Microalgae: a green source of renewable H_2

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This article summarizes recent advances in the field of algal hydrogen production. Two fundamental approaches are being developed. One involves the temporal separation of the usually incompatible reactions of O_2 and H_2 production in green algae, and the second involves the use of classical genetics to increase the O_2 tolerance of the reversible hydrogenase enzyme. The economic and environmental impact of a renewable source of H_2 are also discussed.

Hydrogen (H_2) metabolism is primarily the domain of bacteria and microalgae. It occurs in many, taxonomically diverse, species, takes place by a variety of biochemical mechanisms and processes, and has many physiological adaptations[1–4]. Microbial H_2 formation is catalysed by either nitrogenases or hydrogenases, enzymes that can only function under anaerobic conditions. Nitrogenases are used by certain cyanobacteria (blue–green algae) and photosynthetic bacteria, whereas green algae use hydrogenase(s) to photoevolve H_2. Nitrogenase-catalysed H_2 evolution has been examined as a means of producing H_2 gas commercially[5–8]. However, this has limitations including the low catalytic turnover rate of the enzyme[9,10] and the high energy requirement (two or more ATP molecules per electron transported[11]).

The H_2 metabolism of green algae was discovered in the early 1940s by Hans Gaffron[12]. He observed that green algae (under anaerobic conditions) can either use H_2 as an electron donor in the CO_2-fixation process or evolve H_2 in both dark and the light. These original observations were later extended to many other green algae including *Chlamydomonas reinhardtii*[13–15], *Chlorella fusca*[16] and *Scenedesmus obliquus*[12,17].

H_2 production in eukaryotic green algae requires a period of several minutes to a few hours of anaerobic incubation in the dark[14,18–20]. This apparently induces the biosynthesis and/or activation of the reversible hydrogenase, and probably of other H_2-metabolizing enzymes, and enables the cells to photoproduce H_2. The function of the reversible hydrogenase is to combine protons (H^+) (in the medium) and electrons (from reduced ferredoxin) to form and release molecular H_2. Thus, microalgae possess the genetic, enzymatic, metabolic and electron-transport machinery to photoproduce H_2 gas. Such release of H_2 permits a sustained electron flow through the electron-transport chain for the generation of ATP by the cell[21,22].

In principle, the process of algal photosynthesis could be used to oxidize H_2O and evolve O_2 [driven by light absorbed by photosystem II (PSII)], followed by transport of electrons to ferredoxin [driven by light absorbed by photosystem I (PSI)]. The reversible hydrogenase is poised to accept electrons directly from reduced ferredoxin and to generate molecular H_2 (Refs 23,24). Electrons might also originate from a metabolic substrate such as starch or acetate[15,25,26]. Given the high quantum yield of photosynthesis, photoproduction of H_2 gas by eukaryotic microalgae is of keen interest because it holds the promise of generating a renewable fuel from the most plentiful resources of nature: light and water.

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Two fundamental approaches for photosynthetic H_2 gas production from water (often referred to as 'biophotolysis'[27,28]) are being examined. The first are processes in which photosynthetic O_2 and H_2 gas production are temporally and/or spatially separated. In such a two-stage process, CO_2 is first fixed into H_2-rich endogenous substrates during normal oxygenic photosynthesis (stage 1), followed by light-mediated generation of molecular H_2 when the microalgae are incubated under anaerobic conditions (stage 2). A recent development of this approach was based on a Stage 1 → Stage 2 process and has allowed substantial and sustained photobiological H_2-gas production in the green alga *C. reinhardtii*[29].

The second approach involves processes in which photosynthetic O_2 and H_2 gas production occur simultaneously. In this case, electrons that are released upon photosynthetic H_2O oxidation feed into the hydrogenase-mediated H_2-evolution process, without involving intermediate CO_2 fixation and energy storage as cellular metabolites. Theoretically, the 'single-stage' H_2-production process should be superior to the 'two-stage' process. Indeed, when the entire electron-transport capacity of the photosynthetic apparatus was directed towards H_2 production under low-light conditions, energy conversion efficiencies of 5–10% were attained[30]. However, currently, the single-stage mechanism has encountered several limitations, including the suppression of H_2 production in the presence of O_2, which is evolved by the water-splitting reactions of PSII (Ref. 31). State-of-the-art technology and current efforts to overcome the mutually exclusive O_2 and H_2 production are discussed below.

Two-stage photosynthesis and H_2 production in green algae

Anaerobiosis in C. reinhardtii *cultures upon sulfur deprivation*

As mentioned above, wild-type cells must be incubated anaerobically for a certain period in order to induce the expression of the gene that encodes the reversible hydrogenase and of other genes that may be essential for

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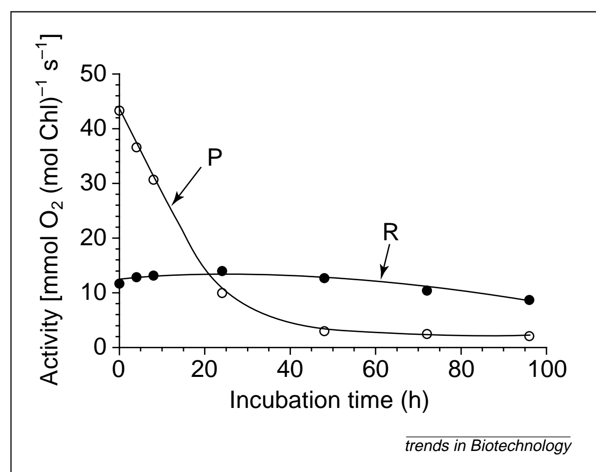

Figure 1
Absolute activity of oxygenic photosynthesis and respiration in *Chlamydomonas reinhardtii* CC-124 suspended in a medium devoid of sulfur (S). Cells were first grown on S replete Tris–acetate–phosphate (TAP) medium[39]. Incubation under S deprivation started at 0 h. The rate of cellular respiration (R) was recorded in the dark, followed by measurement of the light-saturated rate of photosynthesis (P). Rates of photosynthesis were corrected for the rate of dark respiration. Cultures at 0 h contained 2.2×10^6 cells ml^{-1}.

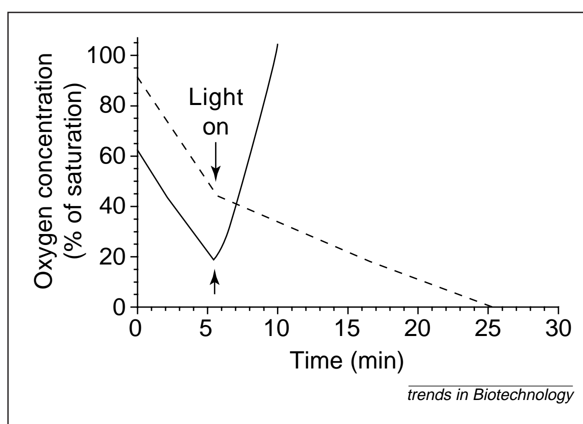

Figure 2
Recorder traces of O_2 concentration in aliquots removed from *Chlamydomonas reinhardtii* cultures, immediately following suspension under S-deprived conditions (solid line) and after incubation in this medium for 30 h (broken line). Aliquots placed in a Clark-type O_2-electrode chamber were exposed to saturating actinic illumination, with the light being turned on after 5.5 min (arrows). At the time of S deprivation, cultures contained ~3.3 10^6 cells ml^{-1}.

H_2 production. It is known that the O_2 evolved by photosynthesis is sufficient to lower or to inhibit completely the activity of the reversible hydrogenase, which makes an efficient single-stage process difficult to sustain. This incompatibility between the simultaneous O_2- and H_2-production reactions can be overcome by separating the two reactions in time by physiological and fully reversible means. This can be achieved by incubating the green algae in the absence of sulfur-containing nutrients.

Sulfur deprivation exerts distinctly different effects on oxygenic photosynthesis and mitochondrial respiration in green algae. When *C. reinhardtii* cultures are deprived of inorganic S, the light-saturated rates of O_2 evolution and CO_2 fixation decline significantly within 24 h in the light[32]. The absolute activity of photosynthesis, measured from the light-saturated rate of O_2 evolution in *C. reinhardtii* (Fig. 1), declines bi-exponentially from ~44 (mmol O_2) (mol chlorophyll)$^{-1}$ s^{-1} initially to ~2 (mmol O_2) (mol chlorophyll)$^{-1}$ s^{-1} after 100 h. The reason for this loss of activity is traced to the requirement for high rates of *de novo* protein biosynthesis in the chloroplast, needed for the frequent replacement of the D1 32 kDa reaction center protein in the H_2O-oxidizing PSII complex[33]. In the absence of S, which is an essential component of cysteine and methionine, protein biosynthesis is impeded and the PSII repair cycle is blocked[32].

Cellular respiration, measured as the rate of O_2 consumption in the dark (Fig. 1), remains fairly constant at 12–14 (mmol O_2) (mol chlorophyll)$^{-1}$ s^{-1} over the 0–50 h period and declines slightly thereafter[29]. It is important to realize that the absolute activity of photosynthesis decreased below that of respiration in *C. reinhardtii* after ~22 h of S deprivation. Some time after ~22 h of S deprivation, a sealed *C. reinhardtii* culture is expected to become anaerobic in the light, because of the significantly greater respiratory (compared with photosynthetic) activity of the cells. This has been observed in direct measurements with a Clark-type O_2 electrode.

Figure 2 shows O_2 electrode traces that monitor the O_2 concentration in *C. reinhardtii* cultures immediately following S deprivation and after a 30 h incubation in the absence of S. In both cultures, cellular respiration caused a linear decrease in the concentration of O_2 in the dark (0–5 min). The onset of actinic illumination, after 5.5 min, caused a significant change in the direction of the trace in the 0 h sample, showing a steep increase in the O_2 concentration in the control culture. In the culture incubated for 30 h in the absence of inorganic S, the onset of actinic illumination caused a partial compensation in the negative slope of O_2 consumption owing to the onset of residual photosynthesis in the sample. However, the O_2 concentration continued to decrease linearly in the culture. The culture incubated in the absence of S for 30 h became anaerobic within ~20 min, even though it was maintained under continuous illumination.

Thus, after ~22 h of incubation in the absence of inorganic S, the O_2-evolution activity of photosynthesis falls below the O_2-uptake activity of respiration. In sealed cultures, this leads to anaerobiosis, even under saturating illumination. Consequently, photosynthetic phosphorylation and oxidative phosphorylation are largely inhibited in S-deprived, sealed green-alga cultures.

Photoproduction of H2 upon S deprivation in C. reinhardtii

Under conditions of S-deprivation, most electrons for H_2 production seem to originate from the residual H_2O oxidation activity in PSII (unpublished results). However, *C. reinhardtii* might also resort to the consumption of endogenous substrate to generate extra electrons for the reversible hydrogenase[25,26]. Such electrons would pass through plastoquinone, the cytochrome *b–f* complex and PSI, with electron transport coupled to the reversible hydrogenase pathway, in order to generate ATP and molecular H_2.

To test this, *C. reinhardtii* cells in a Roux bottle were incubated in S-deprived media under continuous

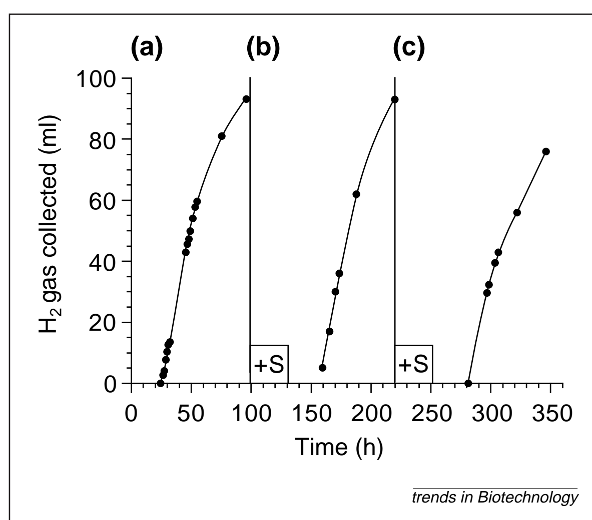

Figure 3

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

illumination and the cultures were sealed after 24 h of S deprivation, at which time the rate of photosynthetic O_2 evolution was equal to, or less than, that of respiration. H_2 gas accumulation was observed in the light (Fig. 3a) but not in the dark[29]. The rate of H_2 accumulation was constant at ~2.5 ml h^{-1} for the first 25–35 h before starting to level off. Gas chromatographic analyses revealed

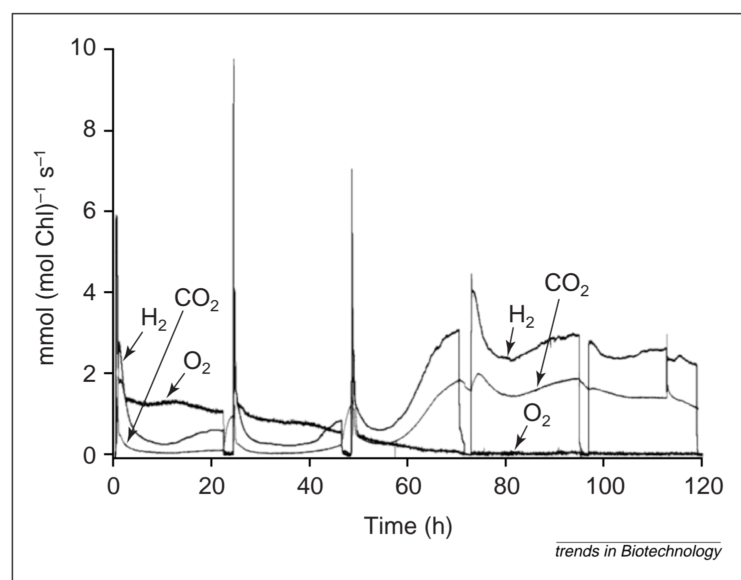

Figure 4

Simultaneous recordings of H_2, O_2 and CO_2 photoproduction by sulfur-deprived *Chlamydomonas reinhardtii*. Cells were first grown on replete medium and were then incubated in the absence of sulfur under anaerobic conditions in the dark for 3 h. At 0 h, the samples were purged continuously with inert helium gas to facilitate the removal of H_2, O_2 and CO_2 from the reaction mixture. The concentration of each of these gases was monitored downstream from the reactor.

that the composition of the headspace in the culture bottle after 100 h was ~87% H_2 and 1% CO_2, with the remainder being mostly nitrogen (N_2) with traces of O_2.

The initial rate of H_2 gas accumulation (~2.5 ml h^{-1}) was equivalent to 7 (mmol H_2) (mol chlorophyll)$^{-1}$ s^{-1}. This rate is significantly lower than the capacity of electron transport in the photosynthetic apparatus, which can be estimated from the rate of light-saturated O_2 evolution at the onset of S deprivation to be 44 (mmol O_2) (mol chlorophyll)$^{-1}$ s^{-1} (Fig. 1). The estimated molar H_2:O_2 ratio of 0.16:1 suggests a low yield of H_2 production compared with the capacity of the thylakoid membranes for electron transport.

The S-deprivation–H_2-production sequence of events was shown to be reversible and reproducible by cycling a single *C. reinhardtii* culture between the two stages (oxygenic photosynthesis in the presence of S and H_2 production in its absence) for up to three full cycles (Fig. 3). At the end of H_2 production in the first cycle, after 100 h, the culture was supplemented with inorganic S. This caused prompt inhibition of H_2 production because of the ensuing S-induced activation of oxygenic photosynthesis (100–130 h). Subsequently, the culture was driven to anaerobiosis upon S deprivation (130–160 h) and H_2 production (160–220 h). A similar pattern was seen in the third cycle (Fig. 3c).

Figure 4 shows the result of a different approach, developed by Greenbaum[14], in which the amounts of H_2, O_2 and CO_2 are recorded simultaneously in *C. reinhardtii* cultures suspended in S-deprived media. In this case, cells are suspended in the absence of S and are incubated under externally imposed anaerobic conditions in the dark for 3 h to induce the expression of the reversible hydrogenase. Commencing with the simultaneous recording of H_2, O_2 and CO_2, samples were purged continuously with inert helium (He) gas to prevent the accumulation of photosynthetic gases, particularly O_2, in the reactor.

During the first period of illumination (Fig. 4; 0–22 h), O_2 production by the photosynthetic apparatus is the dominant activity of the chloroplast. However, subsequently and concomitant with the decline in the activity of the H_2O-oxidizing enzyme, the production of H_2 and CO_2 increased in parallel (60 h). Interestingly, the molar H_2:CO_2 ratio remained constant at about 1.6:1 throughout the transient changes in the kinetics of the process. These results provide evidence for the involvement of endogenous substrate in the H_2-production process[29], with CO_2 being the byproduct of a catabolic process that uses O_2 from the residual photosynthesis (Fig. 1) and that might generate some of the electrons for the reversible hydrogenase pathway.

Thus, S deprivation might be the key with which to alter the dynamic relationship between the cellular processes of oxygenic photosynthesis, aerobic respiration, chlororespiration and H_2 photoproduction. Detailed analyses of this dynamic interplay might hold the answer to the quest for increased yields of H_2 production by the S-deprivation method.

Single-stage photosynthesis and H_2 production in green algae

The simultaneous photoproduction of H_2 and O_2 from H_2O in green algae depends on the O_2 tolerance of the reversible hydrogenase enzyme. When oxygenic photosynthesis is active, and unless strict measures are

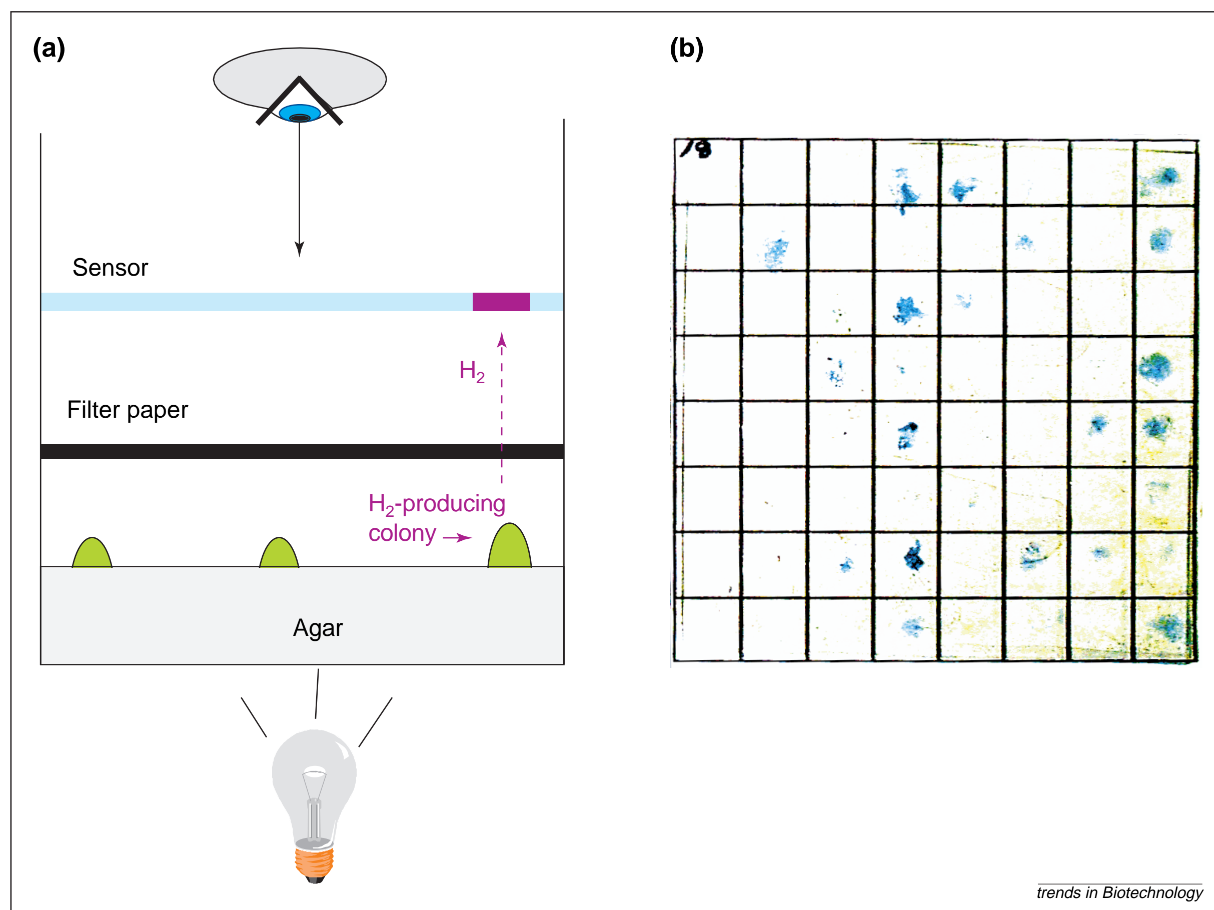

Figure 5
Chemochromic screening for individual H_2-producing *Chlamydomonas reinhardtii* colonies on a grid in agar plates. (**a**) Cross-section of how H_2 produced by individual colonies (green) goes through a filter paper to reach the chemochromic sensor, where it causes a chemical change, resulting in the appearance of a bluish-purple color that can be detected visually. (**b**) Actual appearance of the sensor for the detection of O_2-tolerant H_2-producing clones. The bluish-purple spots on the sensor indicate the grid position of individual clones that can photoproduce H_2.

taken to remove O_2 (Ref. 34), green-algal H_2-production activity is transient owing to the rapid inactivation of the reversible hydrogenase by photosynthetic O_2. Mutant prokaryotic organisms containing hydrogenases that tolerate increased partial pressures of O_2 have been described[13,35], suggesting that the enzyme is amenable to manipulations that affect its O_2 tolerance.

One approach to address the O_2-sensitivity problem of the enzyme in green algae is to use classical genetics[36]. The goal of this approach is to generate *C. reinhardtii* mutants that are sufficiently tolerant to O_2 to permit H_2 production under aerobic conditions. The availability of such mutants could lead to the development of an aerobic photosynthetic H_2-production process. This process should be superior to the two-stage process described previously in terms of yield, cost and effectiveness, because H_2 would be produced continuously.

The classical mutagenesis approach takes advantage of the reversible activity of the green-algal hydrogenase. Mutagenized cells are subjected to one of two different selective pressures. The first selective pressure requires that cells metabolize H_2 (H_2-uptake selective pressure) and the second requires cells to evolve H_2 (H_2-production selective pressure[31]) in the presence of O_2 concentrations that usually inactivate the wild-type strain. Given the generally low specificity of these selective pressures, the surviving organisms must subsequently be subjected to an additional screening procedure using a chemochromic sensor that detects algal H_2 evolution activity. The screen is based on the ability of a multilayer, thin film sensor containing tungsten oxide and palladium to change color (from transparent to bluish-purple) upon exposure to nanomolar quantities of H_2 (Fig. 5). The usefulness of the film in detecting H_2 produced by individual algal colonies on agar plates[37] and its application in the isolation of mutants with higher tolerance to O_2 are shown by quantitative measurements.

Table 1 shows the concentrations of O_2 that produce a 50% inhibition in the initial rate of algal H_2 production (O_2 I_{50}) for wild-type *C. reinhardtii*. Table 1 also shows the O_2 I_{50} of two mutants (strains 104G5 and 155G6) obtained through H_2-uptake selective pressure and of two mutants (strains 76D4 and 141F2) obtained through H_2-production selective pressure. All mutants have a significantly higher O_2 I_{50} than the wild type. These observations suggest that it might be possible to use genetics to alleviate the extreme O_2 sensitivity of the green algal reversible hydrogenase and to sustain the photoproduction of H_2 in the presence of O_2.

Future prospects

For experimental purposes, in the past, the activity of the reversible hydrogenase enzyme was induced in the cells after dark anaerobic incubation. However, in

Table 1. O_2 I_{50}[a] values for H_2 evolution by *Chlamydomonas reinhardtii* mutant clones[b]

Chlamydomonas reinhardtii	I_{50} value[a] (% O_2)
Wild-type	0.22
H_2-uptake selective pressure	
Strain 104G5	0.57
Strain 155G6	1.22
H_2-production selective pressure	
Strain 76D4	0.80
Strain 141F2	2.00

[a]The O_2 I_{50} is the concentration of O_2 that causes a 50% inhibition of H_2 production.
[b]The clones were obtained by using a mutagenesis, selection and screening procedure. Light-induced rates of hydrogenase activity were measured polarographically with a Clark-type electrode set up for the detection of molecular H_2. Clones were pre-exposed to different concentrations of O_2 in the dark for 2 min to inactivate the hydrogenase and were then illuminated to induce H_2 evolution[31,37].

wild-type organisms, such activity was rapidly lost upon illumination as a result of the immediate inactivation of the enzyme by photosynthetically generated O_2. Consequently, the absence of a physiological method to surmount the O_2 sensitivity of hydrogenases has discouraged research on applied algal H_2-production systems. However, there are novel physiological methods to separate O_2 evolution from H_2-production activities in time, thus enabling H_2 production for extended periods, as well as classical genetic methods that can be applied to develop an O_2-tolerant H_2-production process.

Could green algae and the photosynthetic production of renewable H_2 contribute to a future H_2 economy? This remains to be seen but it is clear that the advent of H_2 as a renewable energy carrier will have important economic implications, provided that scientific and technological challenges are addressed successfully. Furthermore, H_2 will have a positive impact on the environment, alleviating atmospheric pollution and mitigating the greenhouse effect. However, more fundamental research is needed. With respect to two-stage algal photosynthesis and H_2 production, there is a need to increase the yield of H_2 production by a factor of ten or more[29], and to develop a protocol that works with minimal media before the process can be viable in commercial applications.

One approach might be to test other microscopic algae (green, blue–green, brown and red) for H_2 production and to elucidate the mechanism of H_2 production by S deprivation. Similarly, the O_2 tolerance of the reversible hydrogenase in the single-stage algal photosynthesis and H_2-production system must be increased before this process can be viable in commercial applications. Optical problems associated with the size of the chlorophyll antenna and the light-saturation curve of photosynthesis must also be addressed[38] before green algae can be used successfully in mass culture for H_2 production. Finally, the combined application of selection and breeding by classical genetics with the powerful recombinant DNA and gene technology will undoubtedly increase the H_2 productivity and/or O_2 tolerance of the reversible hydrogenase in green algae.

Acknowledgment

Our work was supported by the US Department of Energy, Hydrogen Program.

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Vaccine cookbook

Vaccine Adjuvants: Preparation Methods and Research Protocols
edited by Derek O'Hagan, Humana Press.

The 'new generation' of vaccines rely on recombinant proteins, synthetic peptides and DNA to prevent infectious diseases, cancer, fertility, allergies and autoimmune diseases. However, they are often poorly immunogenic on their own and are increasingly dependent on adjuvants for enhanced humoral and cellular immunogenicity. *Vaccine Adjuvants: Preparation Methods and Research Protocols* is a textbook designed to help vaccine researchers to select and prepare optimal formulations of vaccine antigens and adjuvants.

Increased scientific understanding of the immune correlates of protection and of the interplay between immune cells (antigen-presenting cells, T and B cells), cytokines and chemokines that controls the type of vaccine-induced immune responses have placed more stringent requirements on the impact of adjuvant formulations. In addition to a requirement for the 'type' of immunity generated (e.g. neutralizing antibody, cytotoxic T cells), there is increasing need to target these immune responses to specific mucosal sites (e.g. respiratory, intestinal, genital tracts) for protection from mucosally transmitted pathogens.

Extensive preclinical evaluation of various adjuvant formulations in small animals and non-human primates are conducted in order to select the best adjuvants for progression to clinical testing. For this reason, it is extremely important to remove laboratory-specific variations in the efficiency and optimization of various adjuvant formulations. This book has a primary objective of providing detailed protocols to enable individual scientists to prepare optimized adjuvant formulations, thereby facilitating interlaboratory consistency.

This book takes several steps to reach these goals. Each chapter is devoted to a specific adjuvant currently undergoing preclinical or, in some cases, clinical evaluation. The authors (often the scientists who developed the adjuvant) provide a brief history of the adjuvant and then describe its composition, discuss its mechanism of action, metabolic degradation and reactogenicity profiles (animal and/or human), and provide brief reviews and references to preclinical and/or clinical studies.

In some cases, additional information is included about the stability of the adjuvant formulation, its potency (duration and number of immunizations), utility via alternate routes of immunization, cost of production, ease of use, ease of production on a large scale (particularly with respect to various global vaccine initiatives that target millions), safety concerns and regulatory issues. Most important, however, are the detailed descriptions of and notes about the materials required and methods used to preparate the adjuvant, and to prepare the antigen–adjuvant formulation. Importantly, in most chapters, suggestions and detailed descriptions are included of various quality-control procedures to ensure the success and efficiency of the formulation.

Although the book targets the major adjuvants in preclinical and clinical evaluation, the chapters do vary in quality and in their focus on laboratory materials and methods. This text is an excellent companion to previous books such as *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M. and Newman, M., eds), which provided detailed descriptions and summaries of preclinical and clinical experiences of over 100 adjuvants but no detailed protocols for their preparation. The detailed protocols and extremely helpful notes in this book will provide scientists with the internal information to optimize their use of these adjuvants with their antigens of interest. For these reasons, I would recommend this book for those involved in enhancing and/or specifically targeting humoral or cellular vaccine-induced immune responses.

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0167-7799/00/$ – see front matter © 2000 Elsevier Science Ltd. All rights reserved.