HAWAII NATURAL ENERGY INSTITUTE
UNIVERSITY OF HAWAII

REPORT TO THE U.S. DEPT. OF ENERGY HYDROGEN PROGRAM

BIOHYDROGEN PRODUCTION

FINAL SUMMARY REPORT

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ABSTRACT

The Hawaii Natural Energy Institute (HNEI) at the University of Hawaii has carried out R&D on biological hydrogen production since the early 1990's, supported by the U.S. Dept. of Energy Hydrogen R&D Program. Initially, this project investigated the genetics of cyanobacterial (blue green algae) hydrogenases. A new R&D phase was initiated in 1996 to develop a microalgal indirect biophotolysis process, in which water is converted in separate stages into O2 and H2.

In this conceptual process, an initial photosynthetic stage, an open pond microalgae cultivation system, fixes CO2 into starch (green algae) or glycogen (cyanobacteria). This is followed by a second stage, where the algae are concentrated as needed, become anaerobic (through respiration), induce the hydrogenase enzyme and start evolving H2 from their stored carbohydrates in a dark fermentation. Finally, a light-driven H2 production stage would complete the conversion of the biomass to H2, using only part of the photosynthetic apparatus of the algae. A single organism, a green alga or a cyanobacterium, would carry out all these reactions, and be reused through several such cycles of CO2 fixation and H2 evolution (with CO2 recycled between stages).

The research proposed to develop this process at HNEI was to have two parallel tasks:

1) Laboratory research of the physiology of hydrogen production by the algae, and
2) Development of outdoor, pilot-scale, photobioreactors for use in H2 production.

The organism chosen for initial work on this project was a strain of Spirulina (Arthrospira platensis) already being commercially grown in Hawaii and used in the prior biohydrogen research at HNEI. A culture collection, mainly of cyano- and photosynthetic bacteria, originating from the University of Florida, was transferred to Hawaii, for use in strain surveys and related studies. The photobioreactors, based on a design developed at the University of Florence, consisted of two sets of 8 parallel thin flexible plastic tubes of appx. 4 cm diameter and 20 m in length, inclined at a slight angle (appx. 10% slope), connected by manifolds and provided with internal gassing, for gas exchange and air lift circulation. The photobioreactor R&D was to demonstrate microalgal productivities, gas exchange and other engineering attributes required for indirect biophotolysis.

The major part of the research carried out under this project from 1996 to 2000 was the operation and engineering studies of the photobioreactors. These reactors receive a near-maximal solar flux per reactor area and allow relatively large unit sizes (100-200 m²). Their internal gas exchange also serves for air-lift mixing. Temperature control is provided by water spray systems. Initial work demonstrated the ability to produce Spirulina in the reactors. An afternoon decline in productivity was observed, correlating with high solar irradiation. Measured gas transfer rates suggest this as a possible limiting factor in high productivity biophotolysis processes. Laboratory work confirmed that Spirulina produces H2 by dark fermentations, but not in the light. Culture collection strains were used to demonstrate that dark H2 production is a common property among cyanobacteria. In conclusion, some significant advances were made during this project, although an indirect biophotolysis process using cyanobacteria and using the photobioreactors was not demonstrated.

Any future R&D project in biohydrogen production at HNEI should aim to maximize the yield of H2 from endogenous substrates by dark fermentations in microalgae and, in particular, by bacteria using exogenous waste substrates. Such processes could produce H2 fuel in commercial amounts at acceptable costs in the near-term, and larger quantities in the long-term.
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1. INTRODUCTION

Hydrogen R&D has been a long-term focus of research at the Hawaii Natural Energy Institute (HNEI). In reviewing potential renewable hydrogen resources for Hawaii, Takahashi (1986) concluded that: "For the long term, research on direct conversion of solar energy to hydrogen and other chemicals, particularly through photochemical and photobiological processes, offer promise of providing inexpensive hydrogen." He called for increased long-term R&D funding of this field of applied research, which could produce renewable hydrogen without requiring electrolysis of water. The Spark M. Matsunaga Hydrogen Research, Development and Demonstration Program Act was passed by Congress and signed into law in 1990, resulting in new funding for H2 research in general, and supporting a broad R&D initiative.

Biological hydrogen production R&D was initiated in Hawaii, to develop a solar-driven process for water decomposition, using microalgae as catalysts. Initially the work emphasized the development of a genetics system for cyanobacteria, specifically Spirulina, to develop a direct biophotolysis system (in which H2 and O2 are simultaneously produced from water). With the untimely death of the Principal Investigator, Dr. Kelton McKinley, this research shifted towards genetics of nitrogen-fixing cyanobacteria. See Section 2 for a brief discussion of these early researches. The next phase of the research, under a new P.I., Dr. Oskar Zaborsky, started in 1996 and aimed to develop an indirect microalgal biophotolysis process, as proposed by Benemann (1995, 1996, 1998a), and described in Section 3. This concept involves a multistep process: first, photosynthetic CO2 fixation into starches (and O2 evolution) in open ponds; second, the concentration of the biomass as needed; third a dark anaerobic H2 fermentation; and, finally, a light-driven photobioreactor stage, in which the algae release most of the H2. Although more complex than a direct biophotolysis process (where O2 and H2 are directly and simultaneously produced by photosynthesis without intermediate CO2 fixation) such a process would not suffer from the severe problem of O2 inhibition of the H2 production process (Section 3).

The work carried out under this project is summarized in the following sections:
Section 4. Design, construction and microalgae cultivation in the photobioreactors.
Section 5. Engineering studies (gas transfer, hydraulics, etc.) of the photobioreactors.
Section 7. Hydrogen production by Spirulina under dark fermentative conditions.
Section 8. Strain survey for dark hydrogen production among cyanobacteria.

The work carried out under this project was only an initial step in the development of the proposed indirect biophotolysis process. Physiological studies of Spirulina H2 evolution did not demonstrate a photobiological H2 producing reaction. The cultivation of Spirulina in the photobioreactors was only an initial effort, however the engineering studies resulted in some advances.

Most recently, the feasibility of the indirect biophotolysis process has been called into question (Benemann and San Pietro, 2001), mainly based on the view that the photobiological stage would require several-fold the area envisioned in the earlier studies (Benemann, 1998a) and because of the limited gas exchange capacity of low-cost photobioreactors (this Report). This led to the conclusion that future research in biohydrogen should emphasize dark fermentations, both with microalgae and, in particular, fermentative bacteria (Benemann and San Pietro, 2001, Section 9).

The initial research carried out by the HNEI biohydrogen project involved the development of the genetic tools required to investigate and improve H₂ production by hydrogenases in cyanobacteria. Spirulina (now called *Arhrospira*) was chosen as the organism for this project, as it did not fix nitrogen (and, thus hydrogen evolution would not be dominated by this enzyme), but did produce hydrogen, at least under anaerobic conditions in the dark, that is by some type of fermentations (Bylina, 1994, and literature cited therein). As discussed later, cyanobacterial hydrogenases evolve H₂ primarily if not exclusively in dark fermentations. The regulation, reductant source and electron transport pathway, of such cyanobacterial hydrogenases have so far been little studied.

The specific strain selected was *Spirulina pacifica*, the tradename given to the Spirulina cultured commercially in Hawaii by Cyanotech, Inc. The immediate requirements in the development of a genetics system, allowing genetic manipulation and engineering of this organism, were five-fold:

1. characterize DNA restriction-modification enzymes in *S. pacifica* (needed for gene transfers).
2. determine inhibitory antibiotics and isolate resistant strains (to provide genetic markers),
3. construct a library of chromosomal Spirulina DNA, to serve as raw material for gene transfers;
4. design a DNA vector which will be stably maintained in Spirulina, to allow gene transfers, and
5. develop physical or biological methods to introduce foreign DNA into Spirulina cells

Significant progress was made by this project during its initial stage (Bylina, 1994):

1. Three different restriction enzymes were purified and characterized of recognition sites. They were isoschizomers of known restriction enzymes (e.g. they cut the same DNA sequence). Identifying these enzymes allows construction of vectors that will not be destroyed in these cells.
2. Spirulina was most sensitive to chloroamphenicol and erythromycin. Vectors engineered from strains selected for resistance to these antibiotics can be used as selectable markers in Spirulina.
3. A Spirulina genomic DNA cosmid library was constructed, as a source of Spirulina genes.
4. Exploratory research was carried out in vector development and DNA gene transfer.

With the untimely death of the P.I., Dr. Kelton McKinley, this research effort was transferred to Prof. Dulal Borthakur, under whom the work to identify and characterize the Spirulina restriction enzymes continued. This resulted in the identification of an additional restriction enzyme, for a total of four, all known isoschizomers of known restriction enzymes (Tragut et al., 1995). This provided a basis for initiating gene transfer studies in this organism.

At this point, the focus of this research changed to hydrogenases in *Anabaena* sp. PCCC 7120, a heterocystous cyanobacterium. Like almost all such algae, *Anabaena* has an uptake hydrogenase involved in recycling the H₂ produced as a by-product of nitrogenase action. Like all such uptake hydrogenases, it is a Ni-Fe containing enzyme, and there are several genes (and proteins) involved in their assembly. One such gene is hubB, which was isolated from a genomic DNA library using the polymerase chain reaction (PCR) with so-called degenerate primers obtained from hupB genes from other bacteria. Although of scientific interest, neither this organism nor this uptake hydrogenase are of direct interest in the development of a practical biophotolysis process. In general, nitrogenase-based systems are not of interest in biohydrogen (Benemann, 1998b). With the appointment in 1995 of a new P.I. for this project, Dr. Oskar Zaborsky, the direction of the research changed, to the development and demonstration an indirect biophotolysis process.
3. THE "HAWAII" INDIRECT BIOPHOTOLYSIS PROCESS

The arguments for and against direct and indirect biophotolysis systems have been presented before (Benemann, 1996). Essentially the fundamental feasibility of a practical direct biophotolysis process (simultaneous O\textsubscript{2} and H\textsubscript{2} production without intermediate CO\textsubscript{2} fixation) requires a highly O\textsubscript{2} resistant hydrogenase reaction, including reductant supply, not just an O\textsubscript{2} stable enzyme. Such a metabolic process would need to produce H\textsubscript{2} and O\textsubscript{2} simultaneously at atmospheric pressures. Such an enzyme systems have not been described. O\textsubscript{2} absorbers, reversible or irreversible have been suggested as a way to overcome the limitations of such direct biophotolysis processes. However, irreversible O\textsubscript{2} absorption requires a consumable substrate, which most plausibly means glucose or a derivative. Simply stated, this would double the amount of photosynthetic area required: for each square meter of direct biophotolysis, at least as large (and in practice somewhat larger) area would be required to photosynthetically produce the oxygen absorber. Essentially reducing overall solar energy conversion by over half, compared to a truly oxygen resistant direct process. Reversible O\textsubscript{2} absorbers would be limited by the process energy inputs and capital costs required to efficiently recycle the absorber. A recent Workshop (Benemann and San Pietro, 2001) concluded that direct biophotolysis processes required basic and fundamental investigations, rather than applied and process oriented, R&D.

Another suggested biophotolysis process is to the use of heterocystous cyanobacteria, in which simultaneous O\textsubscript{2} and H\textsubscript{2} production is coupled through CO\textsubscript{2} fixation (Benemann and Weare, 1974). However, this approach can also be rejected, on various grounds. First based on the energy inefficiency of the nitrogenase reaction, and second, and more fundamentally problematic, the high energy required to maintain heterocyst metabolism, including respiration (Benemann, 1978b). Finally, another major objection in any direct (or heterocystous cyanobacterial) biophotolysis process, would be in the need to separate the two gases being created simultaneously. Although technically feasible, gas handling and separation represent a significant (though not yet well quantitated) costs, as well as a safety concern. And these arguments do not even address the most fundamental issue in such single-stage biophotolysis concepts: the costs of the closed photobioreactors required to cover the entire areas of the process.

These fundamental problems of direct biophotolysis, led to suggestions, already many years ago, for indirect processes, separating the O\textsubscript{2} and H\textsubscript{2} reactions into separate reactors, coupled through an oxygen stable reductant, such as the pyridine nucleotides (e.g. NADPH), which are also involved in CO\textsubscript{2} reduction (Benemann and Weissman, 1976). More logically, the intermediate would be a CO\textsubscript{2} fixation product, particularly one which could be relatively easily converted by intermediate metabolism to H\textsubscript{2}, specifically a polyglucose such as starch (accumulated by green algae) or glycogen (found in cyanobacteria). Nitrogen limitation is known to result in the accumulation of large amounts of such storage carbohydrates in green algae and cyanobacteria. A key issue is how to convert the stored glucose into H\textsubscript{2}. Some of it can be produced in the dark, the remainder would need to be evolved in a light-driven reaction. The loss of O\textsubscript{2} evolving capacity during nitrogen limitation is well known, and provides a mechanism for light-driven H\textsubscript{2} evolution without O\textsubscript{2} inhibition. This was observed in the alternating cycles of N\textsubscript{2} fixation and O\textsubscript{2} production in non-heterocystous cyanobacteria (Weare and Benemann, 1974). Such cyclic metabolism is a model for the indirect biophotolysis process proposed by Benemann (1994, 1996) and selected for process development under this project.
This indirect biophotolysis process was subjected to an initial conceptual design and cost analysis (Benemann, 1998a). The main components of such a process were
1. Open algal ponds for production of algal biomass under N-limitation but at high productivity;
2. An algal settling (harvesting) tank, which becomes anaerobic by endogenous respiration;
3. A fermentation tank for dark production of about one third of the H₂;
4. A photobioreactor, for completion of the H₂ production in a light-driven anaerobic reaction;
5. Gas separation (removal of CO₂), clean-up, storage and other gas handling subsystem; and
6. Support systems, such as inoculum ponds, waste treatment, water supply, etc.

The economic analysis was based on a number of very favorable assumptions: that the algal biomass could be produced at very high productivities (essentially near the theoretical limit of about 10% solar conversion efficiencies), that essentially 100% of the reductant could be recovered in the form of H₂ fuel (about one third in the dark, two-thirds in the photobioreactor stage), and that the light-driven stage would require only one photon per H₂ evolved. The costs of the ponds (and subsidiary systems) was based on prior studies of large-scale algal production, the cost of the gas handling also derived from prior work, and the photobioreactor costs were assumed to be some $100/m² ($130/m² including contingencies and engineering costs). Even though the photobioreactors would only cover one-tenth the area of the open ponds, they represented about half the total capital costs (the remainder roughly divided between the open ponds and gas handling).

For the HNEI project proposal, Spirulina, was again chosen as the immediate organism of choice, for similar reasons as before: it contains hydrogenase, but is not nitrogen fixing, and there is a great amount of information available on this species, from genetics (see above) to mass cultivation. Indeed, there was little need to carry out open-pond cultivation with Spirulina, as this is well-established technology. The key objective in Spirulina cultivation proposed under this project was to develop techniques for simultaneously maximizing both productivity and glycogen accumulation. The main objective in the proposed hydrogen production research was to demonstrate both a relatively high-yielding dark fermentations and, more importantly, a highly efficient light-driven reaction. As no such reaction had been reported in the literature, that was one of the specific goals of this research.

A major emphasis for the new HNEI biohydrogen project was the demonstration and development of a closed photobioreactor design suitable for biohydrogen production. Selection of such a reactor was based on the likely lowest cost system. Based on a review of the photobioreactor technologies (Benemann, 1998c), the design of Prof. Mario Tredici of the University of Florence was selected. This system has many potential advantages in photobiological hydrogen production. It provides the largest practicable photon capture per photobioreactor area. Its internal gas exchange avoids the need for an external gas exchange device. It can be of relatively large scale, with each unit being some 100 to 200 m² in size, and it is potentially of very low cost (Tredici et al., 1998). A general schematic of such the proposed indirect biophotolysis "Hawaii Process", is shown in Figure 3.1.
FIGURE 3.1. SCHEMATIC OF THE HAWAII INDIRECT BIOPHOTOLYSIS PROCESS.
4. DESIGN, CONSTRUCTION AND OPERATION OF THE PHOTOBIOREACTORS.

Figure 4.1 shows a schematic of the Tredici photobioreactor design (Tredici et al., 1998 and references therein). It consists of thin flexible tubular plastic sleeves filled with water and ganged together with top and bottom distribution pipes (footers and headers). The tubes are positioned in a corrugated plastic roofing sheet, which keeps them straight and even. The system is inclined (facing south) at approximately a 10% slope, to allow gas bubbles to rise freely. The footer includes a compressed air line that allows supply of air at the bottom of the reactor into selected tubes. The header (serving as degasser) is of enough volume to allow for containment of the fluid displaced during aeration, degassing, and to provide some head for the return tubes. Every third to fourth tube is not gassed, to serve as a fluid return, setting up an efficient air-lift type of recirculation. Cooling is by water spray, a process most suitable for dryer climates. Any other type of cooling would be prohibitive for present applications. The tubes can be of considerable length, with an optimal length likely to be between 20m and 50 m, depending on factor being optimized. This design was chosen because of its simplicity (internal gas exchange and hydraulic mixing combined) and high photon capture per unit of enclosed area, compared to most other alternatives.

Another major reason for selection of the Tredici-type of photobioreactor for this project was the relatively low projected cost compared to other designs, with projections as low as $50/m² (Tredici et al., 1998). However, that cost projection was rather optimistic and did not include many important components. In any event, this was the only photobioreactor design considered to have the potential to meet the cost of $130/m² of sunlight interception area, as assumed in the Benemann (1998a) cost analysis for indirect biophotolysis. Considering the need for highly efficient hydrogen capture, it is still uncertain if the plastic tubular material used in the Tredici design would allow sufficient retention of the H2 produced in a biophotolysis process. If not, the alternative would be glass tubes, which could be of similar costs as the plastic tubes, but would require expensive connections and field assembly. One of the objectives of the proposed research with this photobioreactor was to allow an assessment of the optimal dimensions of such a reactor important from an economic perspective. The larger objective of this research was to determine the engineering characteristics of this photobioreactor design - hydraulics, mass transfer, temperature control, etc. The photobioreactors were also to be used for demonstrating high productivity microalgae cultures. The final objective of the photobioreactor research was to adapt these reactors for applications in photobiological hydrogen production. Prof. Tredici agreed to collaborate with this project and provided the footers, headers, connectors and plastic tubular material for these units, and assisted with installation, training, operations and experimental work.

The Tredici tubular photobioreactor installed in Hawaii was approximately 20 m in length, with two sets of 8 tubes, each with a diameter of 4 cm. This was believed to be sufficient to allow determination of scale-up performance in a realistic fashion. The system was installed on a platform erected with construction scaffolding, 5 m wide and 20 m long, with wooden walkways on the outside and middle. The tubes were placed on corrugated plastic sheeting between the walkways. Eventually wire mesh had to be installed over the tubes for protection from stray cats. The design of the headers and footers is shown in Figure 4.2, with the final assembled reactor shown in Figures 4.3 and 4.4. A pH monitor controlled the CO₂ flow in response to pH and a computer collected the temperature data and activated the water spray in response to temperature. Dissolved O₂ was also measured, but was not controlled.
FIGURE 4.1. SCHEMATIC OF THE TREDICI PHOTOBioreACTOR
FIGURE 4.2.
FOOTER AND HEADER DESIGN OF THE "TREDICI" PHOTOBIOREACTOR
(Top: header, total volume of about 30 liters; Bottom: footer containing a compressed air line).
FIGURE 4.3. TREDICI-TYPE PHOTOBIOREACTOR IN OPERATION AT HNEI.
(Note: bottom of figure by ladder is the top part of the reactor platform, about 3 meters high).
FIGURE 4.4.
CLOSE-UP OF THE PHOTOBIOREACTOR TUBES SHOWING GAS BUBBLES.

Notes: 1. Dark cover on left was used to shade the culture during inoculation.
2. Tube on right is return tube without air, small bubbles are O2 produced by the algae.
The basic photobioreactor installation was completed in March of 1997, and over the next year initial operations and further development of the photobioreactor system took place. (Zyper et al., 1998; Zaborsky et al., 1998). The only organism grown in the system was Spirulina (*Arthrospira*), specifically a strain isolated from a pond culture obtained from Cyanotech Corp. The inoculated culture was first covered up with screens to reduce light intensity, as dilute Spirulina cultures are very sensitive to high light intensities. A single tube was used to produce sufficient inoculum for the two eight tube photobioreactors. The total liquid volume of each eight-tube photobioreactor was about 230 liters. The growth media used was Zarrouk's, a high bicarbonate medium. Dry weights were measured to determine the growth of the culture.

Figure 4.5 (Zyper et al., 1998) shows some typical operating parameters during early operation of the reactor. Maximum daily temperatures were kept under 40°C by means of the automatic water sprays. Dissolved O₂ (not particularly high in these experiments, also note the low DO levels at night) and the pH and effect of CO₂ addition (this was before the operation of the automatic CO₂ controllers) are also shown. An initial six day replication experiment of the two reactors showed that the reactors were reproducible within 10% or less. Maximum daily productivities were 1.3 to 1.6 g/l/d, with sustained productivities over six days of about 1 g/l/d, with a density of 3.5 g/l. Significant day-to-day fluctuations in cell densities and productivities were noted, likely due to sub-optimal operating conditions, in particular the uncontrolled and intermittent CO₂ supply. With the further development of this system, and a pH-CO₂ controller, longer-term stable operations were achieved. Figure 4.6 (Zaborsky et al., 1998, Radway et al., 1998) shows the results of an experiment which ran for almost two months in which the culture was diluted daily to maintain two fixed cell densities, initially about 3 g/l and then at 5 g/l for the last half of the experiment. In spite of the large change in cell density, the two reactors exhibited almost identical productivities, about 0.43 g/l/d. This was considerably lower than the maximal daily productivities seen in the initial experiments. The differences could be attributed to several factors, such as the use of two different strains and questions about the dry weight determinations in the initial experiments.

Another experiment carried during this first year of operations, involved a comparison of N₂ vs. air sparging at night. The idea was to reduce night-time respiration, which could account for 25 to 40% of daytime productivity. Indeed, night-time productivity losses were much lower (0.16 vs. 0.26 g/l/dark period) for the N₂ vs. air gassed reactors but the subsequent daytime productivities were about 0.1 g/l/day lower in the N₂ gassed tubes. Overall, this resulted in the same overall (0.40 g/l/d) productivity for both treatments. The inhibitory effect of night-time anaerobic conditions remains to be explored and is of obvious relevance to any indirect biophotolysis process, in which the cultures would be made anaerobic at night (mainly by respiration) to initiate H₂ production.

In further experiments, diluting the cultures in the morning vs. diluting in the evening had no significant effect on productivity (again 0.4 g/l/d). It is clear that Spirulina has a rather broad optimum in terms of cell density/dilution and productivity. Figure 4.7 shows the two major limitations on productivity: an afternoon growth slowdown, during the period of highest solar incidence, and a significant (almost 50%) night-time loss of the prior daytime production. The afternoon decline in productivity has been observed before by indirect methods (fluorescence) but this is probably the first direct demonstration of this effect. How to avoid both limitations in a practical sense remains for future research.
FIGURE 4.5.
EXAMPLES OF DIEL CYCLES OF MONITORED PARAMETERS IN THE TREDICI PHOTOBIOREACTORS.

a

Temperature

b

Dissolved Oxygen mg/l

c

pH

CO₂ added

Time of Day
FIGURE 4.6
DAILY SPIRULINA PRODUCTIVITY AT TWO BIOMASS DENSITIES.

FIGURE 4.7.
DIURNAL CHANGES IN BIOMASS DENSITY AND LIGHT INTENSITY
5. BIOENGINEERING STUDIES OF THE PHOTOBIOREACTOR

One of the major objectives of this project was to carry out an engineering evaluation of the basic hydraulic, gas transfer and heat balance of such a photobioreactor. The key issue in this design was that the bubbles generated from the small holes in the aeration (compressed air) tube inside the footer quickly coalesced (within the first meter) into large bubbles that slowly traveled up the remaining 19 m incline of the photobioreactor. Figure 5.1 shows the shape of the typical bubbles in the riser tubes of the photobioreactor. The bubble generates a turbulent wake, initially thought to increase gas exchange between the liquid and gaseous phases. This proved not to be the case.

Figure 5.2 and Figure 5.3 (Radway et al., 1999; Malda et al., 1999) shows the average bubble rise velocity and liquid velocities, respectively, in the photobioreactor vs. the gas flow rate. The gas flow range used in these experiments corresponded to roughly 0.01 to 0.1 volume gas/volume reactor/min, the practical lower and upper limits. A much higher volume would displace too much liquid, while a lower rate would result in insufficient liquid mixing (air lift). Note that the bubble rise velocity (Figure 5.2) is the sum of the liquid flow (Figure 5.3) and the slip velocity (essentially a constant, Figure 5.4). Expressing gas flow as a superficial velocity (for the six of eight tubes sparged with air), a correlation between air flow and liquid flow is obtained (Figure 5.3), with some differences among the various tubes (tubes 1 and 8 were used for return flows).

From the changes in pH upon injection of CO2 into the reactor tubes it’s possible to calculate an effective mass transfer coefficient as a function of the gas sparging rate (superficial gas velocity), Figure 5.5. No significant differences in mass transfer coefficient were observed between tap water, seawater or culture medium. As expected the rate of gas transfer increases with increasing gas flow. However, overall, the gas transfer coefficient is very low, compared to literature reports for bubble columns (Figure 5.6). Note that the data from Figure 5.5, transformed to O2 transfer, hugs the X axis of this logarithmic chart. The major difference between this reactor and bubble columns is the size of the bubbles. If the bubble size were assumed to be 6 mm, then mass transfer would be quite high, even higher than for bubble column reactors (Figure 5.6, top "actual, surface corrected"). It was concluded that the large bubble size greatly reduced gas transfer coefficients.

Table 5.1 summarizes the characteristic times for the Tredici photobioreactor at 20 ºC and a gas flow rate of 10 l/min. A comparison of the time constants for O2 or CO2 liquid-gas phase transfer with gas phase retention in the reactors clearly shows that there is not enough time for liquid-gas (or vice versa) equilibration. Indeed, bubble saturation with O2 takes hundreds of times longer than the transit time for the bubbles, making O2 build-up in the media a major problem in these reactors. Bubble exhaustion of CO2 is a lesser problem, but still significant amounts of CO2 will be lost out of the system. Finally, comparing the time constant of mixing with the time constants for mass transfer of the gasses shows that gradients of pH, pCO2, and pO2 will likely occur in these reactors. One proposal to increase gas transfer is to add a short (1 m) initial vertical section to the tubular reactors, where gas bubbles would not immediately coalesce and, thus, would provide additional gas exchange. Similarly the headers could be redesigned to provide additional gas exchange. However, fundamentally, this photobioreactor design, as do most others, suffers from gas transfer limitations. In the case of photobiological H2 production, a major factor will be the high overpressure of this gas, with outgassing likely limited by nucleation. This would require further investigation.
FIGURE 5.1.
SHAPE OF THE BUBBLES IN THE RISER TUBES OF THE PHOTOBIOREACTOR

FIGURE 5.2.
AVERAGE BUBBLE RISE VELOCITY IN PHOTOBIOREACTOR VS. GAS FLOW RATE
FIGURE 5.3.
LIQUID VELOCITY IN THE RISER TUBES VS. SUPERFICIAL GAS VELOCITY

FIGURE 5.4.
AVERAGE SLIP VELOCITY OF BUBBLES VS. SUPERFICIAL GAS VELOCITY
FIGURE 5.5. $k_{\text{COLa}}$ VS. SUPERFICIAL GAS VELOCITY.

FIGURE 5.6. $k_{\text{OLA}}$ VS. SUPERFICIAL GAS VELOCITY
<table>
<thead>
<tr>
<th>Phenomena</th>
<th>Characteristic time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass transfer oxygen</td>
<td>690</td>
</tr>
<tr>
<td>Mass transfer carbon dioxide</td>
<td>1130</td>
</tr>
<tr>
<td>Bubble saturation</td>
<td>21380</td>
</tr>
<tr>
<td>Bubble exhaustion</td>
<td>1250</td>
</tr>
<tr>
<td>Gas phase retention</td>
<td>50</td>
</tr>
<tr>
<td>Circulation</td>
<td>360</td>
</tr>
<tr>
<td>Mixing</td>
<td>3600</td>
</tr>
</tbody>
</table>
6. H₂ EVOLUTION BY SPIRULINA UNDER ANAEROBIC CONDITIONS.

One of the objectives of this project was to carry out laboratory experiments on cyanobacterial hydrogen production, to develop the indirect biophotolysis process. One of the central issues was the ability of Spirulina to produce H₂ under anaerobic conditions, both in the dark and also in the light. As the photobioreactor and culture collection work consumed most of the staff time available for this project, only limited experimental work on this goal was carried out.

These studies were carried out with strains of Spirulina (Arthrospira) isolated from a mixed culture retrieved in early 1998 from the Cyanotech water/media storage ponds. Isolate C was used for the reported experiments (carried out by Dr. JoAnn Radway and reported in Rocheleau et al., 1999). In the experimental protocol, the cells were grown, as usual, on Zarrouk's medium, both high nitrogen (H-N, with 400 mg/l nitrate N) and low nitrogen (L-N, only 5 mg/l nitrate-N). The L-N cultures had some significant nitrogen carryover from their inoculum, accounting for some of the growth. The L-N cultures were chlorotic when harvested, by screening. They were washed and resuspended in No-N Zarrouk's medium to a cell density of about 1.5 g/l. The reactions were carried out 1.9 ml of culture in a small Fernbach flask (headspace about 5 ml). The flasks were flushed with N₂ and incubated in the dark on a shaker. Some cultures were treated with methyl viologen (MV) and dithionite, a direct assay for intracellular hydrogenase activity.

Figure 6.1 shows the results of that experiment. Within about 4 hours all cultures started to evolve significant amounts of H₂. There was no significant difference between the L-N and H-N cultures. The MV-dithionite treated cultures had a higher initial rate of H₂ production, but peaked earlier than the cultures in which H₂ evolution took place only from endogenous reductants. This suggests that hydrogenase is present early on, but limited by reductant in the dark anaerobic cells. The reason for the cessation of hydrogenase activity in the MV-dithionite treated cells after some 6 hours is not clear. The maximum extrapolated rate for endogenous H₂ production is about 0.1 umoles H₂/hr/mg dry weigh (roughly 2 ul H₂ /hr/mg dry weight), which is similar to the rate reported by Aoyama et al. (1997) and others with Spirulina platensis. Light did not stimulate hydrogen production, and even inhibited it (data not shown).

The major issue in these experiments is identification of the factors limiting H₂ production in the dark, both in terms of rate and yield, and the lack of a light-driven H₂ producing response. The work of Aoyama et al. (1997) provides some information. They also used nitrogen limitation as technique to attempt to stimulate H₂ production. A three day incubation in nitrogen-free medium increased cell densities from 0.8 to 1.1 g/l, but glycogen concentrations increased from about 10% to over 50% of dry weight. The nitrogen-starved cells (at 1.6 g/l concentration) had almost ten-times a higher yield and rate of dark fermentative hydrogen production as the nitrogen-sufficient cultures. This is contrary to the results in Figure 6.1, which shows no major difference in the two types of cultures. However, both rates in Figure 6.1 are similar to those for the nitrogen-limited cultures in Aoyama et al. (1997). The reason for this discrepancy is not clear. In conclusion, H₂ production can be demonstrate in cyanobacteria, but thus far only in the dark and at rates that are at least an order of magnitude below that required by the indirect biophotolysis process envisioned for cost effective H₂ production.
FIGURE 6.1.
HYDROGEN EVOLUTION BY SPIRULINA CULTURES IN THE DARK
7. MICROALGAE CULTURE COLLECTION – TRANSFER, MAINTENANCE, USE

Another significant activity of this project was the transfer (from Miami, Florida to Hawaii), maintenance and curating, and use of the culture collection that Prof. Mitsui collected during his lifetime. The transfer of this culture collection to HNEI was actually initiated before the period covered by this report (Zaborsky et al., 1996), but essentially the entire process was carried out during the 1997-1999 period. This represented a significant effort because of the size, provenance, condition and documentation of the collection and the strict import restrictions imposed by the State of Hawaii on any importation of living organisms, including bacterial and algal cultures.

During this same period, a second collection was transferred to HNEI, the NREL (National Renewable Energy Laboratory) Algae Culture Collection, which was collected over several years by a number of researchers and used extensively for the Aquatic Species Program on microalgae oil production supported by the U.S. Department of Energy during the 1980 to 1995 period (Sheehan et al., 1998). This culture collection was the better documented but perhaps presented even more challenging logistical and maintenance problems.

The transfer of these two culture collections to HNEI provides a solid base for future research in practical applications of microalgae. While imposing a significant maintenance cost. The two cultures are now part of the Hawaii Culture Collection, HCC. (See www.Hawaii.edu/hicc) for the background, description, list of strains, media composition, bibliography, and ordering information). Strains can be ordered for $70 per strain, with the signing of a Materials Transfer Agreement.

The Mitsui collection was originally collected starting in the 1970's and through much of the 1980's, by Prof. Mitsui and his students and colleagues, including Dr. JoAnn Radway, who was the person responsible for the transfer of these collections and is now their curator. The culture collection originally contained almost 3,000 different isolates, but about two thirds were only enrichment cultures for cyanobacteria, not further identified or characterized, and were abandoned. Of the almost 1,000 isolated strains of cyanobacteria and photosynthetic bacteria, a number were lost before transfer to Hawaii, some were not transferred, and some were lost during and since transfer. The web site lists some 169 cyanobacteria and 106 photosynthetic bacteria in inventory, but this may not be up-to-date or complete. This collection contains some of the strains that Prof. Mitsui published on during his extensive research on biohydrogen production. A bibliography of Prof. Mitsui's work can be found on the web site.

The NREL culture collection, contains several hundred strains of diatoms, green algae and flagellates (an exact count is not available) that were isolated by several groups working for the Aquatic Species Program (see references listed on the web site and Sheehan et al., 1998, for a detailed review of their isolation and studies carried out by the Aquatic Species Program with these strains).

The Mitsui strains in the HCC were used for the purposes of the DOE-funded hydrogen project only at the end of this project (see Section 8, below).
8. CYANOBACTERIAL STRAIN SURVEY FOR DARK H₂ FERMENTATIONS

The Mitsui cyanobacterial strains were proposed to be used in a strain survey to identify promising strains for hydrogen production. A detailed screening protocol was presented in the original proposal, to measure H₂ production by nitrogen sufficient and deficient cultures under flowing inert gas (N₂ or Ar). The reason for sparging with inert gas is to assist in the anaerobic adaptation (in the dark) and in the transfer of H₂ out of solution into the gas phase, to reduce liquid side H₂ partial pressures. For illustration purposes, at the gassing rate of 0.05 v/v/min and a plausible production rate of 10 ul/hr/mg dry weight, that would, for an algal biomass density of 1 g/l, this result in a gas phase H₂ concentration of 0.3%, and a likely liquid side H₂ partial pressure of about 1-3%. This low H₂ partial pressure would favor H₂ production over uptake. Cultures not sparged experience very high liquid side H₂ concentrations (up to one thousand-fold higher than in well sparged cultures). Only for irreversible reactions (methane fermentations, nitrogenase), and for low or absent uptake hydrogenase activities, would such high H₂ concentrations not inhibit the process. Thus a reasonable strategy for an initial screening effort, where the potential for hydrogen evolution is the primary and initial selection criterion, is to sparge the cultures. Experiments with a H₂ electrode were also proposed to determine the actual rate of in-situ H₂ production. The proposed screening effort was to avoid nitrogen-fixing cyanobacteria. However, it was never carried out as proposed.

The final activity under this project was a strain survey using the culture collection (Wachi, 2000). From the above (Section 7) reported lack of light-driven hydrogen production in Spirulina, and similar reports from other cyanobacteria, the strain survey tested only for dark H₂ fermentations by cyanobacteria. The testing protocol was first developed with Spirulina (Arthrospira). It involved placing a concentrated algal cell suspension into test tubes with a only a small head space (about 1 to 2 ml of headspace for a 15 ml culture volume), placing these in the dark at 32°C, and mixing magnetically. Figure 7.1 shows the time course of H₂ and O₂ production with Spirulina – showing a reduction in O₂ levels and increase in H₂ over the first 14 hours, followed by a rise in O₂ and a decrease in H₂ over the next twenty hours. The first phase is explained by endogenous respiration creating anaerobic conditions (though note that significant O₂ remains in the head space) and induction of the H₂ fermentation reactions. The subsequent decline in H₂ and, in particular, rise in O₂, suggest air contamination (during sampling or actually diffusing into the head space). The maximal rate of H₂ production was 82 ul/14 hours, equivalent to about 0.1 ul/mg dry weight/hour.

Next a total of 118 filamentous cyanobacteria from the culture collection were surveyed for H₂ production in the dark, for both nitrogen sufficient and deficient cultures, using a similar protocol to that above, but in smaller tubes. H₂ production was measured by the accumulation of H₂ in the headspace. About two-thirds of the strains produced some H₂, but only 9 produced more than 1% in the gas phase, with the best being, Nostoc sp. 1083, which produced 27% H₂ in the gas phase. In the follow-up experiment, the best strains were examined using 25 ml Earlenmeyer flasks with magnetic stirring. In this case the best strain was Nostoc 1069, under conditions of nitrogen deficiency, suggesting N₂ fixation. However, no direct measure of nitrogen fixation was available. Further measurements on effects of salinity, bicarbonate, etc., on one strain, Nostoc 1083, were not conclusive. A suitable method for screening strains for H₂ production remains to be demonstrated.
FIGURE 8.1.
H₂ AND O₂ PRODUCTION AND UPTAKE IN THE DARK BY SPIRULINA.
9. CONCLUSIONS AND RECOMMENDATIONS

This project made some significant contributions to the advancement of photobiological hydrogen production. It was the first project that attempted to develop an indirect biophotolysis process, as proposed conceptually by Benemann (1995, 1996, 1998). The concept was to use nitrogen-limited microalgal mass cultures to maximize CO₂ fixation into carbohydrates and then to produce H₂ in both the dark and light. Nitrogen limitation would shut down O₂ evolution by the PSII system, in analogy with a process observed in nitrogen-fixing cyanobacteria (Weare and Benemann, 1974).

However, for various reasons, demonstration of such a process was not accomplished by this project. For one, in the physiological experiments (section 7), no light-driven H₂ evolution was observed, and no such a reaction has thus far been reported in the literature for any cyanobacteria.

It should be noted that an indirect biophotolysis process using green algae has also not been yet demonstrated. A recent report of such a system (Melis et al., 2000, Ghirardi et al., 2000), using S as the limiting nutrient (to downregulate PSII activity), was not successful: residual O₂ evolution from PSII can account for essentially all the H₂ produced (Benemann, 2000). This was confirmed by the strong DCMU inhibition of H₂ evolution (Ghirardi, 2000). Thus, an indirect biophotolysis process using microalgae remains to be demonstrated, even at the bench scale. A small (appx. 5 m²) demonstration project of a two-organism process (a green alga and a photosynthetic bacterium) was carried out in Osaka, Japan, with low overall efficiencies (Ikuta et al., 1998).

Another objective of the present research project was to demonstrate high productivities with microalgal cultures under relatively controlled outdoor conditions. However, that goal also was not even attempted during this project, and remains as a challenge for the future. Laboratory work in the U.S. (Neidhardt et al., 1997, Melis et al., 1998, 1999; Polle et al., 2000, 2001) and in Japan (Kajima and Ueno, 1999, 2000), has demonstrated one approach to this goal: the use of reduced antenna size mutants. Such mutants were shown by the Japanese workers to exhibit increased productivity in laboratory continuous cultures at high light intensities. However, the demonstration of such an enhanced productivity process with actual outdoor cultures remains a goal for the future.

The major accomplishment of this project was the development and study of the photobioreactors, in particular their engineering aspects. Development of the photobioreactors as research tools, both in design and operations, required a significant initial investment in resources and time. The outcome of that research supported the choice of these reactors for biophotolysis applications, but also determined that gas transfer rates (H₂ and CO₂ outgasing) would likely be limiting any such process. This would make spontaneous outgasing of H₂ the major route for H₂ gas transfer, which implies a liquid side partial pressures of 10 and 100 atmospheres H₂ (see Pauss et al., 1990).

Of course, before a photobioreactor could produce H₂, a light-driven reaction would need to be demonstrated. This was not achieved during this project with cyanobacteria, and remains to be demonstrated in any practical sense with green algae. In conclusion, indirect biophotolysis is still far from being realized practically, and even experimentally.

Recently the assumptions on which the indirect biophotolysis process concept was based were called into question. A Workshop sponsored by the U.S. Department of Energy Hydrogen R&D
Program (Benemann and San Pietro, 2000), rejected a major underlying assumption of this process: that one photon would be sufficient to produce one mole of H₂ in the photobiological stage. Rather, the Workshop participants concluded that at least two photons per H₂ would be required. This would essentially require doubling the size of the photobioreactors. As these were estimated to account for over 50% of total capital and operating costs of this process (Benemann 1998a), doubling their size would increase overall costs by at least half, putting this technology well beyond the $15/GJ, the goal set by the DOE Hydrogen Program. As that analysis was already based on the most favorable assumptions plausible, there is little basis to predict future cost reductions. The key issue is the cost of photobioreactors, which, as discussed above, will likely be at least $100/m².

The likely high partial pressures of H₂ present in the would require generation of a very low potential reductant to allow achievement of reasonable rates. This would, for a photobiological process, be a further argument for a requirement of at least one photon per electron transferred, as already concluded on mechanistic grounds (see above). In any event, the Workshop review concluded that though somewhat more plausible than direct biophotolysis, any photobiological H₂ production, would, at present, be rather speculative and too problematic to justify a major applied R&D effort. The Workshop did, however, recommend that the Tredici photobioreactors work be completed, to provide a more definitive answer regarding their utility and costs. However, the main recommendation of the Workshop was for greater emphasis in biohydrogen R&D of dark fermentations, both with microalgae and, in particular, with bacterial waste conversions.

The R&D challenge in dark fermentations is to achieve much higher rates and, most importantly, yields, than up to now reported. High yields, near stoichiometric, from glucose can be obtained through operation of the pentose phosphate cycle, as was recently demonstrated experimentally by Woodward et al. (2000) with a purified enzyme (in vitro) system. However, that reaction started with a phosphorylated sugar, exhibited a slow rate and was operated at very low H₂ partial pressures. None of these would be acceptable in a practical process. In microalgae, starch breakdown could phosphorylate glucose, a possible advantage over bacterial fermentations. However, even in such cases, the overall thermodynamics of dark hydrogen fermentations could argue against such a process. Mechanistic and evolutionary arguments against high yielding hydrogen fermentations can also be made: bacterial metabolism has evolved to avoid such wasteful reactions as excessive H₂ production. This is the reason for the often stated "theoretical" limit of a maximum yield of four moles of H₂ (and two moles of acetate) per mole of glucose, rather then the stoichiometric 12. However, the evolutionary arguments are not decisive, as both conventional selection and modern molecular tools (Keasling et al., 1998) can be used to overcome such metabolic limitations. The thermodynamic barriers could be overcome through operation at higher temperatures (favoring H₂ evolution), by "reinvesting" some of the metabolic energy derived from the initial glucose breakdown, and/or by supplying an exogenous source of metabolic energy (e.g. oxygen, for facultative bacteria) to help drive the reaction.

In conclusion, although the biohydrogen R&D project at HNEI did not achieve all its original goals, it did significantly advance the state-of-the-art. While the photobioreactors drew attention to the practical aspects of this technology, the physiological experiments focused it on dark fermentations. Future R&D at HNEI could continue these studies while emphasizing bacterial fermentations as a more immediately practical, and near-term, approach to biohydrogen.
10. REFERENCES


