CHAPTER 3

Solar-Driven Hydrogen Production in Green Algae

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Abstract

The twin problems of energy security and global warming make hydrogen an attractive alternative to traditional fossil fuels with its combustion resulting only in the release of water vapor. Biological hydrogen production represents a renewable source of the gas and can be performed by a diverse range of microorganisms from strict anaerobic bacteria to eukaryotic green algae. Compared to conventional methods for generating H_2, biological systems can operate at ambient temperatures and pressures without the need for rare metals and could potentially be coupled to a variety of biotechnological processes ranging from desalination and waste water treatment to pharmaceutical production. Photobiological hydrogen production by microalgae is particularly attractive as the main inputs for the process (water and solar energy) are plentiful. This chapter focuses on recent developments in solar-driven H_2 production in green algae with emphasis on the model organism *Chlamydomonas reinhardtii*. We review the current methods used to achieve sustained H_2 evolution and discuss possible approaches to improve H_2 yields, including the optimization of culturing conditions, reducing light-harvesting antennae and targeting auxiliary electron transport and fermentative pathways that compete with the hydrogenase for reductant. Finally, industrial scale-up is discussed in the context of photobioreactor design and the future prospects of the field are considered within the broader context of a biorefinery concept.

I. INTRODUCTION

Global energy consumption in 2008 was estimated at ~16TW-years (5.1×10^{20} J) and is predicted to rise by 44% to ~23TW-years (7.4×10^{20} J) by 2030 (IEA, 2010). Concomitantly, 60–80% cuts in total CO_2 emissions relative to 1990 levels are thought to be required by 2050 to avoid the worst impacts of climate change (Stern, 2006). Therefore, bridging the energy gap without increasing CO_2 emissions will require radical changes to the way energy is produced and consumed.

A. The hydrogen economy

One possible solution is a carbon-free, ''hydrogen economy'' (Blanchette, 2008; Lattin and Utgikar, 2007; Marbán and Valdés-Solís, 2007; McDowall and Eames, 2007). However, unlike oil, gas, or coal, hydrogen is a secondary energy carrier and must therefore be generated from alternate sources. The majority of hydrogen is currently created from fossil fuels which would make any change in energy supply unsustainable (Bartels *et al.*, 2010), but can also be generated from renewable sources

including hydroelectric, wave, wind, solar, geothermal, biomass (Bartels *et al.*, 2010), or biological approaches, which can be classified as dark fermentative (Das, 2009; Guo *et al.*, 2010; Hallenbeck and Ghosh, 2010), photofermentative (Akkerman *et al.*, 2002; Kars and Gündüz, 2010), or biophotolytic (Bothe *et al.*, 2010; Ghirardi *et al.*, 2009; Kruse *et al.*, 2005b).

II. BIOPHOTOLYTIC H_2 PRODUCTION

By far the most abundant renewable energy supply available on earth is in the form of solar radiation, which totals $\sim 5 \times 10^{24}$ J/annum (Miyamoto, 1997). This is $\sim$11,000$\times$ more than the total energy demand in 2008, making solar-driven H_2 production processes an attractive prospect.

Biophotolytic H_2 production is the process whereby the light-driven oxidation of water by the photosystem II (PSII) complex of oxygenic photosynthesis Eq. (3.1) is coupled to the enzymatic reduction of protons to H_2 Eq. (3.2) to give the net reaction shown in Eq. (3.3).

$$2H_2O \rightarrow 4H^+ + O_2 + 4e^- \tag{3.1}$$

$$2H^+ + 2e^- \rightarrow H_2 \tag{3.2}$$

$$2H_2O \rightarrow 2H_2 + O_2 \tag{3.3}$$

The formation of hydrogen gas is catalyzed in green algae by a ferredoxin-dependent [FeFe]-hydrogenase (Forestier *et al.*, 2003; Happe and Kaminski, 2002), in unicellular cyanobacteria via a NADPH-dependent [NiFe]-H_2ase (Volbeda *et al.*, 1995) and in nitrogen-fixing cyanobacteria in the absence of N_2 via a nitrogenase (Benemann, 1996; Bothe *et al.*, 2010; Nath and Das, 2004).

The advantage of biophotolytic processes over conventional photovoltaic technologies includes absolving the need for expensive rare metals and the potential for coupling to a range of processes such salt or waste water treatment, carbon capture (Sheehan *et al.*, 1998), human and animal food provision, production of health supplements, biopolymers, cosmetics, high-value molecules (Spolaore *et al.*, 2006; Stephens *et al.*, 2010), or therapeutic proteins such as vaccines and antibodies (Rasala *et al.*, 2010).

Here, we highlight recent advances in understanding the molecular basis of hydrogen production in green algae, in particular, the model alga *Chlamydomonas reinhardtii*, and possible strategies to improve the yield both in terms of development of new strains and bioreactor design. Cyanobacterial H_2 production has been reviewed elsewhere (Angermayr *et al.*, 2009; Bothe *et al.*, 2010; Lopes Pinto *et al.*, 2002; Sakurai and Masukawa, 2007).

III. H_2 PRODUCTION IN GREEN ALGAE

The presence of H_2 metabolism in green algae has been known since the 1930s (Gaffron, 1939), although extensive screening of culture collections has since shown that it is not a universal trait (Ben-Amotz *et al.*, 1975; Brand *et al.*, 1989; Greenbaum *et al.*, 1983; Healey, 1970; Meuser *et al.*, 2009; Skjånes *et al.*, 2008; Stuart and Gaffron, 1972; Timmins *et al.*, 2009a).

C. reinhardtii is currently the main model organism for studying algal H_2 production (Hemschemeier *et al.*, 2009). A typical *C. reinhardtii* cell is $\sim$10 μm in diameter and possesses a single large chloroplast in addition to a nucleus containing a 121 Mbp genome, multiple mitochondria, and two flagella for motility and mating (Merchant *et al.*, 2007). Strains exist as either mating type positive (*mt*+) or mating type minus (*mt*−), which together are capable of sexual reproduction, allowing for classic genetic analysis. Additionally fully sequenced and transformable mitochondrial, chloroplast, and nuclear genomes (Boynton *et al.*, 1988; Maul *et al.*, 2002; Mayfield and Kindle, 1990; Merchant *et al.*, 2007; Randolph-Anderson *et al.*, 1993; Vahrenholz *et al.*, 1993), microarrays (Gonzalez-Ballester *et al.*, 2010; Nguyen *et al.*, 2008), and a large collection of expressed sequence tags (Asamizu *et al.*, 1999) and mutants are available to facilitate genetic analysis.

A. Sources of reductant for hydrogen production in *C. reinhardtii*

1. PSII-dependent pathways

In this process, PSII catalyzes the photolysis of water, producing O_2 and releasing electrons into the photosynthetic electron transport (PET) chain, ultimately generating reduced ferredoxin that can be used by hydrogenase to reduce protons to hydrogen (Fig. 3.1; Greenbaum *et al.*, 1983). This process offers the maximum theoretical efficiency of converting solar to H_2 energy (Kruse *et al.*, 2005a), but because [FeFe]-hydrogenases are irreversibly inactivated by O_2 (Stripp *et al.*, 2009b), as soon as cells begin to produce oxygen at high rates, hydrogen evolution rapidly stops (Ghirardi *et al.*, 1997), meaning it is not currently a viable process under standard conditions.

2. PSII-independent pathways

Starch reserves accumulated through photosynthetic activity can undergo autofermentation to produce NADH and pyruvate, both of which can be oxidized to provide electrons for hydrogen production. In the case of pyruvate, a chloroplast-located pyruvate:ferredoxin oxidoreductase (PFOR) is likely to be involved in reducing ferredoxin in the dark, and

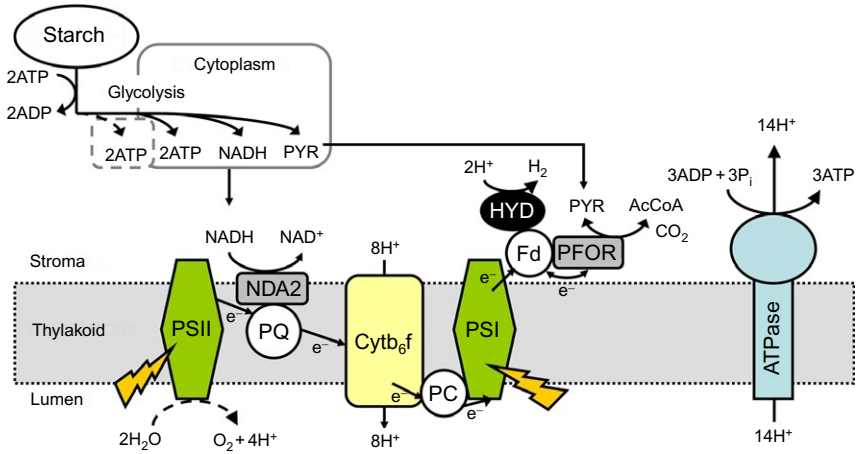


FIGURE 3.1 Schematic representation of potential sources of reductant for hydrogen production during (1) transient illumination of dark-adapted cultures, (2) dark fermentation, and (3) sulfur depletion. Abbreviations: $Cytb_6f$, cytochrome b_6f complex; Fd, ferredoxin; HYD, [FeFe]-hydrogenase; NDA2, type II NADH dehydrogenase; PFOR, pyruvate:ferredoxin oxidoreductase; PSI, photosystem I; PSII, photosystem II; PC, plastocyanin; PQ, plastoquinone. For simplicity, the Q cycle at the $Cytb_6f$ complex is not shown.

possibly the light, with the concomitant production of CO_2 and acetyl-CoA (Atteia *et al.*, 2006; Mus *et al.*, 2007; Fig. 3.1). Oxidation of NAD(P)H occurs in the light via NDA2, a thylakoid-associated type II NADH dehydrogenase (Desplats *et al.*, 2008; Jans *et al.*, 2008) which feeds electrons into the PET at the point of the plastoquinone (PQ) pool (Fig. 3.1).

B. Sulfur depletion

The most commonly used protocol for achieving sustained H_2 evolution in the light involves starving cultures of sulfur (Melis *et al.*, 2000), which can be described as passing through five phases: aerobic, oxygen consumption, anaerobic, hydrogen production, and termination (Kosourov *et al.*, 2002).

Aerobic and O_2 consumption phases last between 20 and 30 h (Kosourov *et al.*, 2002) but are strain (Chochois *et al.*, 2009, 2010) and condition dependent (Kosourov *et al.*, 2002). Initially, cells continue to evolve oxygen (Ghirardi *et al.*, 2000; Kosourov *et al.*, 2002; Melis *et al.*, 2000) and accumulate energy reserves in the form of starch (Timmins *et al.*, 2009b; Zhang *et al.*, 2002) and triacylglycerides (TAGs; Timmins *et al.*, 2009b), which is a well-characterized response to nutrient limitation

and can also be seen during nitrogen starvation (Ball *et al.*, 1990) and salt stress (Siaut *et al.*, 2011). These changes are followed by the rapid degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) suggesting a decline in CO_2 fixation rates (Zhang and Melis, 2002; Zhang *et al.*, 2002) and cultures enter a phase of oxygen consumption, as by depriving cells of sulfur, synthesis of the PSII reaction center protein D1 is impaired, reducing the cell's ability to repair PSII (Nixon *et al.*, 2010; Wykoff *et al.*, 1998). Taken together, the lack of Calvin cycle activity leading to the overreduction of PQ (Antal *et al.*, 2003), and impaired D1 turnover, causes a decrease in the number of active PSII centers to about 5–10% of normal levels (Zhang *et al.*, 2002), as determined by chlorophyll fluorescence measurements (Antal *et al.*, 2003; Zhang *et al.*, 2002) and the rate of photosynthetic oxygen evolution (Wykoff *et al.*, 1998). Initially, respiratory activity in the mitochondrion remains largely unimpaired (Zhang *et al.*, 2002) causing the rate of photosynthesis to drop below respiration and sealed cultures to go anaerobic (Melis *et al.*, 2000). These changes are accompanied by a decrease in photorespiratory and TCA cycle intermediates (Timmins *et al.*, 2009b), as O_2 and CO_2 become limiting, and a decline in chlorophyll levels as cells seek to limit photodamage (Melis *et al.*, 2000; Zhang and Melis, 2002).

A brief lag period ensues where cells consume dissolved oxygen and induce hydrogenase expression, followed by hydrogen evolution which lasts several days (Melis *et al.*, 2000). In the absence of CO_2 fixation, H_2 production acts to allow continued PET for the generation of ATP by photophosphorylation albeit at a reduced rate (Melis, 2007). Additionally, limited O_2 availability causes reduced rates of oxidative phosphorylation in the mitochondria; resultantly, glycolysis and anaerobic fermentation also become major pathways for ATP formation (Timmins *et al.*, 2009b). This suggestion is supported by the decrease in starch concentration during the hydrogen production phase (Zhang *et al.*, 2002) and the excretion of formate (Hemschemeier *et al.*, 2008b; Timmins *et al.*, 2009b; Tsygankov *et al.*, 2002) and ethanol (Hemschemeier *et al.*, 2008b; Kosourov *et al.*, 2003; Timmins *et al.*, 2009b) along with minor amounts of succinate and amino acids with the exception of glutamate (Timmins *et al.*, 2009b) which allows for the reoxidation of NADH formed by glycolysis. The amino acids excreted are found to originate at least partly from *de novo* synthesis (Timmins *et al.*, 2009b), which coupled to the uptake of ammonium via the glutamine synthetase/glutamate synthase cycle also oxidizes one molecule of NADPH.

In the final period, hydrogen production gradually declines to a stop despite the continuing presence of energy reserves in the form of starch, TAGs, and acetate, which could be due to the toxic nature of the accumulated metabolites or as a result of the long-term consequences of sulfur depletion (Timmins *et al.*, 2009b).

1. Contribution of PSII to H_2 production during sulfur starvation

The precise contribution of the PSII-dependent and -independent pathways to hydrogen production is still unclear and a range of values have been proposed (Antal *et al.*, 2009; Chochois *et al.*, 2009; Kosourov *et al.*, 2003) with the most recent suggestion of up to 90% of electrons coming from residual water splitting (Chochois *et al.*, 2009, 2010), but this value is likely to be strain and condition dependent, which could provide an explanation for the differences reported (Chochois *et al.*, 2009).

The importance of PSII activity for the process was first discerned by the inability of mutants lacking PSII to evolve H_2 (Hemschemeier *et al.*, 2008a; Zhang *et al.*, 2002). Similarly, addition of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at the beginning of sulfur depletion led to an inhibition of H_2 evolution (Hemschemeier *et al.*, 2008a). However, if PSII inhibition was delayed until after the aerobic and oxygen consumption phases, hydrogen evolution was observed, although at a vastly reduced level ($\sim$10% uninhibited; Chochois *et al.*, 2009) indicating that, as with light-induced hydrogen production, there is a PSII-independent source of reductant for the hydrogenase. This indirect pathway is dependent on PSII activity during the initial stages of sulfur depletion, suggesting it is based on degradation of the energy reserves formed in the aerobic phase (Chochois *et al.*, 2009; Hemschemeier *et al.*, 2008a). In addition, the indirect pathway was found to be absent in starch-deficient mutants, indicating carbohydrate reserves are the sole source of reductant for PSII-independent H_2 production and not protein or TAGs (Hemschemeier *et al.*, 2008a). Further evidence for this theory came from mutants affected in starch catabolism, which showed a general decrease in the amount of PSII-independent H_2 production, and those with a reduced rate of starch degradation which showed delayed H_2 evolution (Chochois *et al.*, 2010).

2. Role of starch and acetate during H_2 production

NADH produced from starch breakdown plays a dual role in hydrogen production: it is oxidized via the mitochondrial respiratory chain, to keep oxygen levels sufficiently low to allow expression of active hydrogenase for the PSII-dependent pathway (Melis, 2007), and it feeds electrons into the PET chain in the indirect pathway (Bamberger *et al.*, 1982; Melis, 2007). However, experiments using the ADP-glucose pyrophosphorylase deficient mutant, *sta6*, which is unable to accumulate starch, found wild-type levels of H_2 evolution during sulfur depletion (Chochois *et al.*, 2009). This result suggests that starch is dispensable for PSII-dependent hydrogen production and can be explained by the fact that, unlike *wt* cultures, *sta6* consumes acetate during the hydrogen production phase, which could act as a replacement for carbohydrate in maintaining respiration (Chochois *et al.*, 2009).

Similarly, although acetate is usually included in the growth medium (Melis *et al.*, 2000), it is dispensable for H_2 production when cultures are grown under a defined light regime, with CO_2 bubbling to promote starch accumulation (Kosourov *et al.*, 2007; Tolstygina *et al.*, 2009; Tsygankov *et al.*, 2006). This suggests the role of starch or acetate in the maintenance of respiration is interchangeable, although CO_2 bubbling and acetate are required for maximum H_2 yields (Kosourov *et al.*, 2007).

IV. IMPROVING H_2 PRODUCTION IN *C. REINHARDTII*

It has been calculated that if coupled to the production of high-value products, biophotolytic energy conversion efficiencies of around 5% will be required for economic viability (Kruse *et al.*, 2005b). Currently achievable light to hydrogen conversion efficiencies, as demonstrated in a laboratory environment, are in the region of 1% postsulfur deprivation for *C. reinhardtii* mutant stm6 (Kruse *et al.*, 2005b). Therefore, as with dark and photofermentative processes (Hallenbeck and Ghosh, 2010), further increases in efficiency are still required.

A. Choice of WT strain

Different laboratory strains of *C. reinhardtii* display a great variation in rates of hydrogen production; thus it is important to choose the right strain to work with. Long-term maintenance in culture collections has presumably allowed for the accumulation of mutations—with strains supposedly of the same genotype from different laboratories displaying markedly different phenotypes (ranging from ~55 to 95 ml/l H_2 reported for different CC-124 isolates; Chochois *et al.*, 2010). The precise reasons why strains differ in their ability to produce hydrogen are unknown, and careful genetic analysis may be a means of identifying mutations important for the process.

The choice of strain for study is important not only for H_2 production but will also determine the ease of genetic analysis. The best H_2 production rates have been reported using derivatives of the 137c strain (CC-124, CC-125), which possess a cell wall that can be transformed by biolistic bombardment (Debuchy *et al.*, 1989; Kindle *et al.*, 1989) or electroporation, but transformation efficiencies are low (Brown *et al.*, 1991).

Alternatively, wild-type strains treated with autolysin, which degrades the cell wall, or cell-wall-deficient strains of *C. reinhardtii* (*cw*) can be easily transformed by glass beads (Kindle and Sodeinde, 1994) or electroporation (Brown *et al.*, 1991; Shimogawara *et al.*, 1998).

B. Improving the genetic tools

Although *C. reinhardtii* possesses many genetic advantages, the lack of effective, reproducible methods for generating targeted mutants via homologous recombination (HR; Zorin *et al.*, 2009) makes the construction of knockout mutants time consuming. Reverse genetic analysis can be performed through isolation of knockout mutants by PCR screening random mutagenesis libraries created by marker gene insertion (Pootakham *et al.*, 2010). However, this requires the labor-intensive screening and maintenance of ~50,000 transformants to provide complete genome coverage. Alternatives include the creation of knockdown mutants by RNA interference (Jans *et al.*, 2008; Mussgnug *et al.*, 2007; Petroutsos *et al.*, 2009; Rohr *et al.*, 2004) or artificial microRNA technology (Molnar *et al.*, 2009; Schmollinger *et al.*, 2010; Zhao *et al.*, 2009), but the stability of the knockdown of expression needs to be carefully evaluated.

Additionally, some approaches to improving H_2 production might require the induction or repression of particular genes at specific stages of growth (see Section IV.E). A number of inducible promoter systems are currently available (Kucho *et al.*, 1999; Ohresser *et al.*, 1997; Quinn *et al.*, 2003; Schroda *et al.*, 2000), but each has drawbacks which could be detrimental for use in H_2 production systems, variously including lack of tight control (Quinn *et al.*, 1998) and use causing physiological stress (Schroda *et al.*, 2000) or repression by ammonium (Ohresser *et al.*, 1997)—which is required for optimum H_2 production. Alternative possibilities include utilizing the potential of other controllable expression systems such as riboswitches (Croft *et al.*, 2007) or promoters that are switched on by anoxia (Chen *et al.*, 2010; Nguyen *et al.*, 2008; Terashima *et al.*, 2010).

C. Engineering an O_2-tolerant hydrogenase

As previously stated, maximum efficiencies are achieved through direct photolysis; therefore, the ultimate goal is to engineer an O_2-tolerant hydrogenase. There are a few known examples found in nature of the [NiFe] type (Burgdorf *et al.*, 2005), but they tend to favor H_2 uptake (Maroti *et al.*, 2009). *C. reinhardtii* contains two highly similar, differentially regulated, oxygen-sensitive [FeFe]-hydrogenases, HYDA1 and HYDA2 (Forestier *et al.*, 2003), of which only HYDA1 is thought to play a role in H_2 production (Godman *et al.*, 2010). The assembly and proper functioning of [FeFe]-hydrogenases is an O_2-sensitive process dependent on accessory factors HydE, HydF, and HydG (Nicolet *et al.*, 2010; Posewitz *et al.*, 2004). The active site H-cluster is a [4Fe–4S] cubane linked to a 2Fe subcluster via cysteine (Adams, 1990; Peters, 1999). The mechanism of assembly is not fully characterized, but HydE and HydG belong

to the radical SAM class of enzymes involved in radical based catalysis (Sofia *et al.*, 2001) and are proposed to play a role in H-cluster synthesis (Rubach *et al.*, 2005) while HydF possess GTPase activity (Brazzolotto *et al.*, 2006) and is thought to act as a scaffold for assembly (McGlynn *et al.*, 2008). Three main approaches can be taken to modify the O_2 tolerance of the enzyme, but any modified hydrogenase would also require a means of assembly in the presence of oxygen:

(1) *Random mutagenesis*. The *C. reinhardtii* [FeFe]-hydrogenase can be expressed heterologously in *E. coli* using the *Clostridium acetobutylicum* hydrogenase assembly factors HydE, HydF, and HydG (King *et al.*, 2006). In principle, such a system is amenable to a high-throughput mutagenesis screen, although ideally the species-specific ferredoxin donor should be used in assays as the ability for ferredoxin–hydrogenase electron transfer can vary widely between different isoforms (Jacobs *et al.*, 2009). Examples of research on the algal hydrogenase include random gene shuffling (Nagy *et al.*, 2007) and directed evolution (Stapleton and Swartz, 2010), the latter of which managed to identify a version of *C. reinhardtii* HydA1 with a fourfold increase in catalytic activity but none yet with improved O_2 tolerance.

(2) *Intelligent design*. In contrast to high-throughput random mutagenesis approaches, it may be possible to use existing structural and mechanistic information to mutate specific amino acid residues within the hydrogenase structure to improve catalytic properties. For example, it has been suggested that narrowing gas channels leading to the active site (Posewitz *et al.*, 2009) or potentially modifying the protein environment around the H-cluster itself (Stripp *et al.*, 2009b) may be means of preventing inhibition by O_2. There are now a number of X-ray crystal structures of [FeFe]-hydrogenases (Cohen *et al.*, 2005; Peters *et al.*, 1998; Shima *et al.*, 2008) including the partially assembled *C. reinhardtii* enzyme (Mulder *et al.*, 2010). A homology model of the *C. reinhardtii* HYDA2 has been constructed (Chang *et al.*, 2007) and HYDA1 has been isolated and characterized by electron paramagnetic resonance (EPR) spectroscopy (Kamp *et al.*, 2008) as well as by X-ray absorption spectroscopy (XAS) which has revealed the structure of the active site H-cluster (Stripp *et al.*, 2009a), and the means by which O_2 inhibition occurs (Stripp *et al.*, 2009b). However, these data have not yet been successfully applied to making beneficial mutations to reduce the O_2 sensitivity of the algal hydrogenase.

(3) *Bioprospecting*. The vast diversity of hydrogenase sequences remains underrepresented in databases (Beer *et al.*, 2009), and there may yet be a suitable sequence present in nature. The establishment of metagenomics has allowed for identification of novel hydrogenases from the

environment including analysis of termite gut microbiota (Warnecke *et al.*, 2007) and through the Craig Venter Institute's Global Ocean Sequencing program (Maroti *et al.*, 2009; Rusch *et al.*, 2007), but as yet the oxygen tolerance of these enzymes is unknown.

D. Hydrogenase–ferredoxin interactions

Ferredoxins play a central role in allocating high energy electrons in the chloroplast and are involved in distributing electrons to CO_2 fixation (Kitayama *et al.*, 1994), nitrite (Fernández *et al.*, 1989; Hirasawa *et al.*, 2009) and sulfite reduction (Gonzalez-Ballester *et al.*, 2009), glutamate synthesis (García-Sánchez *et al.*, 2000), cyclic electron flow (CEF; Finazzi *et al.*, 2002; Rochaix, 2011), and reduction of thioredoxins for regulation of biosynthetic pathways (Lemaire *et al.*, 2003) in addition to the role in hydrogen production.

C. reinhardtii encodes six differentially regulated [Fe_2S_2] ferredoxins: PetF (the dominant Fd reduced in the light reactions) and Fdx2–Fdx5 (Mus *et al.*, 2007; Terauchi *et al.*, 2009; Winkler *et al.*, 2010) of which Fdx5 was found to be induced by anoxia as well as copper stress (Jacobs *et al.*, 2009; Lambertz *et al.*, 2010; Mus *et al.*, 2007; Terauchi *et al.*, 2009) but which is unable to donate electrons to HYDA1 (Jacobs *et al.*, 2009). PetF is thought likely to be the substrate for H_2 production *in vivo*, although the Fd specificity of the hydrogenases, HydA1 and HydA2, has yet to be fully evaluated (Happe and Naber, 1993; Lambertz *et al.*, 2010).

In principle, hydrogen production could be enhanced by increasing the specificity of electron transfer from the ferredoxin to the hydrogenase over other competing pathways. Progress has been made in identifying potentially important residues involved in the interaction between the Fd and the hydrogenase (Chang *et al.*, 2007; Long *et al.*, 2008; Winkler *et al.*, 2009, 2010) which opens up the possibility of manipulating the affinity of binding and kinetics of electron transfer.

E. Dissipating the thylakoid proton gradient

Disrupting the transthylakoid proton gradient (ΔpH) through heterologous expression of a proton channel in the thylakoid membrane is another potential way of increasing supply of reductant to the hydrogenase. ΔpH is thought to limit H_2 production by causing a decrease in proton concentration in the stroma and a reduction in electron transport at the point of the $Cytb_6f$ complex (Antal *et al.*, 2009; Kramer and Crofts, 1993). The proton gradient uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) does indeed stimulate H_2 evolution (Antal *et al.*, 2009; Cournac *et al.*, 2002; Lee and Greenbaum, 2003); however, addition

prior to anaerobic induction abolished H_2ase activity *in vivo* (Chochois *et al.*, 2009), suggesting that the proton gradient is also important for hydrogenase expression. Therefore, although a potential means of increasing yields, any attempts at decoupling electron transport from ΔpH, such as integration of a proton channel in the thylakoid membrane (Lee and Greenbaum, 2003), will likely require an inducible element to allow for hydrogenase expression.

F. Targeting auxiliary electron transport pathways

Switching off auxiliary electron transport pathways offers another way of increasing the supply of electrons to the hydrogenase, as they dissipate excess reducing pressure in competition with H_2 production. Pathways include nonphotochemical reduction of electron carriers in a process known as chlororespiration, the oxidation of PQ or photosystem I (PSI) electron acceptors by O_2 in a process known as the Mehler reaction and CEF around PSI (Fig. 3.2; Peltier *et al.*, 2010; Rochaix, 2011). The precise effect of the Mehler reaction or chlororespiration on H_2 production is

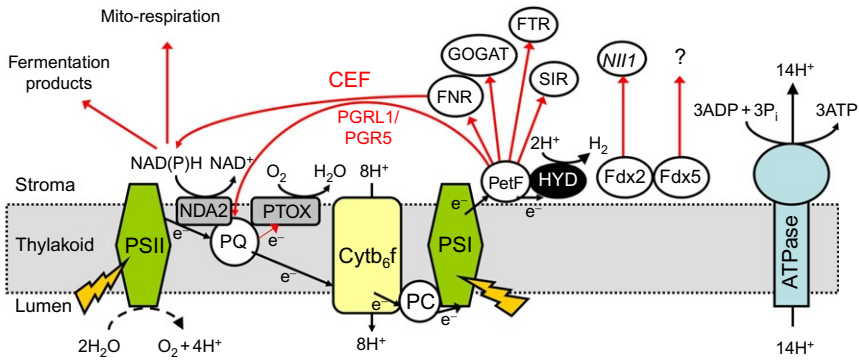


FIGURE 3.2 Schematic representation of processes that potentially compete with the hydrogenase for reductant. Abbreviations: $Cytb_6f$, cytochrome b_6f complex; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ reductase; FTR, ferredoxin–thioredoxin reductase; GOGAT, ferredoxin-dependent glutamate reductase; HYD, [FeFe]-hydrogenase; NDH-2, type II NADH dehydrogenase; NII2, ferredoxin-dependent nitrate reductase; PFOR, pyruvate:ferredoxin oxidoreductase; PGR5, proton gradient regulator protein 5; PGRL1, proton gradient regulator-like protein 1; PSI, photosystem I; PSII, photosystem II; PC, plastocyanin; PQ, plastoquinone; PTOX, plastid or plastoquinol terminal oxidase; SIR, ferredoxin-dependent sulfate reductase. *Note*: during linear electron transport, eight protons are pumped per four electrons coming from the oxidation of one molecule of H_2O. However, the operation of both cyclic and linear electron flow will increase the relative number of protons pumped at $Cytb_6f$ complex (figure based on Winkler *et al.*, 2010).

unclear, but several studies have indicated that competition for reductant occurs between CEF and H_2 production (Antal *et al.*, 2009).

1. Cyclic electron flow

For optimal photosynthetic activity photosynthetic organisms must balance the amount of light energy absorbed by the PSII and PSI reaction centers through transfer of mobile light-harvesting complexes in a process known as state transitions (Lemeille and Rochaix, 2010). In *C. reinhardtii*, this process allows the switch between linear and CEF (Lemeille and Rochaix, 2010). CEF potentially occurs by two pathways in *C. reinhardtii* which can be distinguished by their sensitivity to antimycin A (Ravenel *et al.*, 1994).

a. *Antimycin A-sensitive pathway.* This involves the reduction of PQ using electrons from ferredoxin. The precise mechanism is unclear (Rochaix, 2011) but is mediated by a PSI-LHCI-LHCII-Cytb_6f-FNR-PGRL1 supercomplex which has been implicated in CEF in *C. reinhardtii* (Iwai *et al.*, 2010).
b. *Antimycin A-insensitive pathway*. The second pathway is believed to be controlled by the activity of a type II NAD(P)H dehydrogenase, reducing PQ using NAD(P)H generated via the ferredoxin-dependent $NADP^+$ reductase (Rumeau *et al.*, 2007; Fig. 3.2).

The efficacy of disrupting CEF as a means of increasing H_2 yields was demonstrated in short-term inhibitor studies using antimycin A, which resulted in a doubling of H_2 production as a result of antimycin A's activity in blocking CEF (Antal *et al.*, 2009).

It is possible to screen for mutants affected in CEF through chlorophyll fluorescence video imaging, which has allowed the identification of mutants unable to switch between linear and cyclic electron transport (Depège *et al.*, 2003). This screen also led to the discovery a high hydrogen producing strain deficient in the mitochondrial transcription factor *moc*1 known as stm6 (*s*tate *t*ransition *m*utant 6; Kruse *et al.*, 2005a; Table 3.1). stm6 is locked permanently in ''state 1'' meaning in the process of linear electron transport, thereby inhibiting CEF which was cited as one of the major factors contributing to the high hydrogen phenotype. stm6 is also able to accumulate more starch and has a higher respiratory rate due to an upregulated alternative oxidase (AOX) which is beneficial for H_2 production because of more favorable rates of oxygen consumption (Kruse *et al.*, 2005a).

For targeted gene knockout, potential candidates in the antimycin-sensitive pathway include protein gradient regulator protein 5 (PGR5; Munekage *et al.*, 2002; Nandha *et al.*, 2007) and PGRL1 (DalCorso *et al.*, 2008). Knockout of these genes will also likely have the dual effect of disrupting ΔpH formation to help H_2 evolution. However, identifying the

TABLE 3.1 Summary of published mutations affecting H_2 production in *C. reinhardtii*

Target gene	Function	Type of mutation	Mode of action	Effect on H_2 production relative to parental strain	Reference
psbA	D1 subunit of PSII complex involved in water oxidation	Double point mutation L159I-N230Y	Decrease in chlorophyll content Prolonged H_2 production Higher respiration rate Higher photosynthetic capacity relative to quantum yield of PSII	~15× increase Conditions: light, TAP-S Chl content: 12 μg/ml[a] Total yield: 504 ml/l Maximum rate: 5.77 ml/l/h Duration: 285 h Light intensity: 70 μmol/m^2/s	Torzillo *et al.* (2009)
psbA	D1 subunit of PSII complex involved in water oxidation	Amino acid deletion Residues 239–240	Increase in photoinhibition Prolonged H_2 evolution phase Reduced chlorophyll content Higher respiration-to-photosynthesis ratio Increased carbohydrate accumulation Higher synthesis of xanthophyll-cycle pigments	12–18× increase Conditions: light, TAP-S Chl content: 12 μg/ml[a] Total yield: 475 ml/l Maximum rate: 2.6 ml/l/h Duration: 183 h Light intensity: 70 μmol/m^2/s	Faraloni and Torzillo (2010)
hydEF	[FeFe]-hydrogenase assembly factor	Knockout	Inhibition of hydrogenase synthesis	No detectable H_2 production	Posewitz *et al.* (2004)
LHC	Light-harvesting complex	RNAi	78% reduction in chlorophyll content	Not tested	Mussgnug *et al.* (2007)

moc1	Mitochondrial transcription factor	Knockout	Decrease in CEF, increased starch accumulation, prolonged H_2 evolution phase	~5× increase Conditions: light, TAP-S Chl content: 25–30 μg/Chl/ml Total yield: 540 ml/l Maximum rate: 4 ml/l/h Duration: 336 h Light intensity: 100 μmol/m^2/s	Kruse *et al.* (2005a)
moc1 + *hup1*	Mitochondrial transcription factor with hexose uptake protein	Knockout + transgenic expression	In addition to *moc1* mutant phenotype allows glucose transport for use in H_2 production	~7.5× increase Conditions: TAP-S + 1 mM glucose Data given as % of Kruse *et al.* (2005a)	Doebbe *et al.* (2007)
nab1	LHC RNA-binding protein	RNAi	Decrease in antennae size 10–17%	Not tested	Beckmann *et al.* (2009)
pdc3	Pyruvate decarboxylase	amiRNA	Altered fermentation	Not tested	Burgess and Nixon (unpublished)
pfl1	Pyruvate formate lyase	amiRNA	Decrease in formic acid production	No increase Conditions: TAP-S	Burgess and Nixon (unpublished)
pfl1	Pyruvate formate lyase	Knockout	Decrease in formic acid production	~1.5× increase Conditions: dark N_2 purged ~55% decrease Conditions: light N_2 purged	Philipps *et al.* (2011)
pgrl1	Proton gradient regulator-like protein 1	RNAi	Potential alteration to CEF and ΔpH	Not tested	Petroutsos *et al.* (2009)
pgrl1	Proton gradient regulator-like protein 1	amiRNAi	Potential alteration to CEF and ΔpH	Greater than twofold increase Conditions: TAP-S	Burgess and Nixon (unpublished)

(*continued*)

TABLE 3.1 Summary of published mutations affecting H_2 production in *C. reinhardtii (continued)*

Target gene	Function	Type of mutation	Mode of action	Effect on H_2 production relative to parental strain	Reference
hemH, lba	Ferreochelatase (hemH) Leghemoglobin (lba)	Transgene expression	Lower internal O_2 levels due to expression of soya bean O_2 scavenging proteins	4.5× increase Conditions: light, TAP-S (sparged N_2 to induce anaerobiosis) Chl content: 12.5 μg/ml Total yield: ~55 ml/l Maximum rate: 2.0 μl/mgChl/h Light intensity: 50 μmol/m^2/s	Wu *et al.* (2010, 2011)
sulP	Chloroplast localized sulfate permease	RNAi	Knockdown of chloroplast sulfate transporter. Triggering sulfur starvation response in replete media	H_2 production in sulfur-replete media, overall yields not given	Chen *et al.* (2005)
tla1	Control of light-harvesting antennae size	Knockout	~48% decrease in chlorophyll content	6× increase Conditions: light, immobilized TAP-S Maximum rate: 8.5× increase Duration: 250 h Light intensity: 350 μmol/m^2/s	Berberoglu *et al.* (2008), Kosourov *et al.* (2011), Mitra and Melis (2010), Polle *et al.* (2003)

[a] Similar number of cells was used in H_2 measurements as in Kruse *et al.* (2005a).

precise enzyme(s) responsible for the antimycin-insensitive CEF pathway in *C. reinhardtii* has been more difficult as the genome encodes six type II NADH dehydrogenases, including a plant type NDB (NDA2), a yeast type NDE (NDA3), one that grouped with plant type NDAs (NDA1), and three cyanobacterial type NDCs (NDA5, NDA6, and NDA7; Desplats *et al.*, 2008; Jans *et al.*, 2008). *Arabidopsis* also has multiple type II NADH dehydrogenases with differing physiological roles (Michalecka *et al.*, 2003); six out of seven are targeted exclusively to the mitochondria (Elhafez *et al.*, 2006; Michalecka *et al.*, 2003), highlighting the importance of identifying the correct isoform or isoforms for study. In *C. reinhardtii*, RNA hybridization only detected transcripts for NDA2 and NDA6 under mixotrophic conditions and further analysis revealed NDA2 was located in the chloroplast. An RNAi study knocked down NDA2 showing a reduction in CEF and reduced ability for state II transition when oxidative phosphorylation was inhibited (Jans *et al.*, 2008). Despite this, the NDA2 knockdown resulted in an ~50% decrease in H_2 production under sulfur depletion (Jans *et al.*, 2008) which was proposed to occur as NDA2 may be acting as the point of entry for reductant from the breakdown of starch reserves, and therefore NDA2 overexpression may actually be a means of increasing the supply of reductant for H_2 production (Jans *et al.*, 2008). It is unclear why NDA2 knockdown resulted in such a dramatic decrease in overall H_2 yields when the indirect pathway is proposed only to contribute 10% of reductant to H_2 production, but could perhaps be attributed to the knockdown of additional targets by siRNAs generated from the inverted repeated RNAi construct (Jans *et al.*, 2008).

G. Targeting fermentative pathways

Switching off fermentative pathways is also a potential method of increasing flow of reductant to the hydrogenase (Doebbe *et al.*, 2010; Mus *et al.*, 2007; Timmins *et al.*, 2009b). This is based on the assumption that eliminating sinks for NADH could potentially increase the amount of reductant fed into the PET by a type II NADH dehydrogenase, and redirecting carbon flux to a PFOR may be a means of increasing the amount of reduced ferredoxin available to the hydrogenase (Fig. 3.3).

Key enzymes for targeting are thought to include pyruvate formate lyase (PFL1), bifunctional acetaldehyde/alcohol dehydrogenase (ADH1), and pyruvate decarboxylase (PDC; Mus *et al.*, 2007). This is based on a working model of fermentative metabolism (Fig. 3.3) constructed from genomic (Grossman *et al.*, 2007), biochemical (S.J. Burgess and P.J. Nixon, unpublished data; Kreuzberg *et al.*, 1987), transcriptomic (Mus *et al.*, 2007; Nguyen *et al.*, 2008), metabolomic (Doebbe *et al.*, 2010; Timmins *et al.*, 2009b), and proteomic (Atteia *et al.*, 2009; Chen *et al.*, 2010; Terashima

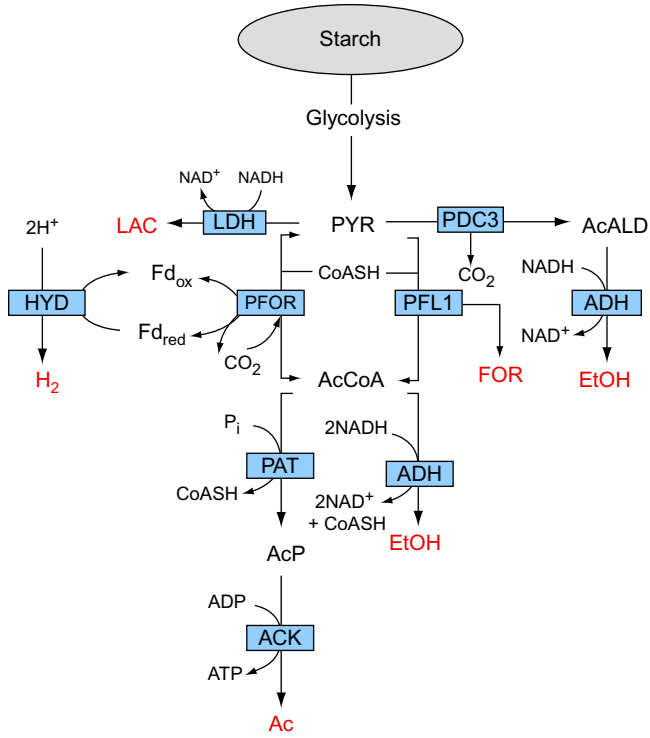


FIGURE 3.3 Proposed pyruvate metabolism in *C. reinhardtii* (adapted from Grossman *et al.*, 2007). Metabolites are labeled in red—AcALD, acetaldehyde; Ac, acetate; AcCoA, acetyl-CoA; EtOH, ethanol; Fd, ferredoxin; LAC, lactate; PYR, pyruvate. Enzymes present in the *C. reinhardtii* genome are in blue boxes—ACK, acetate kinase; ADH, alcohol dehydrogenase; HYD, [FeFe]-hydrogenase; LDH, lactate dehydrogenase; PTA, phosphotransacetylase; PDC, pyruvate decarboxylase; PFL1, pyruvate formate lyase; PFOR, pyruvate ferredoxin oxidoreductase. It must be noted that, in a hydrogenase knockout mutant, additional pathways are activated during anaerobic incubation, as reviewed (Grossman *et al.*, 2010).

et al., 2010) analysis of anoxic pathways in *C. reinhardtii* (reviewed by Grossman *et al.*, 2010).

Pyruvate can be broken down to formate and acetyl-CoA by PFL1 (Atteia *et al.*, 2006; Hemschemeier and Happe, 2005; Hemschemeier *et al.*, 2008b), and the acetyl-CoA converted into acetate by a phosphate acetyltransferase (PAT)–acetate kinase (ACK) catalyzed pathway or potentially into ethanol by a bifunctional aldehyde/ADH consuming two molecules of NADH (Fig. 3.3). Additionally, pyruvate can be converted into lactate by a D-lactate dehydrogenase (LDH; Husic and Tolbert, 1985) with the

cost of one molecule of NADH (Fig. 3.3), or into acetaldehyde by a pyruvate decarboxylase (PDC3) which could then be converted to ethanol by an ADH (Mus *et al.*, 2007) catalyzed pathway, also consuming NADH (Atteia *et al.*, 2003; Fig. 3.3).

The feasibility of knocking down fermentative pathways as a means of increasing hydrogen evolution was recently demonstrated by studies of a PFL1 insertion line, which showed improved H_2 evolution in the dark, suggesting flux was redirected toward PFOR providing reduced ferredoxin for hydrogen production (Philipps *et al.*, 2011; Table 3.1). This additionally caused an increase in the production of lactic acid, meaning some carbon flux was directed to the LDH making it a good target for future efforts at genetic engineering.

However, the link between fermentation and hydrogen production under varying circumstances is still not fully understood. While improving yields in the dark, PFL1 knockout actually decreased H_2 evolution in the light, possibly due to reduced hydrogenase transcription (Philipps *et al.*, 2011). Additionally, analysis of the hydrogen production ability of a PFL1 knockdown line during sulfur depletion revealed little change in overall yields (S.J. Burgess and P.J. Nixon, unpublished data). This suggests a greater understanding is required of the more complex metabolic response to nutrient starvation and hypoxia caused by sulfur starvation, as fermentative pathways interact with respiration, ammonium assimilation, and amino acid synthesis through metabolic intermediates (Doebbe *et al.*, 2010; Timmins *et al.*, 2009b).

H. Modifying the photosynthetic apparatus

1. Decreasing the light-harvesting antennae

When exposed to high irradiances of light, *C. reinhardtii* dissipates up to 80% of absorbed photons as heat or fluorescence to protect against photodamage (Polle *et al.*, 2002), in a process known as feedback de-excitation (Holt *et al.*, 2004). This photoprotective mechanism automatically reduces the energy conversion efficiency of PET. Decreasing the light-harvesting capacity of the photosystems reduces excess light absorption by individual cells, thereby increasing photon and energy conversion efficiencies as well as light penetration in a culture (Melis *et al.*, 1998; Mussgnug *et al.*, 2007; Polle *et al.*, 2002).

Mutants with a truncated light-harvesting antennae (such as the *tla* mutants) have been identified on account of their pale phenotype (Berberoglu *et al.*, 2008; Melis, 2009; Mitra and Melis, 2010; Polle *et al.*, 2002) or through reverse genetic approaches, such as by downregulation of light-harvesting complexes through RNAi (Mussgnug *et al.*, 2007) or overexpression of the RNA-binding protein, NAB1, known to block translation of light-harvesting subunits (Beckmann *et al.*, 2009).

Preliminary results have found that the *tla1* mutant could not establish anaerobiosis after sulfur depletion in a bioreactor (Kosourov *et al.*, 2011). However, recent results indicate that, when immobilized in an alignate film and deprived of both sulfur and phosphorous, H_2 production rates are increased four to six times relative to the parental strain at light intensities ranging from 285 to 350 $\mu E/m^2/s$, demonstrating the feasibility of this strategy for improving yields under the optimized conditions (Kosourov *et al.*, 2011; Table 3.1).

2. Altering photosynthetic rates

Decreasing O_2-evolution rates further may have beneficial impacts in shortening the lag phase between sulfur deprivation and H_2 evolution, as well as increasing hydrogenase expression and activity. This could potentially be achieved by targeting photoprotective mechanisms (Li *et al.*, 2009), components of the PSII repair cycle (Nixon *et al.*, 2010), or altering photosynthetic electron transfer rates.

The efficacy of this approach is largely untested, but mutations in the Q_B-binding site of D1 which is responsible for optimal electron transport from PSII (Johanningmeier *et al.*, 2000; Lardans *et al.*, 1998; Rose *et al.*, 2008) resulted in substantial increases in hydrogen evolution compared to wild-type cultures (Faraloni and Torzillo, 2010; Torzillo *et al.*, 2009; Table 3.1). The precise reason for the increase is unclear but is likely the result of a number of contributing factors (Table 3.1).

However, not all photosynthetic mutations are likely to be beneficial. For example, directly targeting photosynthetic activity appeared to cause a decline in overall hydrogen yields (Makarova *et al.*, 2007), possibly as a reduced water-splitting activity affected starch accumulation in the aerobic phase, and may represent a problem for this approach in certain cases.

I. Creating anoxic conditions

1. Improving upon sulfur starvation

As mentioned in Section III.B, sulfur depletion is the commonly used process for downregulation of photosynthetic activity, but centrifugation, as used in laboratory protocols as a means of cycling cultures from sulfur-replete to sulfur-deplete media, is unfeasible for scale-up. One method to circumvent this problem is to restrict sulfate transport into the chloroplast. This was done by targeting the *C. reinhardtii* chloroplast sulfate transporter s*ulP* using RNAi, resulting in the establishment of anaerobiosis in stationary phase cultures triggering H_2 production (Chen *et al.*, 2005; Table 3.1). Alternatively, optimization of the sulfate content in the growth media would allow cells to reach optimal density for H_2 production at the point when sufficient sulfur is consumed during growth to trigger the starvation response.

2. Optimizing culturing conditions

Under standard conditions, sulfur-deprived cultures of *C. reinhardtii* CC-124 produce H_2 at maximum specific rates of ~4–6 μmol/mgChl/h (Kosourov *et al.*, 2002). Since its establishment in 2000, experimental parameters have been optimized, although mainly in isolation, through synchronization of cultures (Tsygankov *et al.*, 2002), re-addition of low levels of sulfate (Kosourov *et al.*, 2002), optimization of pH (Kosourov *et al.*, 2003), light intensity (Laurinavichene *et al.*, 2004), medium composition (Jo *et al.*, 2006), and growth conditions (Kosourov *et al.*, 2007), with maximum rates of up to 9.4 μmol/mgChl/h reported (Kosourov *et al.*, 2003). The duration of hydrogen evolution has also been extended through cycling between sulfur-replete and -deprived media (Ghirardi *et al.*, 2000; Kosourov and Seibert, 2009; Laurinavichene *et al.*, 2006), the use of a two-staged bioreactor setup for continuous production (Fedorov *et al.*, 2005) and immobilization of cells in an alignate film (Kosourov and Seibert, 2009; Laurinavichene *et al.*, 2006). Immobilizing cells also provided a higher tolerance to O_2, greater achievable cell densities, and better light utilization, leading to maximum recorded rates of H_2 production at around 12.5 μmol/mgChl/h and an increase in light energy conversion efficiency from 0.24% in liquid cultures to ~1% (Kosourov and Seibert, 2009).

3. Regulation of PSII activity through inducible promoters

The disadvantages of sulfur depletion include that it ultimately results in cell death, is only suitable as a batch process, and requires continuous illumination over several days for maximum H_2 production (Oncel and Sukan, 2011). Therefore, controllable expression of PSII could be used to reduce oxygen evolution to a rate below respiration and drive cultures anaerobic. Surzycki and colleagues used the copper-sensitive cytochrome c_6 promoter to repress PSII assembly (Surzycki *et al.*, 2007), thereby inducing anoxia and hydrogen production when cells were transferred from copper-free to copper-replete media. However, it was also found that the promoter used was stimulated by anoxia, even in the presence of copper and resultantly aerobic conditions were reestablished shortly after the initiation of H_2 evolution, bringing the process to a stop (Surzycki *et al.*, 2007). Despite this setback, this approach did demonstrate that controlling photosynthesis through inducible promoters is an effective method of stimulating H_2 evolution.

4. Mutants with altered rates of photosynthesis to respiration

A different approach could be taken to identify mutants with altered rates of photosynthesis to respiration (P/R) which would automatically go anaerobic when placed in sealed containers. A forward genetics screen

was used to identify such strains based on a colorimetric analysis of dissolved oxygen concentrations, finding one which was named *apr*1 for *a*ttenuated *p*hotosynthesis to *r*espiration, showing dramatically reduced photosynthetic rates and a slight increase in respiration (Ruhle *et al.*, 2008). However, despite going anaerobic when placed in sealed containers, H_2 was only produced in the light after the Calvin cycle was inhibited through the addition of glycoaldehyde (Ruhle *et al.*, 2008). These results suggested downregulation of the Calvin cycle is a necessary step for stimulating H_2 production which otherwise acts as a preferential electron sink (Ruhle *et al.*, 2008).

5. Rubisco mutants

Mutant CC-2803, which lacks Rubisco, a key enzyme of the Calvin cycle involved in the fixation of CO_2, has a light-sensitive phenotype and a dramatically reduced rate of photosynthesis. Consequently, cultures go anaerobic and produce H_2 in sealed containers even in the presence of sulfur (Hemschemeier *et al.*, 2008a).

Control of the Calvin cycle or Rubisco activity therefore represents a potentially novel method of inducing H_2 production (Marín-Navarro *et al.*, 2010) by removing the major sink of electrons for reduced ferredoxin generated by the light reactions. This could be achieved through inducible control of Rubisco or Calvin cycle enzyme expression or control of CO_2 supply to carbon-concentrating mutants (Spalding, 2008).

Rubisco may also make an interesting target to decrease the specificity of carboxylation to oxygenation reactions as has previously been demonstrated (Chen *et al.*, 1988; Genkov *et al.*, 2006; Satagopan and Spreitzer, 2004), which would result in a higher respiratory rate as well as reduced flux through the Calvin cycle.

6. Direct reduction in O_2 levels

In addition to altering the specificity of Rubisco to increase the respiratory rate, direct reduction of internal O_2 levels without affecting PSII activity could be achieved by overexpressing O_2 consuming enzymes, such as a plastid/plastoquinol terminal oxidase (PTOX) in the chloroplast or an AOX in the mitochondrion, although it remains to be seen whether the benefits of decreasing O_2 levels would outweigh the loss of electrons which could potentially be fed to the hydrogenase. Another approach for the direct reduction of O_2 levels has been demonstrated to improve yields where O_2 scavenging proteins from soya bean root nodules were expressed in *C. reinhardtii*, causing a 4.5× increase in H_2 production relative to the parental strain during sulfur depletion (Wu *et al.*, 2010, 2011; Table 3.1).

V. ENGINEERING CHALLENGES: PHOTOBIOREACTORS

A. Overview

In addition to improving yields through generation of novel strains, processes for production of hydrogen from algae or cyanobacteria will require scaled-up photobioreactor (PBR) systems (Giannelli *et al.*, 2009; Morweiser *et al.*, 2010; Posten, 2009). Inexpensive open systems such as natural ponds, circular ponds with a rotating arm for stirring, and raceway ponds have already been used for commercial growth of algal biomass, in particular of the green algae *Chlorella* and *Dunaliella*, grown for the pigmenting agent astaxanthin and β-carotene, respectively (Akkerman *et al.*, 2002). The disadvantages of such outdoor systems include a lack of control of temperature and hydrodynamics, poor mass transfer and gas exchange within the algal culture, and a strong possibility of contamination and evaporative losses (Carvalho *et al.*, 2006). Additionally, there is significant public concern about genetically manipulated algal species, particularly if the biomass cultivation is open to the environment. Open systems are particularly unsuitable for H_2 production process due to the need to efficiently harvest a highly mobile and diffusive gaseous molecule. Enclosed PBR systems, however, feature more reproducible cultivation conditions, with better heat and mass transfer control. This increases biomass production rates and H_2 yield, resulting in better product quality as well as providing an opportunity for more flexible technical design (Pulz, 2001). They can enable algal cultivation in arid regions, hence ensuring that algae do not need to compete for land area with food crops, while also opening new economic possibilities in desert countries (Ugwu *et al.*, 2008). The main drawbacks of enclosed PBR systems are their high capital and operating costs (Melis, 2002).

B. Photobioreactors

A PBR is best described as a complex, multiphase system, consisting of the gaseous H_2 product, the liquid growth medium, and the solid algal cells, as well as the superimposed light radiation field (Borowitzka, 1999). Environmental parameters such as the light transfer and fluid dynamics within the PBR have a strong influence on the biohydrogen production reaction. To achieve optimal productivity, the PBR should be operated at appropriate illumination conditions, with an optimal surface-to-volume ratio and light–dark cycle, and with sufficient gaseous mass transfer through the algal culture (Posten, 2009). Both the intensity and wavelength of light incident on the PBR are equally important, along with the parameters that influence the light attenuation, light dilution, and light mixing in the system (Tsygankov, 2001). The types of PBR previously

considered in the literature are the stirred-tank reactor (often with internal illumination), the vertical-column reactor, the horizontal tubular reactor, and the flat-plate reactor (Akkerman *et al.*, 2002; Borowitzka, 1999; Carvalho *et al.*, 2006; Dasgupta *et al.*, 2010; Melis, 2002; Posten, 2009; Pulz, 2001; Tamburic *et al.*, 2011; Ugwu *et al.*, 2008). The different PBR geometries are compared in Table 3.2.

1. Stirred-tank reactors

Stirred-tank fermenter-type reactors have regularly been used for laboratory measurements of biophotolytic H_2 production (Berberoglu *et al.*, 2008); nevertheless, these reactors are characterized by a high degree of back-mixing and poor light penetration through the culture (Pulz, 2001). Agitation is typically provided by means of a mechanical stirrer, which becomes a major source of energy consumption for large volumes. High sheer rates also induce substantial cell death. The possibility of providing internal illumination to stirred-tank systems has been explored as a means of increasing the functionality of these PBRs but becomes a significant engineering challenge for large-scale reactors (Pulz, 2001).

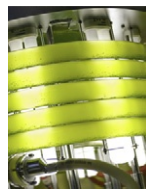
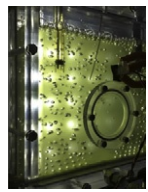
2. Vertical-column reactors

Vertical-column reactors are simple systems consisting of a polyethylene or glass tube, agitated by means of an air-lift loop or a bubble column. They are compact, low cost, easy to operate, and consequently used for domestic (and laboratory) microalgal and plankton growth (Uyar *et al.*, 2007). Due to the reactor orientation, artificial lighting is important; a high degree of turbulence is also required to produce sufficient mixing and light–dark cycling of the culture (Skjånes *et al.*, 2008). The advantages of vertical-column reactors are the high mass transfer rates and good culture mixing with low shear stress on the algae. The main limitation is the relatively small surface-to-volume ratio compared with the flat-plate or stirred-tank reactors. Other drawbacks include the variable light attenuation through the algal culture, the need for sophisticated construction materials to keep the reactor H_2 leak free, and the decrease in illumination surface area upon scale-up (Uyar *et al.*, 2007).

3. Tubular reactors

Tubular photobioreactors consist of straight, coiled, or looped transparent tubing laid out in a specific geometric arrangement designed to maximize light capture (Xu *et al.*, 2002). They come in multiple reactor geometries including horizontal, helical, conical, and α-shape and can be made from a variety of materials ranging from glass capillaries to plastic bags. Tubular reactors are widely available because of the ease of manufacture, process scale-up, and suitability for outdoor use due to their large illumination surface area. Large surface areas are obtained by using thin tubes with a

TABLE 3.2 A comparison of representative photobioreactor geometries used to facilitate green algal H_2 production (adapted from Tamburic *et al.*, 2011)

Reactor geometry		Illumination	Mass transfer	Scale-up	Economics
Stirred-tank[a]		• Poor light penetration and diffusion • Artificial illumination only	• High degree of culture back-mixing • Energy-intensive mechanical agitation	• Impractical geometry • Internal illumination required	• Cheap and effective at laboratory scale • Large applications prohibitively expensive
Vertical-column[b]		• Low surface-to-volume ratio • Artificial illumination for efficient growth	• Air-lift/bubble column provides good mixing • Low shear stress on algal cells	• Illumination area decreases with volume • Multiple reactor units required	• Domestic microalgal and plankton growth • Many large-scale growth applications
Tubular[c]		• Large illumination surface area • Conical/alpha geometries for outdoor operation	• High gas gradients arise along tubes • Algal clustering along tube surfaces	• Additional sections added via manifolds • Difficult to keep reactor H_2 tight	• Commercial *Chlorela* growth • Impractical for H_2 production applications
Flat-plate[d]		• Large surface-to-volume ratio • Inclined design for outdoor operation	• Difficult to control light dilution gradients • Algal fouling possible	• Two dimensions available for scale-up • Multiple units required on industrial scale	• Operational flexibility • Some large-scale growth applications

Photographs are of
[a] SolarBiofuels consortium stirred-tank reactor (http://www.solarbiofuels.org/consortium.php),
[b] Aqua Medic Plankton Light Reactor,
[c] Sartorius Biostat PBR 2s, and
[d] Imperial College flat-plate reactor (Tamburic *et al.*, 2011).

diameter of no more than 10 cm and illuminating the reactor from multiple directions. Horizontal tubular plastic bag reactors have achieved commercial success for the cultivation of *Chlorella* (Ugwu *et al.*, 2008). Helical

designs enclosing a central light source usually aim to attain high surface-to-volume ratios, while conical and α-shape geometries create a better angle relative to the direction of sunlight but have so far proven difficult to scale up (Akkerman *et al.*, 2002). The main disadvantage of tubular reactors is their poor axial mass transfer—long reactors are characterized by significant gas gradients along the tubes (Xu *et al.*, 2002). Tubular reactors are scaled up by the incorporation of additional sections via manifolds (Borowitzka, 1999). However, each additional link increases the possibility of H_2 diffusive losses and the requirement for a H_2-tight reactor also reduces the number of potential tubular reactor materials.

4. Flat-plate reactors

A flat-plate reactor features a rectangular compartment with a depth of 1–5 cm, depending on the quality of agitation in the system (Skjånes *et al.* 2008). The height and width may be scaled up to a practical limit of 2–3 m (Borowitzka, 1999). Flat-plate reactors may be run in both batch and continuous modes and therefore provide operational flexibility. Artificially illuminated flat-plate reactors are typically vertical and the irradiation is incident on one of the large reactor surfaces, while outdoor flat-plate reactors tend to be tilted at an angle corresponding to the mean solar irradiation angle (Akkerman *et al.*, 2002). The reactor region adjacent to the illuminated surface is known as the photic zone. Within the photic zone, light saturation of the algal culture may result in photoinhibition of the cells (Skjånes *et al.*, 2008). Light intensity decreases exponentially away from the photic zone, with a limiting light diffusion length of 0.8 mm for a fully grown culture of *C. reinhardtii* (Janssen *et al.*, 2003). These light gradients may be minimized, and the light–dark cycles experienced by algal cells may be controlled, by introducing effective agitation into the system. Since the space between flat-plate reactor panels is restricted, the gaseous mass transfer rates tend to be low, which reduces the clearance efficiency of the dissolved oxygen produced by photosynthesis (Molina *et al.*, 2000). Gas-lift agitation is therefore required to achieve significant algal biomass production rates. Other limitations for flat-plate reactors include the difficulty in controlling culture temperature and the requirement for multiple compartments and support materials when scaling up the reactors (Molina *et al.*, 2000).

5. Artificial systems

Alternatively, to avoid some of the problems outlined with biological systems, it may be possible to use bio-inspired processes as a solution to the energy problem. Various artificial approaches to harness solar energy using components of biological systems have been envisioned. These include the immobilization of hydrogenase and isolated PSII complexes onto electrodes, spatially separating oxygen evolution and hydrogen

production (Esper *et al.*, 2006) and the construction of hybrid systems ranging from coupling of photosystem complexes to Pt nanoparticles for H_2 production (Grimme *et al.*, 2008; Iwuchukwu *et al.*, 2010; Lubner *et al.*, 2010), attachment of bacterial reaction center complexes to carbon nanotubes for electricity generation (Ham *et al.*, 2010), the coupling of hydrogenases to TiO_2 nanoparticles (Reisner *et al.*, 2009), or the use of viral particles as scaffolds for the assembly and repair of photocatalytic nanostructures (Nam *et al.*, 2010). Efforts have also been directed at biomimetic design (Lewis and Nocera, 2006), creating novel water-splitting catalysts based on the [FeFe]-hydrogenase active site (Gloaguen *et al.*, 2001; Kluwer *et al.*, 2009; Ott *et al.*, 2004). However, there are a number of disadvantages inherent in many of these processes including the short life span of many hybrid systems containing biological components that are rapidly damaged by light and during water oxidation, the requirement for expensive rare metals in the synthesis of many catalysts, and the requirement for energy input in some catalytic systems.

Despite these problems, there have been significant advances in these fields over the past few years (Kanan and Nocera, 2008) and biologically inspired artificial systems represent an exciting prospect for future energy production

VI. CONCLUSIONS AND FUTURE PROSPECTS

Current ideas suggest that the feasibility of algal H_2 production should be considered in terms of the biorefinery concept (Kruse and Hankamer, 2010; Mussgnug *et al.*, 2010) which aims to maximize the biotechnological potential of every fraction of biomass. Synergies exist between the production of a H_2 and variety of algal fuels as the process of sulfur deprivation leads to increased lipid accumulation (Timmins *et al.*, 2009b) and a lower sulfur content aiding biodiesel or biogas production (Mussgnug *et al.*, 2010). Additionally, the economics of the process could be further improved by coupling to a range of biotechnological processes as described in Section II.

The long-term future of society requires drastic changes in how we produce and consume energy, and biological hydrogen production offers one possible alternative to traditional fossil fuels. Knockout of competing processes, integration of dark fermentative, photofermentative, and biophotolytic modes of hydrogen production into a three-step process, and coupling to a range of biotechnological processes within a biorefinery offers promise for increasing efficiencies toward the levels required for economic viability. But much research is still required to improve yields and drive down the costs of culturing and bioreactor construction, in concert with economic and political drive toward a low carbon H_2

economy if the goal of generating clean energy through renewable means is to be achieved.

It is possible, however, given the recent discovery that the marine cyanobacterium *Cyanothecae* can produce significant amounts of H_2 under aerobic conditions via a nitrogenase (Bandyopadhyay *et al.*, 2010), that the solution may lie beyond the limits of established research. This finding illustrates the limitation of using model organisms for biotechnological purposes and suggests the key to photolytic H_2 production could yet lie in the largely untapped biodiversity of cyanobacteria and microalgae in the environment.

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