time than Makkoli wastewater and the remaining broth showed a great deal of variation in carbohydrates composition. When 0.28 $g \cdot l^{-1}$ soluble starch, 0.1 $g \cdot l^{-1}$ sugars and 7.1-8.6 $g \cdot l^{-1}$ total organic acids were present in the remaining broth from anaerobic fermentation of Tofu wastewater, H_2 production was approximately 0.40-0.45 l/l-broth/day and acetate was accumulated during photo-fermentation. However, when the composition of sugar, and total organic acids in the broth were standardized to that of anaerobic fermentation broth from Makkoli wastewater, about 0.2 l H_2/l broth/day was continuously produced for 10 days.

The sewage sludge used in this experiment was the returned sewage sludge which was collected from the aeration tank basin of municipal wastewater disposal utility. Sludge was composed of 98-99 %(w/w) total solid, 1.8 $g \cdot l^{-1}$ volatile solid and less than 0.037 $g \cdot l^{-1}$ NH_3-N. Sugars and soluble starch contents were 0.07 and 0.001 $g \cdot l^{-1}$, respectively (Table 1). Instead of anaerobic fermentation by *C. butyricum* NCIB 9576, sewage sludge was pre-treated with heat for 1 h at 150°C under 10 atm after alkali treatment to pH 14. In our preliminary experiment, the heat treatment without alkali treatment increased the NH_3-N content by 405 times and resulted in the decrease of H_2 production by *R. sphaeroides*. The pH of the fresh sewage sludge was adjusted to pH 14 to strip the ammonia in the gas phase before heat-treatment. This procedure resulted in the decrease of NH_3-N content to 0.04-0.05 $g \cdot l^{-1}$. The pre-treated sewage sludge, which contained 25-40 mM organic acids, 0.04-0.05 $g \cdot l^{-1}$ NH_3-N and a little amount of sugars, continuously produced 0.17-0.28 l H_2/l broth/day during the 30 days of photo-fermentation under the average of 154 μM photon/m^2/sec irradiance at the surface of the reactor using tungsten halogen lamps (Fig.6).

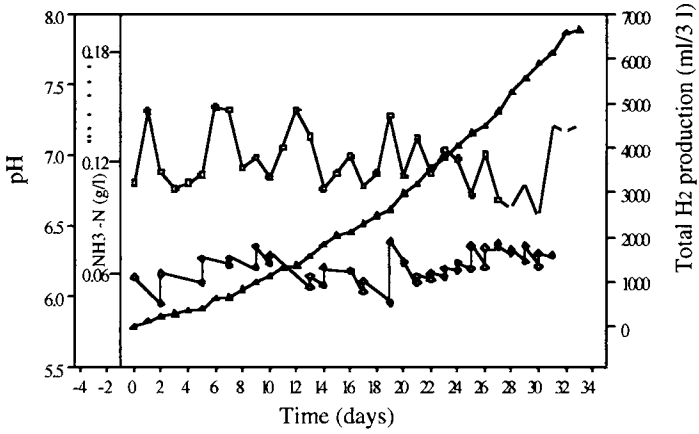


Figure 6: H_2 production by the immobilized *R. sphaeroides* E15-1 from heat-treated sewage sludge. pH; H_2 production; NH_3-N

CONCLUSION

The organic materials in the industrial effluents of Makkoli (raw rice wine) and Tofu (soybean curd) wastewaters and sewage sludge are promising substrates for the H_2 production. The combination of the dark anaerobic fermentation by *C. butyricum* NCIB 9576 with photo-fermentation by purple non-sulfur photosynthetic bacteria is a good technology for the production of H_2 and for the treatment of organic waste and wastewater even though several problems remains to be solved. We will further extend our approach to other food wastes and agricultural wastes for H_2 production after a proper pre-treatment.

ACKNOWLEDGEMENT

Research was supported through the Alternative Energy Research Funds from Ministry of Commerce, Industry and Energy, Korea.

REFERENCES

1. Tanisho, S. (1996). Hydrogen energy progress, Proc. 11th World Hydrogen Energy Conference. Stuttgart. Germany. **3**, 2601-2606.
2. Thangaraj, A. and G. Kulandaivelu. (1994). Biological hydrogen production using dairy and sugarcane waters. *Bioresource Tech.* **48**, 9-12.
3. D'Addario, E., E. Fascetti and M. Valdiserri. (1996). Hydrogen production from organic waste by continuous culture of *Rhodobacter sphaeroides* RV. Hydrogen energy progress,

Proc. 11th World Hydrogen Energy Conference. Stuttgart. Germany. **3**, 2577-2582.

4. Minami, M., T. Uechi, M. Kimura, T. Nishishiro, H. Kuriaki, K. Sano and T. Kawasaki. (1996). Hydrogen production by photosynthetic bacteria using sewage sludge. Hydrogen energy progress, Proc. 11th World Hydrogen Energy Conference. Stuttgart. Germany. **3**, 2583-2589.
5. Ueno, Y., Kawai, T., Sato, S., Otsuka, S., Morimoto, M. (1995). Biological production of hydrogen from cellulose by natural anaerobic microflora. *J. Ferm. Bioeng.* **79**, 395-397.
6. Sigh, S. P. and S. C. Srivastava. (1996). Agrowaste-based bacterial hydrogen production. Hydrogen energy progress, Proc. 11th World Hydrogen Energy Conference. Stuttgart. Germany. **3**, 2613-2618.
7. Tanisho, S., Ishiwata, Y. (1994). Continuous hydrogen production from molasses by the bacterium *Enterobacter aerogenes. Int. J. Hydrogen Energy*, **19**, 807-812.
8. Tanisho, S., and Y. Ishiwata. (1995). Continous hydrogen production from Molassws by Fermentation using urethane foam as a support of flocks. *Int. J Hydrogen Energy* **20**, 541-545.
9. Ueno, Y., Otsuka, S., Morimoto, M. (1996). Hydrogen production from industrial wastewater by anaesobic microflora in chemostat culture. *J. Ferm. Bioeng.* **82**, 194-197.
10. Kalia, V.C. and A.P.Joshi. (1995). Bioresource Technology **53**, 165-168.
11. Singh, S. P., S. C. Sravastava and K. D. Pandey. (1994). Hydrogen production by *Rhodospeudomonas* at the expense of vegetable starch, sugarcane juice and whey. *Int. J. Hydrogen Energy*. **19**, 437-440.
12. Standard method for the examination of water and wastewater (1995) 16th ed., ADHA, AWWA and WPCF .
13. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chem.* **31**, 426-428.
14. Bae, M. and J. K. Lee. (1983). A study on physiological condition for hydrogen evolution by *Rhodopseudomonas sphaeroides* K-7. *Kor. J. Microbiol.* **21**, 109-114.
15. Markov, S.A., M.J.Bazin and D.O. Hall. (1995). *Enzyme Microbiol. Technol.* **17**, 306-310.
16. Omerod, J. G, and H. Gest. (1962). Hydrogen photosynthesis and alternative metabolic pathways in photosynthetic bacteria. *Bacteriol. Rev.* **26**, 51-55.
17. Minami, M., T. Uechi, H. Kimura, T. Nishishiro, H. Kunaki, K. Sano, T. Kawasugi. (1995). Hydrogen Energy Conference. Stutgart. Germany. **3**, 2583-2588.
18. Heyndrix, M., De Vos, P., Thibau, B., Stevens, P., De Ley, Jl. (1987). Effect of various external factors on the fermentative production of hydrogen gas from glucose by *Clostridium butyricum* strains in batch culture. *System. Appl. Microbial.* **9**, 163-168.
19. Heyndrickx, M., A. Vansteenbeeck, and J. DeLeg. (1986). Hydrogen gas production from continuous fermentation of glucose in a minimal medium with *Clostridium butyricum. System. Appl. Microbiol.* **8**, 239-244.
20. Wood, W.A. (1961). Fermentation of carbohydrates and related compounds. in the Bacteria, Gunsalus and Stanier eds., 59-150.

AUTHOR INDEX

Head of Editing Secretariat: Dr. Tatsuki WAKAYAMA

Tissue Engineering Research Center, AIST/METI
Higashi 1-1-4, Tsukuba, Ibaraki 305-8562, Japan.

Email: tatsuki@nair.go.jp
Phone: +81 -298 -61 -2692,
Fax: +81 -298- 61 -3009

This Page Intentionally Left Blank