22
Hydrogen Production by *Chlamydomonas reinhardtii*

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Abstract
This chapter examines some of the advances made in algal hydrogen production over the past decade as an outcome of the discovery of the sulfur-deprivation process. Both the scientific and technical barriers that need to be overcome before hydrogen photoproduction can be scaled-up at an industrial scale are examined. Basic photosynthesis principles behind photobiological hydrogen production are provided in order to orient the reader on how some microalgae under specific conditions can release hydrogen gas. Actual and theoretical limits of the efficiency of the process are also discussed. Emphasis is placed on algal biohydrogen production outdoors, and principles for guiding optimal photobioreactor design are presented. Finally, future prospects for commercial applications are discussed.

Keywords hydrogen; *Chlamydomonas reinhardtii*; photobioreactors; sulfur deprivation; hydrogenase

22.1 INTRODUCTION

In 2050, with continuing population growth, improving conditions in the developing world, and unabated demand, the world could well be using energy at 2.5 times the current rate of over 200 million barrels of oil equivalent per day. Nuclear energy might be part of the solution; however, the March 2011 Japanese disaster at the Fukushima power facility has caused many countries to reassess this path forward. Hydrogen, a valuable energy carrier, could easily be generated from nuclear-driven electrolysis, and this could replace some of the 10^8 m^3 (about 9×10^7 US tons, mostly from fossil sources) used per year in the United States alone. However, a more environmentally positive option is increasing the amount of renewable energy available, and examples include wind, photovoltaics (PV), geothermal, ocean thermal energy conversion (OTEC), wave power, biofuels, and solar thermal technologies that produce electricity, heat, and liquid fuels from biomass. Biohydrogen from microbial action is also included as renewable, and most approaches involve dark fermentation of waste organics. Nevertheless, H_2 photoproduction from cyanobacteria has been known since the 1890s (Jackson & Ellms, 1896) and from microalgae since 1942 (Gaffron & Rubin, 1942). Since the 1940s, interest in the photoproduction of H_2 has been intermittent (Gfeller & Gibbs, 1984; Happe & Naber, 1993; see Weaver et al., 1980, for an early review of the literature) and mostly at the basic research level up until the beginning of the millennium, when researchers at UC Berkeley and NREL reported prolonged H_2 production in the green alga, *Chlamydomonas reinhardtii*, at

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about a 0.1% conversion efficiency (light to H_2) when sulfate was removed from the suspension medium (Ghirardi et al., 2000; Melis et al., 2000). This review will examine some of the advances made in algal H_2 production over the past decade as an outcome of this discovery, but emphasis will be placed on mass cultivation of algae outdoors for biohydrogen photoproduction. A comparison of the different biohydrogen processes has been reported elsewhere (Dasgupta et al., 2010; Hallenbeck et al., 2012).

22.2 ALGAE AS A PLATFORM FOR PRODUCING HYDROGEN

Microalgae are phototrophic microorganisms capable of harvesting solar energy, while converting CO_2 and water photosynthetically to organic macromolecules (e.g., carbohydrates, proteins, and lipids). From a biofuels perspective, the main advantage of algae is that they can produce liquid transportation fuels, chemical feedstocks, high value products (from selected or engineered strains), and H_2. More specifically, microalgae (a) can produce much higher biomass yields per unit area than traditional crops; (b) have a much smaller overall land footprint for H_2 production using direct photosynthetic processes (without an intermediate biomass stage) than alternative schemes that produce H_2 indirectly from biomass; (c) do not compete for arable land or nutrients associated with conventional agriculture; (d) can use waste, saline, or seawater, minimizing the use of freshwater; (e) can recycle waste CO_2 from industrial (including bioethanol plants) emission sources; and (f) are amenable to the integrated production of coproducts along with fuels and fuel precursors.

22.2.1 Challenges for developing an algal biohydrogen system

Notwithstanding hydrogen's abundance in the universe, it is not freely available on Earth. It is in fact an energy carrier (and not a fuel that can be extracted by drilling or mining) and must be generated from chemically combined forms, including traditional fossil fuels, biomass, and most notably water since we have a lot of it. Release of H_2 for use as a fuel takes energy input, which is the first challenge. Electrical, thermal, or light energy can be mobilized as energy sources, but most of these require multiple steps, which add complexity, cost, and thermodynamic losses. Algae and cyanobacteria (blue-green algae) are unique in the biosphere in that they can use light energy directly to convert sunlight and water photosynthetically to H_2, using nitrogenase or hydrogenase enzymes (see next section).

Since outdoor scale-up of algal H_2-producing systems will be emphasized in this chapter, we will first outline both the scientific and technical barriers involved. To examine the former, we will first have to provide some biological background so that the reader can understand how algae produce H_2 from water (next section). Needless to say, many organisms can produce H_2 fermentatively in the dark (Hallenbeck, 2009; Abo-Hashesh & Hallenbeck, 2012) or photofermentatively (Eroglu et al., 2008; Eroglu & Melis, 2011; Adessi & De Philippis, 2012) from biomass or organics fixed by previous oxygenic photosynthetic processes, but these will not be discussed further.

22.3 ALGAL METABOLIC PROCESSES INVOLVED IN HYDROGEN SYNTHESIS

22.3.1 Photosynthesis

Algae use photosynthesis to fix CO_2 for carbohydrate synthesis. The process occurs on thylakoid membranes located within the chloroplasts of algae and involves three primary steps (Fig. 22.1) (Blankenship, 2002): (a) light absorption by chlorophyll and carotenoid pigments associated with each of the two photosystems (PS II and PS I), which harvest light and transfer energy to their respective reaction centers (RCs; P680 and P700 in Fig. 22.1); (b) light-driven charge separation within the RCs, where oxidants and reductants are generated; and (c) electron transport from PS II to PS I (charge equilibration) through a chain of electron carriers, including plastoquinones (PQ), which couple sequential oxidation–reduction reactions to proton translocation across the thylakoid membrane. The charge-separated state generated by the PS II RC ($\sim$1.8 V) is stabilized on the oxidizing side by electron donation from water through the oxygen-evolving complex (OEC) and that generated by the PS I RC ($\sim$1.5 V) is stabilized on the reducing side by electron transfer to an FeS-containing ferredoxin (Fd) molecule. Reduced Fd is the physiological electron donor to $NADP^+$, and Fd is responsible for the accumulation of NADPH necessary for CO_2 fixation by the Benson–Calvin cycle through Rubisco (the first enzyme of the cycle). The other requisite for CO_2 fixation, ATP, is generated upon dissipation of the proton gradient (formed by the release of protons during the water-splitting process and by proton transport across the photosynthetic membrane coupled to electron transport from PS II to PS I) through the chloroplast ATPase enzyme (not pictured in Fig. 22.1).

Under anaerobic conditions, photosynthesis can also produce H_2 directly from water in the following reactions: $2H_2O + \text{light energy} \rightarrow O_2 + 4H^+ + 4e^- \rightarrow O_2 + 2H_2$.

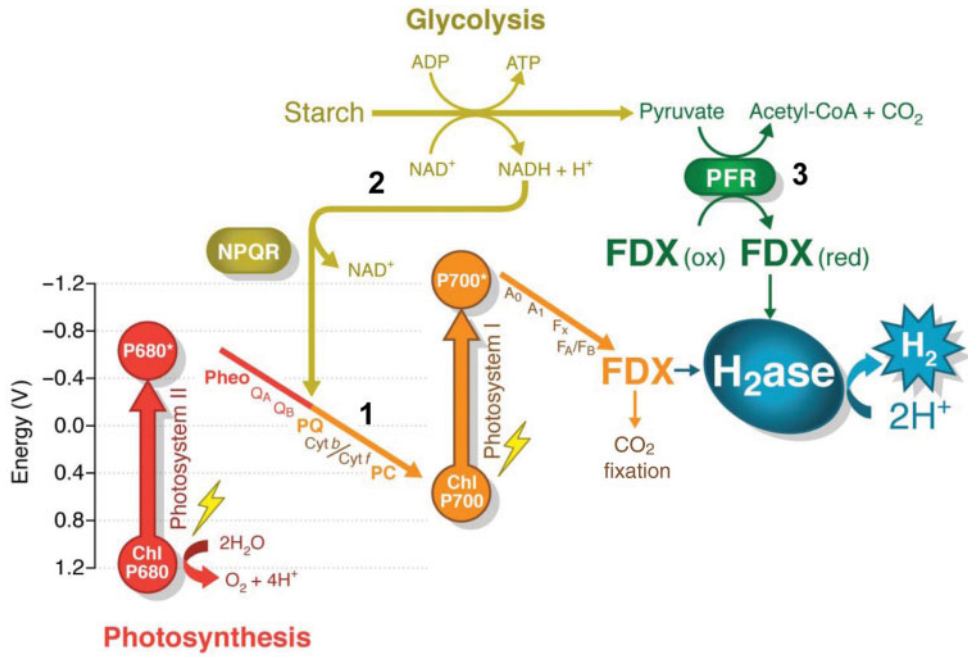

Figure 22.1. Primary pathways for hydrogen production in *C. reinhardtii* cells. Two of them (1 and 2) are driven by light and the third occurs totally in the dark (3). Hydrogen can be produced by both direct water photolysis operated by PS II (1), and indirectly through fermentation of starch (2), which is accumulated during the aerobic phase of the sulfur starvation. In this case, electrons are shuttled to intersystem electron transport carriers via the PQ pool and supplied to hydrogenase through PS I. To supply an electron indirectly to hydrogenase requires the absorption of an additional photon by PS I compared to the direct process.

While three pathways for algal H_2 production (two in the light and one in the dark) are now known, the most direct pathway to H_2 from water employs the normal photosynthetic pathway up to Fd, where reductant (electrons) are redirected to hydrogenases rather than Rubisco. As shown in Figure 22.1, either water or starch reserves previously stored in the algae can supply the electrons for hydrogenase function. The contribution of starch reserves to H_2 production changes with the strain, the ability to accumulate starch during the aerobic phase of sulfur starvation, and also the time after sulfur starvation (Laurinavichene et al., 2004). In one D1 mutant of *C. reinhardtii*, up to 40% of the total H_2 output was due to fermentation of stored carbohydrates (Scoma et al., 2012b).

22.3.2 Hydrogen-evolving enzymes

Both hydrogenases and nitrogenases are biological catalysts known to be involved in H_2 metabolism. Since nitrogenases produce H_2 as a byproduct of N_2 fixation in cyanobacteria, heliobacteria, and photosynthetic bacteria (Weaver et al., 1980; Houchins & Hind, 1984) but are not found in algae, they will not be discussed further (see Vignais et al., 2001 and Tamagnini et al., 2002 for reviews).

Three main types of hydrogenases have been described in the literature: [NiFe], [FeFe], and FeS-cluster-free (Vignais et al., 2001). Recent reviews of the biochemical and structural aspects of these enzymes are available (Lubitz et al., 2007; Fontecilla-Camps et al., 2009; Mulder et al., 2011). All the three types of hydrogenases are almost completely segregated within specific groups of organisms, suggesting convergent evolution (Vignais et al., 2001; Ludwig et al., 2006). [NiFe]-hydrogenase enzymes are present in most facultative anaerobic bacteria, photosynthetic bacteria, and cyanobacteria (Wu & Mandrand, 1993; Albracht, 1994). [FeFe]-hydrogenases, on the other hand, are found only in strictly anaerobic bacteria, hyperthermophiles, algae, fungi, and protists (Happe et al., 2002). The FeS-cluster-free Hmd enzyme (Hartmann et al., 1996; Pilak et al., 2006), although catalyzing H_2 oxidation, is primarily a methylene-tetrahydromethanopterin dehydrogenase (Lyon et al., 2004). A few organisms have been reported to express both [NiFe]- and [FeFe]-hydrogenases, including

Desulfovibrio sp. (Fauque et al., 1988), *Thermoanaerobacter tengcongensis* (Soboh et al., 2004), and some *Clostridial* species (Vignais et al., 2001).

22.4 THEORETICAL LIMIT TO PHOTOBIOLOGICAL HYDROGEN PRODUCTION

Although photobiological hydrogen production using microalgal photosynthesis to capture solar energy and split water into its components, H_2 and O_2, is an inherently appealing process, only a fraction of the total solar light radiation reaching the Earth's surface can be transformed into H_2 energy photosynthetically (Hallenbeck, 2011). Table 22.1 summarizes the expected energy losses of total incident solar radiation during the H_2-production process. It is estimated that about 10% of the photosynthetically active radiation (PAR) is reflected by the cultures. Microalgae, including *C. reinhardtii* when grown under high light also synthesize some protective pigments (xanthophyll cycle pigments) that dissipate excess light as heat. This photosynthetically inactive light absorption, to the extent it occurs, further lowers photosynthetic conversion efficiency. Furthermore, photons above 700 nm and below 400 nm are not utilized by microalgae pigments (and higher plants), and thus about 55% of incident solar light is unavailable to drive photosynthesis. The sum of losses by reflection and more importantly the amount of light out of the PAR range reduces the available light for photosynthesis (and H_2 production) to about 41%. The energy required to drive a charge separation event in PS II is approximately 176 kJ mol^{-1} (i.e., equal to the energy of a 680 nm wavelength photon), and 171 kJ mol^{-1} (i.e., the energy content of a photon at 700 nm) for PS I. If we assume that the mean energy content of photons in the 400–700 nm range is about 218 kJ mol^{-1}, then the loss of energy between absorption and charge separation in the two photosystems will be approximately $\{[218 - (171 + 176)/2]/218\} \times 100$; that is, approximately 20.4% of the incident solar energy is irretrievably lost as heat in the process, because of the relaxation of higher excited states of chlorophyll to the first excited singlet state (Zhu et al., 2008). If we assume that a minimum of 8 quanta are required to produce 2 mol of H_2, the energy content of 1 mol of H_2 is 286 kJ (higher heating value), and the mean energy available for charge

Table 22.1. Summary of the expected energy losses of total incident solar radiation energy in *C. reinhardtii* cultures during hydrogen production, achieved by means of sulfur starvation

Process	Energy radiation losses (%)	Remaining energy (%)
Total solar radiation	–	100
Reflection/scattering	10	90
Radiation outside PAR	55	41
Loss of useful absorbed PAR energy at 680 nm (PS II) and 700 nm (PS I) due to non-photochemical processes	20[a]	32.8
Conversion energy to H_2	59	13.4 (theoretical limit)
Cell maintenance in cultures subjected to diurnal cycle (Pirt et al., 1980)	25	10
PS II downregulation during sulfur starvation	86[b]	1.4

[a]The energy required to drive a charge separation event in PS II is approximately 176 kJ mol^{-1} (i.e., equal to the energy of a 680 nm photon) and 171 kJ mol^{-1} (i.e., a 700 nm photon) for PS I. Therefore, the average for the two photosystems is $171 + 176/2 = 173.5$ kJ mol^{-1}. If we assume that the average energy content of photons in the 400–700 nm range is 218 kJ mol^{-1}, then the loss of absorbed energy due to non-photochemical processes is $(218 - 173.5)/218 \times 100 = 20.4\%$: For blue photons the degradation loss increases to 40%.

[b]Evaluated with measurements of the effective quantum yield of PS II ($\Delta F/F'_m$) at the start of sulfur starvation and during hydrogen production (mean value) (Antal et al., 2003; Faraloni & Torzillo, 2010).

separation at PS II and PS I is 173.5 kJ mol^{-1}, then the efficiency of the process will be (286 kJ $mol^{-1} \times 2$)/(173.5 kJ $mol^{-1} \times 8$) $\times$ 100 = 41.2% (i.e., an energy loss of 59%). Consequently, the theoretical light conversion efficiency (LCE) for H_2 production, attainable by direct biophotolysis, is 13.4% of solar light. Interestingly, this conversion efficiency (the theoretical limit) is significantly higher than the theoretical limit for biomass or biodiesel production (11.2%; Tredici, 2010). This lower value is because the efficiency of the process depends on the number of steps necessary to produce a certain compound (i.e., the more the steps required in the biomass case, the lower the efficiency of the process).

22.4.1 Scientific barriers to reaching the theoretical efficiency limit

With the wealth of biological knowledge, the ability of algae to produce H_2, and the societal need to produce more fuel, including H_2, why is algal biohydrogen not commercial at this point? The answer, of course, is complex, and it turns out that there are still substantial scientific and engineering challenges to be solved.

The O_2 sensitivity of the hydrogenase enzymes, which perform the reaction $2H^+ + 2e^- \leftrightarrow H_2$, is probably the most significant barrier, and this issue has been reviewed thoroughly. Oxygen acts as a transcriptional repressor, an inhibitor of hydrogenase maturation, and an irreversible inhibitor of hydrogenase catalytic activity at the enzyme level in most, but not all, hydrogenases. The source of O_2 is, of course, O_2 from the atmosphere or O_2 from oxygenic photosynthesis itself. The reader is referred to a number of recent sources (Ghirardi et al., 2007; Posewitz et al., 2008), where progress has been reported more in the physical and physiological manipulation of the algae than in the engineering of the hydrogenase enzyme itself. The reason for this is that engineering approaches meant to improve the O_2 tolerance of the enzyme have so far emphasized manipulations of the hydrogenase gas channels (Cohen et al., 2005) in hopes that H_2 gas can diffuse out of the protein, but diffusion of O_2 into the hydrogenase enzyme could be restricted. If O_2 reaches the catalytic site, where H_2 gas is released, it can rapidly inactivate the enzyme. The core problem is thought to be that when engineered enzymes are assembled during the process of protein maturation (i.e., during which the active site metal cluster is inserted), the protein structure does not form properly (P. King, unpublished hypothesis). While there has been evidence of improved O_2 tolerance (Lautier et al., 2011) the activity of the enzyme was severely affected.

Physiological issues that are also known to limit algal H_2 production include (a) the existence of pathways that compete with the hydrogenase for electrons from ferredoxin, such as cyclic electron transfer under anaerobic conditions and CO_2 fixation under aerobic conditions (Fig. 2 in Seibert et al., 2008); (b) downregulation of photosynthetic electron transport under conditions where the proton gradient across the thylakoid membrane is not dissipated (Greenbaum et al., 1995; de Vitry et al., 2004; Kruse et al., 2005); and (c) low sunlight conversion efficiency of H_2 photoproduction due to the large light-harvesting antenna size of the photosystems (Melis, 2005). Issue (a) is under active investigation and new results will appear shortly. Several groups are looking at "leaky" ATPase mutants that will address issue (b). Finally, a recent study addressing issue (c) reports that a *C. reinhardtii* mutant (*tla1*), when immobilized in an alginate film, can produce more H_2 at higher light intensity than the parental strain (Kosourov et al., 2011). This was the first specific experimental evidence confirming Melis' hypothesis that an antenna-truncated mutant should be able to produce more H_2 than its wild type. Another recent exciting advance was the isolation of a multiple-phenotype mutant of *C. reinhardtii* that simultaneously accumulates more starch, is inhibited in cyclic electron transfer (i.e., state transitions are prevented, which allows more electrons to reach the hydrogenase enzyme), and has higher rates of respiration (Kruse et al., 2005). This mutant (*stm6*) might serve as a platform upon which further improvements are made in the future.

Other recent reports have shown significantly higher H_2 production in *C. reinhardtii* Q_B-binding D1 mutants (Torzillo et al., 2009; Faraloni & Torzillo, 2010). Their phenotypic characterization showed some common differences with respect to the wild type, such as (a) a reduced amount of chlorophyll both per dry weight and per cell; (b) a higher respiration-to-photosynthesis ratio, which reduced the length of the aerobic phase; (c) a greater capacity to accumulate carbohydrates; (d) a higher induction of the xanthophyll cycle pigments; and (e) a longer period of H_2 production. These characteristics, in a double amino substitution (L159I-N230Y), translated into an up to 18 times higher H_2-production output as compared to the wild type under the same conditions (Torzillo et al., 2009). With this mutant, the maximum apparent light-to-H_2 conversion efficiency (expressed on the basis of PAR) reached 3.2%, and the PS II efficiency for performing direct biophotolysis was calculated to be 2.03% (Scoma et al., 2012b). A compilation of *C. reinhardtii* mutants with enhanced H_2 production, and their major features has been reported (Esquível et al., 2011).

22.4.2 Technical barriers to reaching the theoretical efficiency limit

22.4.2.1 Sulfur-starvation protocol

The establishment and maintenance of anaerobiosis represents a main obstacle to the attainment of sustainable algal H_2 production. Two methods have been used historically to produce anaerobiosis in illuminated cultures, including (i) inert gas purging, and (ii) additions of exogenous reductants or O_2 scavengers to the culture. Obviously, both of these methods are expensive and impractical, especially when considering the large scale of possible commercial applications. Therefore, until an engineered, O_2-tolerant [FeFe]-hydrogenase is developed, the sulfur-deprivation method discovered by Melis et al. (2000) represents one of the few ways to circumvent the problem of hydrogenase sensitivity to oxygen. The sulfur-starvation strategy is based on the observation by Wykoff et al. (1998) that *C. reinhardtii* can lose as much as 75% of its initial photosynthetic capacity within 24 h following the removal of sulfate from the growth medium. The novel observations of the team at the NREL and the University of California, Berkeley, was that respiratory capacity, which removes O_2, was not affected by sulfur deprivation (Melis et al., 2000) and that illuminated, sulfur-deprived *C. reinhardtii* cultures subsequently become anaerobic and express hydrogenase enzyme function. Sulfur-starved cells cannot re-synthesize the PS II D1 protein, because the D1 repair cycle is blocked by the lack of available sulfate (Melis et al., 2000). When the photosynthesis rate drops below the level of respiration, the culture becomes anaerobic in a short period of time, and then induces hydrogenase activity, resulting in the production of significant amounts of H_2 for several days. Subsequently, H_2 photoproduction is reversible, if sulfate is re-added to the culture medium (reconstituting photosynthetic activity over a period of a day or two). At this point, the cycle can start again if the added sulfate is removed. Sulfur starvation strongly influences H_2 production by stimulating massive amounts of starch accumulation (while the culture is still aerobic), which is a storage sink for excess reducing power and is used by the alga to maintain anaerobic conditions during H_2 production.

A typical example of the kinetics of H_2 production recorded in cultures of *C. reinhardtii* wild type is shown in Figure 22.2. The entire H_2-production process can be divided into four main consecutive phases (Kosourov et al., 2002): (1) an aerobic phase characterized by initial accumulation of O_2, followed by its consumption during which photosynthesis cannot keep up with respiration; (2) a lag phase, during which neither net O_2 nor H_2 is evolved by the culture; (3) a H_2-production phase, during which H_2 is released; and (4) a termination phase, where the accumulation of H_2 ceases. It can be seen that the dissolved O_2 concentration increases during the first 24 h of sulfur starvation. Thereafter, it starts to decline as a result of a decrease in the rate of photosynthesis (due to increased PS II photoinhibition). This is clearly detected by a reduction in chlorophyll fluorescence, measured as $\Delta F/F'_m$, the effective quantum yield of PS II. H_2 production usually starts after a lag phase of about 16 h (but this can depend on the strain and physical conditions of the experiment) during which no increase either in O_2 or in H_2 production is observed. Because a reducing environment is established, the redox potential of the culture (measured with a platinum/gold electrode) which is positive during the aerobic phase, becomes negative (down to −550 mV,) and H_2 production begins after a rapid drop in $\Delta F/F'_m$ from 0.6 to about 0.2, which is observed for 16–50 h (depending on the exact conditions of the experiment) after the start of sulfur deprivation. This precipitous drop in the effective quantum yield of PS II during H_2 production was first reported in the Western literature by Antal et al. (2003), who interpreted the drop as the consequence of a rapid transition from state 1 to state 2 of the photosynthetic apparatus (Finazzi et al., 2002). State 1 to state 2 transitions represent a photoprotective strategy and are accompanied by the partial migration of LHC II antenna from PS II to PS I. This is also known to occur in *C. reinhardtii* under dark, anaerobic conditions (Cournac et al., 2002).

22.5 BIOTECHNOLOGY OF HYDROGEN PRODUCTION

22.5.1 Hydrogen production in laboratory-scale photobioreactors

The discovery of the sulfur-starvation technique in *C. reinhardtii* (Melis et al., 2000) represented an effective model for examining the biotechnology of algal H_2 photoproduction, because it has made it possible to sustain the H_2 process for a long enough time to optimize the culture conditions in order to maximize H_2-gas production. A number of studies have been carried out to enhance and prolong the H_2-production process, such as examinations of the effects of (1) low residual amounts of sulfur at the onset of the experiment (Laurinavichene et al., 2002); (2) re-adding limiting amounts of sulfur to the culture medium in the middle of the H_2-production phase (Kosourov et al., 2005); (3) using light-synchronized cultures (Tsygankov et al., 2002); (4) the initial pH of the medium (Kosourov et al., 2003); (5) using different growth conditions (Kosourov et al., 2007);

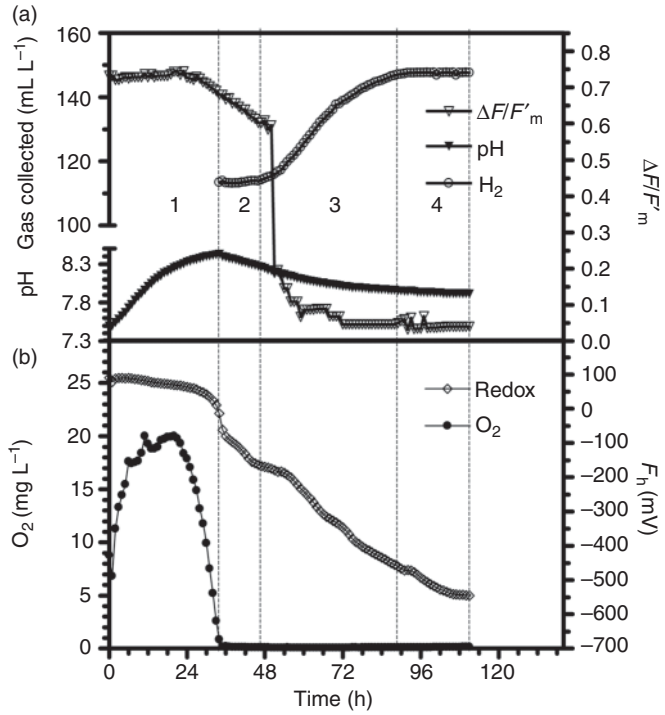

Figure 22.2. Time courses (a) for the output of H_2 gas (O), the effective quantum yield of PS II, ($\Delta F/F'_m$) (▽) and pH (▼); and (b) for redox potential measured with a platinum/gold electrode (E_h) (◊) and dissolved oxygen (pO_2) (●), recorded in laboratory cultures of *C. reinhardtii* wild type.

and (6) light intensity on the production of H_2 (Hahn et al., 2004; Laurinavichene et al., 2004; Kim et al., 2006; Tsygankov et al., 2006). An increase in H_2 production was also achieved by growing *C. reinhardtii* cells in wastewaters characterized by high C/N ratios (Faraloni et al., 2011).

Attempts to improve culture mixing to enhance H_2 photoproduction were also carried out by a French group (Pottier et al., 2005; Pruvost et al., 2006; Fouchard et al., 2008). The result of their research was the design of a complex torus-shaped photobioreactor (PBR) capable of online monitoring of culture behavior. In this PBR design the H_2-production rate reached a maximum of 2.5 mL L^{-1} h^{-1}, but the maximum photosynthetic efficiency attained was only about 0.1%. One of the primary advances achieved with this PBR design, however, was an exhaustive fluid dynamics analysis conducted with Computational Fluid Dynamics (CFD) software, which led the research group to define fully predictable behavior for the fluid inside the reactor.

Giannelli et al. (2009) designed a multistage, rotating impeller that was embedded inside an existing Roux-like PBR, which was similar to the one reported by Kosourov et al. (2002). One of the most important advances observed with this mixing system was a more ordered light–dark (L–D) pattern within the culture bulk volume, achieved by moving the algal cells very rapidly across the light gradient within the culture. An L–D cycle rate within 60 and 500 ms were realized between the center-to-outer wall parts of the PBR. The effect of the mixing system on the H_2 output was most prominent when the cultures were exposed to a combination of high light and high cell density. The improved light-to-dark cycle speed, to which cells were exposed with this impeller design, resulted in a 30% increase in the H_2-production output (Fig. 22.3). In fact, the maximum H_2-production rate was 5.6 mL L^{-1} h^{-1}, and the maximum LCE achieved was 1.64% under white fluorescent light.

Another novel laboratory PBR design, proposed by Tamburic et al. (2011), consisted of two flat compartments constructed from polymethyl methacrylate sheets. Turbulent mixing was achieved by a circulating gas-lift system, operated with a diaphragm pump. A membrane-inlet mass spectrometry system was used to measure the *in situ* concentrations of dissolved gases and volatile organic compounds in the aqueous medium. The LCE with this PBR design was about 0.24%.

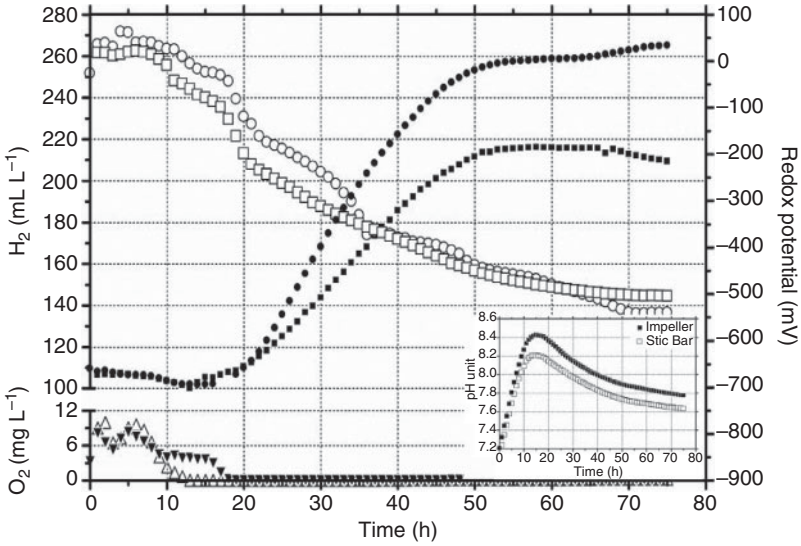

Figure 22.3. Time courses of H_2 photoproduction, recorded in laboratory cultures of *C. reinhardtii* strain CC-124, and mixed with an impeller (●) or a magnetic stir bar (■). Redox potential: mixing with an impeller (○); mixing with a stir bar (□). Dissolved oxygen: impeller (▼); stir bar (△). pH (insert): impeller mixed culture (■), stir bar mixed culture (□).

22.5.2 Hydrogen production in outdoor photobioreactors

The use of solar light is mandatory if one's goal is to scale-up photobiological H_2 production at an industrial level. A number of PBR designs are being proposed, mostly in mass culture of microalgae for biodiesel production (see Morweiser et al., 2010 for review), while information on the production of H_2 outdoors is still scarce. The first attempt to produce H_2 outdoors in a 50 L PBR, using sulfur-deprived *C. reinhardtii* (strain CC124), was reported by Torzillo and coworkers (Scoma et al., 2012a). The experiments were carried out in a fully controlled tubular PBR system (Fig. 22.4) under the climatic conditions of Central Italy (Florence, Latitude 43°N). Here the total light irradiance can reach as much as 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the summer, which is at least 10 times that used for laboratory experiments. The cells were grown mixotrophically outdoors in sterile tris-acetate-phosphate (TAP) medium, but the results indicated that under outdoor conditions, H_2 production was only about 21% of that attained in the laboratory on a culture volume basis. Chlorophyll fluorescence measurements attributed this low rate to a very rapid inactivation of PS II, resulting from extremely high light intensities. In fact, the effective quantum yield of PS II ($\Delta F/F'_m$) dropped below 0.1 in the middle of the day. The complete record of an experiment carried out in the outdoor PBR is shown in Figure 22.5. Scoma et al. (2012a) concluded that the reduced output achieved in the 50 L outdoor PBR compared to laboratory experiments, also reflected the different illumination pattern to which the cultures were exposed (one-sided vs. two-sided illumination used in the laboratory), as well as to the great difference in the culture mixing time (60 min vs. 15.5 s achieved in laboratory-scale PBRs). Mixing time, defined as the time necessary to achieve final equilibration of the culture throughout the medium after dilution (with a variance value of 5%) seems to play a crucial role in the H_2 production of *C. reinhardtii* since mixing affects the H_2 partial pressure in the PBR. An example of a mixing time determination carried out in a tubular PBR made of Pyrex glass tubes is shown in Figure 22.6.

The results of Kosourov et al. (2012) have demonstrated an inhibitory effect of high H_2 concentrations in the PBR headspace on H_2-photoproduction activity in algal cultures and have clearly demonstrated that H_2 output in *C. reinhardtii* cultures depends inversely on the partial pressure of H_2 in the PBR gas phase. Furthermore, the authors found that the ratio of the headspace volume to liquid suspension volume is an important factor for PBR geometry improvements. In fact, increasing the gas phase to liquid phase volume by a factor of 4 increased the total yield of H_2 gas by a factor of 2, which translates in a LCE of over 3% (PAR). These findings are extremely important for the

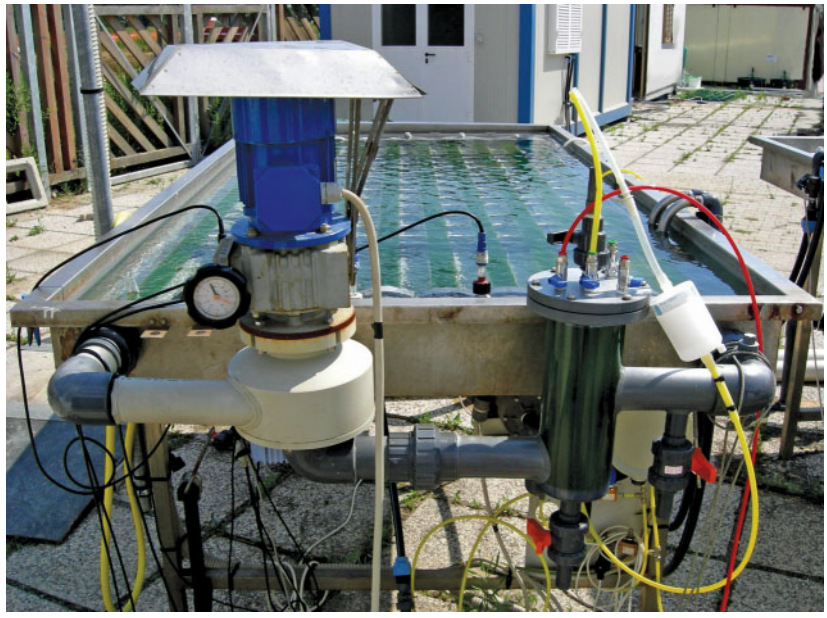

Figure 22.4. Photograph of a 50 L tubular reactor utilized for outdoor H_2-production experiments with sulfur-deprived cultures of *C. reinhardtii*.

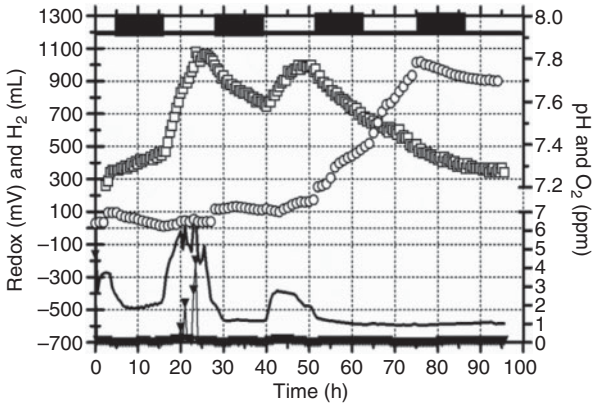

Figure 22.5. Time course of H_2 production in sulfur-deprived cultures of *C. reinhardtii* strain CC-124 grown outdoors in a 50 L tubular photobioreactor. Cultures grown under solar light were centrifuged, and the biomass was then re-suspended in TAP medium deprived of sulfur (TAP-S). The initial chlorophyll concentration immediately after sulfur starvation was 20 mg L^{-1}. White/dark bars at the top represent daily light/dark cycles. Symbols: H_2 accumulation (○), pH changes (□), dissolved O_2 concentration (▼), and redox potential (continuous line).

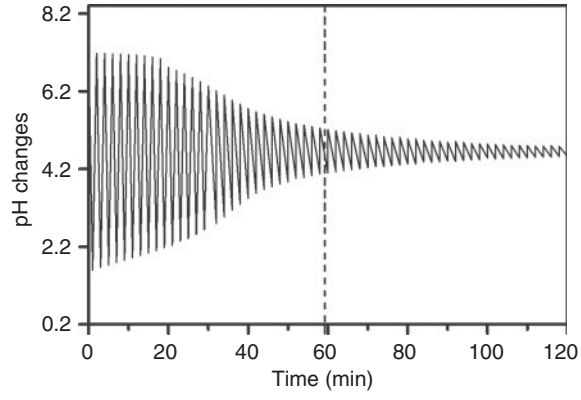

Figure 22.6. Example of a mixing time determination in a tubular photobioreactor (length, 23 m; i.d., 4.85 cm; volume; 50 L), recorded by changes in pH at the inlet and outlet points of the tubular reactor. Concentrated HCl solution (50 mL) was injected at the point where the liquid enters the circulation pump. The time required for the fluid to travel over the whole length of the tubular circuit (i.e., the time between two consecutive spikes or the time cycle (T_c) of the photobioreactor (~60 s)). The vertical line indicates the mixing time, defined as the time it took to achieve an equilibrium pH at final dilution with a variance of 5%.

optimal design of PBRs for H_2 production. Indeed, long tubular PBRs, usually characterized by excessively long mixing times (hours) and little if any headspace, may pose serious limitations on increasing the PBR tube length. Furthermore, the possibility of increasing culture speed to enhance turbulence and to allow a reduction in the mixing time is inconsistent with the necessity to minimize cell damage during mixing and minimize mixing energy cost.

Geier et al. (2012) conducted experiments with *C. reinhardtii* cultures outdoors in a 0.9 L bubble column PBR (4.9 cm internal diameter) using TAP medium. The cells were subsequently tested for H_2 production under laboratory conditions, but the amount of H_2 produced by this PBR was only 10% of that achieved by laboratory cultures.

22.5.3 Lessons learned

The experience gained from experiments with different PBR designs, operated both indoors and outdoors, has provided the following guidelines that may prove useful for optimal design of future PBRs for H_2 photoproduction:

1. **Uniform illumination of the culture**. Illuminating cultures from both sides of the PBR is preferable to illuminating them from just one side. This goal can be more easily achieved using a PBR situated in the vertical position.
2. **Low dark-to-illuminated culture volume ratio**. Some parts in the PBR fabricated with dark materials are unavoidable (e.g., pumps, connectors, degassers); however, unlit parts of the culture cause additional L–D cycles with low frequency, which reduce performance. Therefore, it is advisable that the ratio of dark to total culture volume should be kept as small as possible ($\leq$0.05).
3. **Low H_2 partial pressure**. It is advisable to reduce as much as possible the H_2 partial pressure in the reactor since it inhibits H_2 production by the culture. Indeed, it has been found that a PBR in which the H_2 partial pressure is above 0.05 atm (i.e., 5% H_2 partial pressure in the headspace) strongly reduces the H_2 output (Kosourov et al., 2012). This goal can be reached by increasing the ratio between the gas phase (headspace) and liquid phase (V_{gp}/V_{lp}). Application of rapid mixing and the application of a negative pressure in the PBR can facilitate H_2 degassing of the culture, but the energy cost of this approach must be assessed.
4. **High light surface to volume ratio (*S*/*V*)**. This parameter influences the amount of light available per unit of culture volume, and hence the volumetric productivity. High *S*/*V* ratios can be attained by reducing the average light path. However, the shorter the light path (e.g., narrow tube diameters), the higher the head loss, and consequently, the higher the energy expenditure for culture mixing.
5. **High ratio between illuminated area and ground surface area occupied by the reactor (A_R/A_G)** (Posten, 2009). This ratio gives an indication of the "light dilution factor" that can be sensed by the PBR. In order to benefit from the light dilution effect, sunlight impinging on a given ground area should be spread over a PBR surface area, and this can be accomplished by increasing the illuminated surface of the PBR. In principle, the average daily sunlight irradiance recorded on a horizontal surface should be reduced by a factor corresponding to the A_R/A_G ratio, such that the incident light on the PBR surface falls below the photosynthetic light saturation level of the culture. The optimum value of the A_R/A_G ratio, therefore, will depend on the algal strain and the place where the PBR operates. Inclined or vertical PBRs intercept sunlight at large angles and thus "dilute" light compared to horizontal PBRs. This is why vertical PBRs are expected to be more efficient than horizontal ones in terms of solar energy utilization (Hu et al., 1996; Cuaresma et al., 2011). In close proximity, higher productivity of PBRs per unit land area can be achieved at the expense of higher installation cost (Tredici, 2010).
6. **Orientation of vertical PBRs**. The best orientation of rows aimed at harnessing the highest amount of solar irradiance depends on the latitude. East/west oriented flat plate and vertically arranged (fenced) tubular reactors intercept more light than north–south for latitudes above 35°N, while at lower latitudes the result is the opposite (Sierra et al., 2008).
7. **Reduced mixing time**. This parameter has been reported to affect the H_2 production (Scoma et al., 2012a; Oncel & Sabankay, 2011)). As discussed previously, long mixing times can increase the H_2 concentration level in a PBR, which inhibits hydrogenase activity. Long mixing times may occur more frequently in tubular PBRs, which are characterized by plug flow patterns. Shorter mixing times are more easily attainable in flat PBRs, where mixing involves the whole culture volume simultaneously.
8. **Turbulent mixing**. Mixing is necessary to (a) ensure that all the cells are exposed to light; (b) maintain the nutrient supply throughout the reactor; and (c) diminish the nutritional and gaseous concentration gradients surrounding the cells, which improves the rate of gas exchange (the O_2 and H_2 produced by the algae) at the liquid medium/gas headspace interface. In order to

reach turbulent flow, the Reynolds number should be over 4000.

22.6 MATERIALS FOR HYDROGEN PHOTOBIOREACTOR CONSTRUCTION

The low solar LCE of H_2 photoproduction attainable with current technology translates into a requirement for a larger PBR surface area per unit of energy produced than is desirable, and this is a critical problem since construction of low-cost PBRs are already problematic from an economic perspective (see Chapter 13). Development of sturdy, long-lived materials, which can maintain their transparency and gas impermeability for years, is essential for facilitating biohydrogen production at an industrial scale.

The materials used for PBR construction play a major role in the cost of the H_2-production process. A survey of the material requirements for photobiological H_2 production has been reported by Blake et al. (2008). The main requirements are (1) as high as possible transmittance within the 400–900 nm spectral range (depending on the organism), (2) as much durability as possible (outdoor lifetime), (3) biocompatibility with the medium and metabolites produced by the algae, (4) gas permeability rates (for O_2 and H_2) as low as possible, and (5) maximum physical and mechanical resistance. Glass has many advantages as a glazing material; however, because of its weight and the high cost of low iron glass with high transparency to the solar spectrum, researchers have searched for suitable polymer materials. According to Blake et al. (2008), the best performers, as measured by optical properties after accelerated and real-time weathering tests, were acrylics, polycarbonates, polyesters, and fluorinated polymers such as Teflon^{TM} and related materials. A key property for construction materials for outdoor H_2 production is the rate of H_2 and O_2 permeation through these materials. While data on the permeability coefficient of O_2 are available for a wide range of polymers, similar data for H_2 permeation are rare. Polyesters (PET) and particularly polyvinylidene difluoride (PVDF) including Hylar (Solvay), Kynar (Arkema), and Solef (Solvay), are among the polymers with the lowest known H_2 permeabilities. Silicone has one of the highest H_2 and O_2 permeabilities; therefore, its use as a material in PBR construction should be avoided.

22.7 NET ENERGY RATIO FOR PHOTOBIOLOGICAL HYDROGEN PRODUCTION

The net energy ratio (NER) is a dimensionless parameter, calculated for the lifetime of an energy-producing system, between the total energy output (H_2 produced) and the energy content of all the materials used to construct the production plant (namely their "embodied energy") plus the energy required for operating the plant (mixing, cooling, nutrient supply, etc.). The NER can be also more generally expressed as the ratio between the total renewable energy produced and the primary nonrenewable energy requirement associated with the system LCA, or life cycle assessment (Spitzley & Keoleian, 2004). Therefore, for an energy-generation process to be sustainable and have an ecological benefit, its NER should be greater than 1, and certainly as high as possible. The NER provides a monetary-independent analysis for the viability of an energy-generation process, and it has previously been calculated for H_2 production by steam methane reformation and electrolysis of water with electricity derived from wind energy (Spath & Mann, 2001, 2004). As outdoor photobiological H_2 production is still at the prototype stage, a comprehensive assessment of its NER is not yet possible. Yet, an upper limit of the NER, that is, considering the theoretical H_2 output, has been estimated by Burgess & Fernández-Velasco (2007). These authors calculated the NER for PBRs made with three different materials (glass, low-density polyethylene (LDPE), and acrylic) as a function of the PBR tube diameter and the LCE to H_2. The larger the tube diameter, the higher the NER, because of the reduced energy expenditure for pumping associated with larger diameter tubes. They concluded that the highest NER could be attained with a PBR made from tubes constructed with LDPE (0.18 mm thick). Using this material the NER could be above 1 when the LCE was 1%, when the tube diameter was larger than 6 cm, and this is due to the rather low energy input for mixing. If a 5% LCE could be attained with the same tube diameter, the NER would increase to 5. Acrylic tubes proved to be the worst performers, while glass and low density polyethylene (LDPE) results were very similar (Burgess & Fernández-Velasco, 2007). Similar calculations for H_2 production in flat PBRs are not available; however, NERs $>$ 1 have been reported for flat-plate PBRs using microalgae for biomass production (Rodolfi et al., 2009). In a revised study, based on the actual performance of different culture systems for algal oil production, the NER for a so-called second generation flat-plate PBR was 1.65, for a horizontal tubular PBR the NER was 0.07, and for a raceway pond it was 3.05 (Jorquera et al., 2010). However, the NERs for biomass production in PBRs were 4.51 (flat plate), 8.34 (ponds), and 0.2 (tubular). Nevertheless, it must be pointed out that this study considered neither the cost of different construction materials, nor the costs required for microalgal harvesting and oil extraction, which could significantly

increase the energy demand, particularly in the case of open ponds.

22.8 CONTAMINATION

Contamination may represent a serious concern for H_2 production, particularly with cultures growing in the presence of organic substrates. This is the case with H_2 production, carried out with the current generation of sulfur-deprived *C. reinhardtii* cultures, which use acetate (contamination with bacteria can easily occur when this substrate is available). In the pharmaceutical field, it is common practice to steam sterilize bioreactors up to 20 m^3; however, for H_2 production, necessarily carried out in PBRs with incomparably higher surface to volume ratios, sterilization of such large PBRs becomes impracticable. On the other hand, closed PBRs can represent an efficient barrier against contamination by other organisms, including microalgae, and they can therefore facilitate maintenance of pure, monocultures of algae where growth of the organisms can be maintained at the optimum temperature, pH, and agitation. Although, undesirable organisms might still be present, their growth can be kept at an acceptable level by manipulation of the culture conditions. As a future perspective, the use of autotrophic cultures is preferable due to both the lower cost of the process and a much lower expected level of contamination of these cultures by bacteria. It has been demonstrated that autotrophic, sulfur-deprived *C. reinhardtii* cultures are able to sustain H_2 production (Tsygankov et al., 2006; Kosourov et al., 2007; Tolsygina et al., 2009).

22.9 FUTURE PROSPECTS FOR COMMERCIAL APPLICATIONS

Success of any renewable energy technology, including photobiological H_2 production, is dependent on the ability to develop a cost-effective process. Currently, biohydrogen production with *C. reinhardtii* is a sequential, two-stage process, in which growth and H_2 evolution are separated spatially, and this is the only sustainable way with algae to produce H_2 for several days at a time. However, the efficiency of the process in most cases is below 1% under laboratory conditions, and only in some cases has it reached about 3% PAR by using mutants (Kruse et al., 2005; Scoma et al., 2012b), by immobilizing wild type (Kosourov & Seibert, 2009) algae, or by controlling the partial pressure in the gas phase above the culture (Kosourov et al., 2012). The efficiency is further reduced when H_2 production is carried out under solar light conditions (Scoma et al., 2012a). The two-step process also carries a penalty, in terms of maximum attainable photon conversion, since three photons (instead of two as for direct photolysis) are required to transfer an electron from water to hydrogenase (i.e., one photon to extract one electron from H_2O; one photon to transport the electron through PS I to form NAPH and starch, and one photon to transport the electron from starch to hydrogenase through PS I). However, one advantage of the two-step process is that since O_2 is almost totally absorbed by respiration, the purity of the H_2 produced is very high, approaching 98% if CO_2 is removed by a simple scrubbing system. Furthermore, the direct H_2 conversion process (i.e., using algae with an O_2-tolerant hydrogenase) could reach up to a 10% LCE (Table 22.1). According to James et al. (2009), if this value could be maintained in sunny areas, the cost of algal H_2 could reach as low as $2.99 per gallon gasoline equivalent (gge), while with a LCE of 2% the estimated cost is $8.15 (gge). The same authors have estimated that if sulfur-deprived algae reached 1.5% LCE, the cost of hydrogen would be $8.44 (gge). However, it must be pointed out that in these calculations: (1) neither annual nor daily variations in light intensity were taken into account (indeed, the effect of sudden changes of light irradiance during the day or between consecutive days may cause loss of efficiency due to the necessity for the cells to acclimatize to new light conditions), (2) the cost of algal biomass disposal was not considered, and (3) algal immobilization on alginate (a fermentable film) was not analyzed.

With rises in the cost of energy and the problems of an increasingly carbon constrained world, H_2 is expected to become ever more important as a clean fuel for the future. In fact, microalgal H_2 production has considerable potential to contribute to a sustainable H_2 supply without incurring "food vs. fuel" concerns associated with first generation biofuels. An additional environmental advantage of photobiological H_2 production, compared to bioethanol and biodiesel production, for example, is that the combustion of H_2 (e.g., by an on-site fuel cell system linked to the electricity grid) would allow for the recycling and purification of water (since the combustion of H_2 and O_2 can generate pure distilled water). Finally, the engineering of *C. reinhardtii* (or other appropriate photosynthetic microbial) strains via genetic modifications, which incorporate O_2-insensitive hydrogenases, and the development of high performance, low-cost PBRs will be seminal to improving the feasibility of biohydrogen production processes.

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