Title
Photobiological hydrogen production: Recent advances and state of the art

Journal
Bioresource Technology, 102(18)

ISSN
0960-8524

Authors
Eroglu, Ela
Melis, Anastasios

Publication Date
2011-09-01

DOI
10.1016/j.biortech.2011.03.026

Peer reviewed

Bioresource Technology 102 (2011) 8403–8413

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Photobiological hydrogen production: Recent advances and state of the art

Ela Eroglu [a], Anastasios Melis [b,*]

[a] Centre for Energy, School of Mechanical and Chemical Engineering, The University of Western Australia, Crawley, WA 6009, Australia
[b] University of California, Dept. of Plant and Microbial Biology, Berkeley, CA 94720-3102, USA

ARTICLE INFO

Article history:
Received 28 November 2010
Received in revised form 4 March 2011
Accepted 8 March 2011
Available online 14 March 2011

Keywords:
Biohydrogen
Biophotolysis
Bioreactor
Photosynthesis
Photo-fermentation

ABSTRACT

Photobiological hydrogen production has advanced significantly in recent years, and on the way to becoming a mature technology. A variety of photosynthetic and non-photosynthetic microorganisms, including unicellular green algae, cyanobacteria, anoxygenic photosynthetic bacteria, obligate anaerobic, and nitrogen-fixing bacteria are endowed with genes and proteins for H_2-production. Enzymes, mechanisms, and the underlying biochemistry may vary among these systems; however, they are all promising catalysts in hydrogen production. Integration of hydrogen production among these organisms and enzymatic systems is a recent concept and a rather interesting development in the field, as it may minimize feedstock utilization and lower the associated costs, while improving yields of hydrogen production. Photobioreactor development and genetic manipulation of the hydrogen-producing microorganisms is also outlined in this review, as these contribute to improvement in the yield of the respective processes.

1. Introduction

Photosynthesis is critical for life on earth, as it converts solar energy and inorganic nutrients into organic biomass, thus providing food and fiber. Photosynthesis can also be directed toward the generation of industrially useful bio-products, such as hydrogen, hydrocarbons, lipids, and polymers (starch, polyhydroxyalkanoates, rubber, among others). Hydrogen metabolism by photosynthetic organisms was originally reported for green microalgae by Gaffron (1939) and Gaffron and Rubin (1942), and shortly thereafter for photosynthetic bacteria by Gest and Kamen (1949). It is now well established that hydrogen metabolism is encountered in multiple microorganisms, with molecular hydrogen (H_2) being either the reactant or the end product of diverse independent processes. More specifically, H_2-production includes processes such as (i) direct biophotolysis of water by microalgae and cyanobacteria, (ii) photo-fermentative nitrogen fixation and the attendant hydrogen production by photosynthetic bacteria, (iii) non-photosynthetic hydrogen production from organic compounds by obligate anaerobic bacteria, and (iv) nitrogen fixing fermentative bacteria (Benemann, 1996; Melis and Melnicki, 2006). The scope of the present review is to emphasize photobiological approaches to hydrogen production. Hence only the first two mechanisms (biophotolysis and photo-fermentation) will be expanded upon in the following sections, with cursive connections to the other processes, where integration of H_2-production is concerned.

Photobiological production of hydrogen by photosynthetic microorganisms is of interest due to the promise of generating clean carbon-free renewable energy from abundant natural resources, such as sunlight and water. However, although qualitatively feasible, commercial exploitation of H_2 requires quantitatively better yields. Recent advances and the state of art in photobiological hydrogen production research are reviewed in this article. The discussion includes extensions in hydrogen-related areas such as bioreactor design, hybrid, and integrated systems, metabolic engineering, and associated genetic manipulations that would be needed to make hydrogen a commercially viable fuel for the global economy.

2. Photosynthetic hydrogen production processes

Photosynthesis in green microalgae and cyanobacteria can operate either under oxygenic or hypoxic and anoxic conditions. The evolution of oxygenic photosynthetic organisms, which are proficient in water oxidation, is one of the most important milestones in the Biological History of the Earth. Oxygenic photosynthesis occurs in cyanobacteria, algae, and vascular plants. As

Abbreviations: ATP, adenosine triphosphate; ETS, electron transport system; Fd, ferredoxin; hup$^-$, uptake hydrogenase deficient; LHC, light harvesting complex; PAR, photosynthetically active radiation; PHB, polyhydroxybutyrate; PHB$^-$, PHB synthase deficient; PNS, purple non-sulphur; PS1, photosystem 1; PS2, photosystem 2; TAP, Tris–acetate–phosphate medium; TAP-S, (sulphur deprived) Tris–acetate–phosphate medium; η, light conversion efficiency.

* Corresponding author. Address: University of California, Dept. of Plant and Microbial Biology, 111 Koshland Hall, Berkeley, CA 94720-3102, USA. Tel.: +1 510 642 8166; fax: +1 510 642 4995.
E-mail address: melis@berkeley.edu (A. Melis).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.03.026

oxidation of water is energetically unfavorable, energy input is needed to move the overall process forward. In this case, oxidizing equivalents stored by the Mn_4–Ca cluster, resulting from photosystem-2 photochemistry, are used for the oxidation of water into electrons, protons, and O_2 (Kok et al., 1970; Allakhverdiev et al., 2010). Anoxygenic photosynthetic organisms, given that their single photosystem does not generate a sufficiently oxidizing potential to oxidize water, require a source of electrons from donor molecules such as organic acids. Anoxygenic photosynthesis occurs in microorganisms such as purple non-sulphur bacteria, and green sulphur bacteria (Madigan et al., 2000).

2.1. Biophotolysis

2.1.1. Direct biophotolysis

Organisms of oxygenic photosynthesis oxidize energy-poor but abundant water molecules to extract electrons and protons, and subsequently use them for the reduction of ferredoxin and $NADP^+$. The latter, along with ATP energy, are essential for the metabolic reactions of the chloroplast and the cell. Under anaerobic conditions, the primary products of photosynthesis (reduced ferredoxin, NADPH, and ATP) could also be utilized by green microalgae and cyanobacteria for the production of H_2. In the course of direct biophotolysis, light energy absorbed by PS2 and PS1 helps to transport electrons linearly from water to ferredoxin. Reduced ferredoxin acts as an electron donor to a hydrogenase enzyme, which reversibly catalyzes the reduction of protons (H^+) to molecular hydrogen (H_2), according to the following reaction (Melis et al., 2000)

$$2H^+ + 2FD^- \leftrightarrow H_2 + 2FD \quad (1)$$

Green microalgae are noted for their highly active [FeFe]-hydrogenase enzyme, the relatively high 12–14% solar-to-H_2 energy conversion efficiency (Melis, 2009) and the ability to both oxidize water and to generate H_2 in their chloroplast (Melis and Happe, 2001; Melis, 2002). A problem of such direct biophotolysis, however, is that O_2, generated as a byproduct of the function of PS2, is a powerful suppressor of all H_2-related reactions, including gene expression, mRNA stability and enzymatic catalysis. Thus, direct biophotolysis can operate for short periods of time upon the onset of illumination (a few min), before the accumulating O_2 inactivates the H_2-production process. When photosynthetically-generated oxygen is removed, either upon purging the reaction mixture was inert gases (Greenbaum, 1982, 1988), or directing the cell's own mitochondrial respiration to consume photosynthetic oxygen (Melis et al., 2000; Ghirardi et al., 2000), then sustained H_2-production can be attained for days in green microalgae.

Several green microalgae are genetically endowed with the [FeFe] hydrogenase and associated hydrogenase assembly and hydrogen metabolism genes (Posewitz et al., 2004; Lambertz et al., 2010). Positively identified in this respect are strains such as *Chlamydomonas reinhardtii*, *Chlorella fusca*, *Scenedesmus obliquus*, *Chlorococcum littorale*, *Platymonas subcordiformis*, among others (Melis, 2007; Das and Veziroglu, 2008). The evolutionary origin of the hydrogen metabolism genes in green microalgae is the subject of interesting and intense speculation. The chloroplast of microalgae is assumed to have originated from the endosymbiosis of a cyanobacterial progenitor with a primitive unicellular eukaryote (Tomitani et al., 1999). However, no present-day cyanobacteria (unicellular or filamentous) have been found to contain genes encoding for the green microalgal hydrogen metabolism and its associated enzymes. On the other hand, genes encoding for proteins similar to those in the green microalgal hydrogen metabolism are encountered in obligate anaerobic bacteria, such as *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* (Peters et al., 1998; Melis and Happe, 2001), raising the prospect of a lateral gene transfer from obligate anaerobic bacteria to green microalgae.

2.1.2. Indirect biophotolysis

Cyanobacteria are a diverse group of photoautotrophic microorganisms, and they usually exist in the aquatic (marine or fresh water) environment in a variety of ecotypes. Cyanobacteria that produce hydrogen are mostly filamentous and nitrogen fixing in specialized cells known as heterocysts, i.e., the genus *Nostoc*, *Anabaena*, *Calothrix*, *Oscillatoria*, among others, or non-nitrogen fixing, i.e., the genus *Synechocystis*, *Synechococcus*, *Gloebacter*, among others (Das and Veziroglu, 2008). Indirect biophotolysis and H_2-production is carried out by filamentous cyanobacteria, in which oxygenic photosynthesis and hypoxic nitrogen fixation reactions are spatially separated from each other. The heterocysts of filamentous cyanobacteria are suitably differentiated to promote an anaerobic environment for nitrogen fixation. During the N_2 fixation process, catalyzed by the nitrogenase enzyme, H_2 is also generated, according to the following overall biochemical reaction:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (2)$$

Notable in reaction Eq. (2) is the high energetic requirement of the overall nitrogenase/hydrogenase catalysis, entailing not only the reducing equivalent of eight high potential-energy electrons but, importantly, the consumption of 16 mol ATP per mol N_2 fixed and H_2 produced. This substantial ATP requirement is met in the heterocysts of cyanobacteria via cyclic photophosphorylation, driven by light in the modified thylakoid membranes of these specialized cells.

Cyanobacteria that produce hydrogen can also be non-nitrogen fixing (i.e., the genuses *Synechocystis*, *Synechococcus*, *Gloebacter*) (Das and Veziroglu, 2008). This type of cyanobacteria may possess two different kinds of [NiFe] hydrogenases with different properties and function. The first group is the so-called uptake [NiFe] hydrogenases, encoded by *hup* genes. These enzymes have primarily evolved to capture and recycle hydrogen produced by the nitrogenase. They are co-expressed and probably co-regulated in tandem with the nitrogenase. A multi-subunits bidirectional [NiFe] hydrogenase, encoded by *hoxFUYH* genes, has the capacity both to uptake or generate hydrogen (Tamagnini et al., 2002). Non-nitrogen fixing cyanobacteria can produce hydrogen via the [NiFe] bidirectional (*hoxEFUYH*) hydrogenase (Tamagnini et al., 2002; Baebprasert et al., 2010). The bidirectional enzyme consists of a hydrogenase and a diaphorase subcomplex, and the corresponding 5-hox genes are not always clustered or co-transcribed (Tamagnini et al., 2007). The biosynthesis/maturation of [NiFe]-hydrogenases as well as their phylogenetic origin and evolutionary history were recently discussed (Tamagnini et al., 2007).

2.2. Photo-fermentation

Under anaerobic conditions, photosynthetic bacteria use sunlight as a source of energy and assimilate small organic molecules (i.e., succinate, malate) into biomass with H_2 and CO_2 as the byproducts. Purple non-sulphur bacteria (PNS) are promising photosynthetic microorganisms for H_2-production due to their ability to (i) perform high substrate conversion efficiencies, (ii) operate anaerobically, bypassing the oxygen sensitivity issue that adversely affects the [FeFe] hydrogenase, the *hoxEFUYH* [NiFe]-hydrogenase, and nitrogenase enzymes (iii) utilize sunlight proficiently, i.e., being able to absorb and utilize both the visible (400–700 nm) and near infrared (700–950 nm) regions of the solar spectrum, (iv) show flexibility in organic substrate utilization, including small organic acids from a wide variety of waste matter (Das and Veziroglu, 2001). Purple non-sulfur phototrophic bacteria

are Gram negative prokaryotes that are highly capable of photofermentation (Madigan et al., 2000), and species like *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* have been widely used for genetic and physiological studies in bacterial photosynthesis and H_2-production. Compared to algal hydrogen production emanating upon the oxidation of water, purple non-sulphur bacteria are known to require less free energy to decompose organic substrates, i.e., +8.5 kJ mol^{-1} H_2 from lactate decomposition (Basak and Das, 2007).

Hydrogen production by PNS bacteria is the byproduct of nitrogenase activity in these organisms, induced under nitrogen-deficiency conditions, and facilitated by sunlight as the energy source and small organic molecules as the carbon substrate. The photosynthetic apparatus of anoxygenic phototrophs absorbs sunlight and performs electron transport, which generates the proton motive force required for ATP synthesis (Madigan et al., 2000). High relative amounts of ATP are needed to drive the N_2-fixation and H_2-production reaction (Eq. (2)) in these organisms.

3. Enzymes for hydrogen production

3.1. Hydrogenases

Hydrogenase enzymes are encountered in microalgae, cyanobacteria, and anoxygenic photosynthesis and fermentative bacteria. They are responsible for carrying out either the initial (uptake) or terminal (evolution) act of the respective H_2 metabolism. As already alluded to earlier, there are several types of hydrogenases: (i) *hup*-encoded [NiFe]-uptake hydrogenases, (ii) *hox*-encoded [NiFe]-bidirectional hydrogenases (iii) [FeFe]-hydrogenases, (iv) [NiFeSe]-hydrogenases (as one of the Ni-bound cysteine residues of [NiFe]-hydrogenases is replaced by selenocysteine), and (iv) [Fe]-only hydrogenases. Out of these types, [NiFe]-hydrogenases (in bacteria and cyanobacteria), and [FeFe]-hydrogenases (in obligate anaerobic fermentative bacteria and green microalgae) are most well known (Hatchikian et al., 1992; Shima et al., 2008). [Fe]-only hydrogenases, formerly named as iron sulfur cluster-free hydrogenase, are different from [FeFe]-hydrogenases, as they do not contain Fe–S clusters, but only have a mononuclear Fe active site. They are encountered in a number of hydrogenotrophic methanogenic archaea only (Shima et al., 2008).

In green microalgae, [FeFe]-hydrogenases are enzymes localized in the chloroplast and serve to release electron (in the form of H_2) under hypoxic photosynthesis conditions (Winkler et al., 2002). *C. reinhardtii* encodes two [FeFe]-hydrogenases (Forestier et al., 2003), which exhibit a high enzymatic turnover rate (Happe et al., 2002). [FeFe]-hydrogenases are monomeric or dimeric enzymes with an average molecular weight of 50 kDa. The active site of the enzyme contains a 6Fe–6S cluster, the so-called HC cluster (Peters et al., 1998), arranged in a 4Fe–4S cubane iron-sulfur subcluster to which a 2Fe–2S extension is covalently attached. The iron atoms of the 2Fe–2S subcluster are bridged to each other by three non-protein atoms, probably two sulfide atoms and one carbonyl or cyanide molecule (Peters et al., 1998; Shima et al., 2008; Allakhverdiev et al., 2010). [FeFe] hydrogenases are oxygen sensitive enzymes; their catalytic activity is irreversibly inhibited in the presence of even small amounts of oxygen, a property that hinders H_2-production under oxygenic photosynthesis conditions in green microalgae. Under anoxic or hypoxic conditions, [FeFe]-hydrogenases catalyze the reversible reduction of protons to H_2, according to Eq. (1), in which ferredoxin plays a role as electron carrier (Melis et al., 2000).

Many nitrogen-fixing bacteria and cyanobacteria have an uptake [NiFe]-hydrogenase, by which they efficiently recover energy-rich electrons from the hydrogen released as a byproduct of the nitrogenase. The active site of the [NiFe]-hydrogenases contains a nickel atom, which is linked to a $Fe(CN)_2CO$ molecule (Shima et al., 2008; Allakhverdiev et al., 2010). The uptake hydrogenases are composed of two subunits encoded by the *hupS* and *hupL* genes. The larger hupL subunit has four conserved cysteines in its active site, and the small hupS subunit contains three 4Fe–4S clusters that transfer electrons from the hupL active site, through the hupS 4Fe–4S centers to the electron acceptor molecule. The latter shuttles electrons from the [NiFe]-hydrogenase to components of the cell's electron-transport chain (Allakhverdiev et al., 2010). Cyanobacteria contain, in addition to the hupS/L uptake hydrogenase, a bidirectional enzyme (Tamagnini et al., 2002). The bidirectional hydrogenase can either produce or consume hydrogen according to the prevailing redox conditions. Paradoxically, H_2-production by this hydrogenase appears to be sustained in the dark, whereas a subsequent illumination causes a short burst of H_2 production, followed by H_2 uptake.

The [NiFe]- and [FeFe]-hydrogenases are sensitive to chemical agents like CO and O_2 and, in the case of O_2, inactivation of the [FeFe]-hydrogenase is irreversible. Green algal [FeFe]-hydrogenases have a much higher specific activity than cyanobacterial [NiFe]-hydrogenases (Florin et al., 2001). However, rates and yields of H_2-production by either enzymatic system are often limited by cellular metabolism or biochemical considerations, as defined by substrate availability, the bioenergetic status of the cell, and/or the prevailing metabolic flux considerations, rather than by the K_M and V_{max} properties of the specific hydrogenase.

3.2. Nitrogenases

Photobiological production of hydrogen in cyanobacterial heterocysts and purple non-sulphur bacteria is catalyzed by the enzyme nitrogenase (Meyer et al., 1978). Nitrogenases consist of two-parts: (i) the reductase subunit and (ii) the dinitrogenase complex. The reductase subunit is a Fe–S-protein encoded by the *nifH* gene. It is a homodimer with a molecular weight of around 65 kDa, and is responsible for the transfer of electrons from the external electron donor to the dinitrogenase complex of the enzyme. The dinitrogenase complex is a Mo–Fe–S protein encoded by the *nifD* and *nifK* genes. This protein is a $\alpha_2\beta_2$ heterotetramer, with a molecular weight of around 230 kDa. It catalyzes the step-wise reduction of the dinitrogen (N_2) bonds leading to the formation of two molecules of ammonia (NH_3) (Meyer et al., 1978; Allakhverdiev et al., 2010).

The nitrogenase enzyme is also responsible for the reduction of protons into molecular hydrogen, in a reaction that is catalyzed concomitant with the reduction of dinitrogen to ammonia. Depending on the metal cofactor in the catalytic site of the enzyme, nitrogenases are classified as (i) molybdenum, (ii) iron, and (iii) vanadium-types. Wall (2004) reported variable stoichiometries of ammonia and hydrogen generation by the different types of nitrogenases, depending on the metal cofactor in the catalytic site of the nitrogenase complex, as follows:

$$\text{Mo-nitrogenase}: N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 \quad (3)$$

$$\text{Fe-nitrogenase}: N_2 + 21H^+ + 21e^- \rightarrow 2NH_3 + 7.5H_2 \quad (4)$$

$$\text{V-nitrogenase}: N_2 + 12H^+ + 12e^- \rightarrow 2NH_3 + 3H_2 \quad (5)$$

Nitrogenases function to convert N_2 into NH_3 in a process that naturally includes the production of H_2 (Eqs. (2)–(5)). However, in the absence of dinitrogen substrate, they may exclusively catalyze hydrogen production from protons and high potential-energy electrons. The nitrogenase pathway of NH_3 and H_2-production requires expenditure of ATP energy, as some of the electron transfer steps within the enzyme are endergonic. However, the enzymatic

catalysis is unidirectional and irreversible, resulting in the generation of "pressurized" hydrogen, a feature desirable in large-scale biological H_2-production for commercial purposes. On the other hand, the large amounts of ATP required by nitrogenases lower the energetic efficiency of the nitrogenase process, compared to that of the *hoxEFUYH*-encoded [NiFe]-hydrogenases or [FeFe]-hydrogenases. Also, presence of dinitrogen in the medium naturally tends to lower the yield of hydrogen production as, under these conditions, the nitrogenase enzyme generates two products ($2NH_3$ and $1H_2$) from the same pool of high potential energy electrons (Sasikala et al., 1990; Koku et al., 2002).

4. Challenges for improved hydrogen production

Systematic efforts have been undertaken to improve the efficiency and yield of production. Some of these advances are briefly outlined in the sections below. Table 1 provides a comparison of yields and productivities from a number of studies, where rate of H_2-production is given as "units of product volume per culture volume over time" (ml H_2 L^{-1} h^{-1}). It should be mentioned that the multiple variables such as reactor geometry, substrate source, and illumination conditions complicate the comparison process. Accordingly, comparatively higher hydrogen production rates were obtained by photo-fermentative processes that employed (i) continuous culturing of *R. rubrum* (up to 180 ml H_2 L^{-1} h^{-1}) (Zurrer and Bachofen, 1982), (ii) photo-fermentation with an uptake hydrogenase deficient mutant of *R. capsulatus* under enhanced illumination conditions (100 ml H_2 L^{-1} h^{-1}) (Ooshima et al., 1998), and (iii) Δ*hup* and ΔPHB mutants of *R. sphaeroides* (82.6 ml H_2 L^{-1} h^{-1}) (Kim et al., 2006) (Table 1). These rates compare favorably with the approximately 15–25 ml H_2 L^{-1} h^{-1} reported with wild type purple photosynthetic bacteria (Zhu et al., 1999; Barbosa et al., 2001; Melnicki et al., 2008, 2009), obviously because of the physiological and genetic optimization of the systems, enabling (i) continuity of production over a long period of time, (ii) prevention of recycling and loss of H_2 via the naturally-occurring uptake hydrogenase enzymes, and (iii) enhancement of organic substrate availability for H_2-production upon elimination of carbon sinks (ΔPHB mutants) in the cell.

4.1. Immobilization approaches

Immobilization of cells on solid substrate was reported to confer advantages over free cells in suspension, since the immobilized cellular matter occupies less space, requires a smaller volume of growth medium, is easier to handle, and can be used repeatedly for product generation. Several solid matrices have been successfully utilized for immobilization of photoheterotrophic bacteria, such as porous glass (Tsygankov et al., 1994), carrageenan (Francou and Vignais, 1984), agar gel (Zhu et al., 1999), and even clay surfaces (Chen and Chang, 2006).

In addition to photosynthetic bacteria, immobilized green algal cultures were also employed in efforts to increase the yield and efficiency of H_2 production in these eukaryotic oxygenic photosynthesis systems. Improvement of hydrogen production by immobilized *C. reinhardtii* upon sulfur-deprivation was reported (Laurinavichene et al., 2006). Sulphur deprived *C. reinhardtii* cultures showed enhancement of their hydrogen production rates from 2.5 up to 4.3 ml L^{-1} h^{-1} after being immobilized in Al-borosilicate porous glass sheets (Laurinavichene et al., 2006) (see Table 1). Hahn et al. (2007) investigated a two-step hydrogen production process by the immobilized cells of *C. reinhardtii*, attached to silica particles. Two-step process included growth and hydrogen production modes, which are controlled by shifting the cells between sulfur-containing and sulfur-free culture media. This analysis suggested that immobilized cultures more readily shift between the oxygenic photosynthesis (growth) and the hydrogen production modes. Further, Kosourov and Seibert (2009) reported the immobilization of *C. reinhardtii* within alginate films. Immobilized and sulfur/phosphorus-deprived cultures revealed higher cell densities (2000 μg Chl ml^{-1} of matrix) and hydrogen production rates (12.5 μmol mg^{-1} Chl h^{-1}). They also reported that the alginate polymer helped to enhance the hypoxic environment in the vicinity of the cells, thus promoting conditions for H_2-production.

Immobilized *R. sphaeroides* cells in calcium alginate solid substrate were reported to produce 2.1 L H_2 h^{-1} m^{-2} of gels, which yields about 2- to 3-fold higher hydrogen production compare to free cells in liquid suspension, with tofu wastewater used as the growth medium and organic carbon source (Zhu et al., 1999). When *R. palustris* DSM 131 cells were immobilized on agarose and sodium alginate gel, hydrogen production was also enhanced in comparison to non-immobilized cultures (Fiβler et al., 1995). Chen and Chang (2006) reported that photo-fermentative hydrogen production by *R. palustris* WP3-5 improved upon immobilizing cells on solid substrates such as activated carbon, silica gel, and clay. They observed greater efficiency and higher yields of H_2 production, as a result of clay and silica gel addition. For instance, the hydrogen production rate for the clay-supplemented batch cultures were 28.5 ml L^{-1} h^{-1}, while it was 20.9 ml L^{-1} h^{-1} for the carrier-free cultures. When the reactor was additionally illuminated by internal optical fibers, hydrogen rate for the clay-supplemented cultures reached 43.8 ml L^{-1} h^{-1}. In all cases, acetate (1000 mg COD/l) was used as the organic carbon source (Table 1). On the other hand, Tsygankov (2001) noted disadvantages of some purple bacterial immobilized cultures in comparison to their freely suspended counterparts. Immobilized cultures encountered problems due to the inhomogeneous environment and lower mass diffusions (Tsygankov, 2001; Koku et al., 2003).

4.2. Increasing the culture resistance to stress conditions

Since [FeFe]-hydrogenase enzymes are highly sensitive to oxygen, several investigators have worked on enhancing photosynthetic H_2-production upon lowering the concentration of O_2 in the cellular environment. Continuous sparging of cultures with inert gases removes O_2 from the culture medium and thus enables expression of the hydrogenase and of hydrogen metabolism, resulting in simultaneous H_2 and O_2 production by the photosynthetic apparatus (Greenbaum, 1982, 1988). This approach has laboratory value, but cannot be used in scale-up and commercial H_2-production. Sulphur deprivation was the first physiological tool successfully used in cultures of unicellular green algae, causing partial inactivation of PS2 and lowering the capacity of photosynthetic oxygen evolution, so that cellular respiration itself could effectively consume all photosynthetically generated oxygen, shifting the culture medium to anaerobiosis (Melis et al., 2000). This simple physiological treatment was necessary and sufficient to induce the cell's hydrogen metabolism and to enable sustained photosynthetic electron transport toward the [FeFe]-hydrogenase, yielding continuous hydrogen production by photosynthesis for up to 4 days (Melis et al., 2000; Ghirardi et al., 2000; Zhang et al., 2002). In terms of the temporal separation of H_2 and O_2 production by green microalgae, it is now recognized that different approaches could be taken (Kruse et al., 2005), including (i) reversible inactivation of O_2 evolution from PS2, (ii) enhancement of endogenous cellular respiration, and (iii) reduction of oxygen partial pressure via photochemical methods.

A number of non-filamentous and non-heterocystous cyanobacteria temporally separate oxygenic photosynthesis, which occurs during the daytime, from nitrogen fixation and hydrogen production during the night time, when oxygen partial pressures are

Table 1
Comparison of H_2 production rates obtained from various photobiological processes.

Process/challenges	Microorganism	Carbon source	Rate, ml H_2 L^{-1} h^{-1}	References
*Utilizing various substrate sources (*Sections 4.3.3 and 4.3.5*)*				
Photofermentation	*Rhodopseudomonas s.*	Malate (15 mM)	1.1	Barbosa et al. (2001)
		Butyrate (27 mM)	7.6	
		Lactate (50 mM)	10.7	
		Acetate (22 mM)	25.2	
Photofermentation	*R. palustris* R-1	Malate (15 mM)	5.8	
		Lactate (50 mM)	9.1	
		Acetate (22 mM)	2.2	
Photofermentation	*R. sphaeroides* O.U.001	Malate (15 mM)	12.0	Eroglu et al. (1999)
Photofermentation	*R. rubrum*	Succinate (16 mM)	20.0	Melnicki et al. (2008, 2009)
Photofermentation	*R. sphaeroides* O.U. 001	Sugar refinery effluent + malate	5.0	Yetis et al. (2000)
Photoferm., immobilized in agar gels	*R. sphaeroides* RV	Tofu wastewater	15.9	Zhu et al. (1999)
Biophotolysis (heterocystous cyanobacteria)	*Anabaena azollae*	2% CO_2 (v/v) in air	13.0	Tsygankov et al. (1998b)
Enhancing the amount of micronutrients (Section 4.3.4)				
Photoferm., (1× Mo)	*R. sphaeroides* O.U.001	Malate (15 mM)	5.2	Kars et al. (2006)
Photoferm, enhanced Mo (100× Mo)			8.4	
Photoferm, wild type + EDTA (0.5 mM)	*R. rubrum* K100	Lactate (50 mM)	18.9	Kern et al. (1994)
Photoferm., Hup^- + EDTA (0.5 mM)	*R. rubrum* K4A		23.1	
Integrated bioprocesses (Section 4.4.1)				
Dark ferm. → photofermentation (halogen and tungsten lamp)	*Clostridium pasteurianum*/ *Rhodopseudomonas palustris* WP3-5	Sucrose (20 g COD/l)	20.5	Chen et al. (2008)
Dark ferm. → photofermentation (with 'optical fiber' side-lights)			28.3	
Dark ferm. → photofermentation	Activated sludge/*R. sphaeroides* O.U.001	Olive mill wastewater (50% v/v OMW in H_2O)	8.0	Eroglu et al. (2006)
*Immobilization of intact cells (*Section 4.1*)*				
Photoferm., carrier-free	*Rhodopseudomonas palustris* WP3-5	Acetate (1 g COD/l)	20.9	Chen and Chang (2006)
Photoferm., clay supported			28.5	
Photoferm., carrier-free, internal optical fiber illumination			40.6	
Photoferm., clay supported, internal optical fiber illumination			43.8	
Biophotolysis, sulphur deprived algae, immobilization in Al-borosilicate porous glass sheets	*Chlamydomonas reinhardtii*	Acetate (TAP-S medium)	4.3	Laurinavichene et al. (2006)
Photoferm., immobilized in agar gels	*R. sphaeroides* RV	Tofu wastewater	15.9	Zhu et al. (1999)
*Sulphur deprivation (*Section 4.2*)*				
Biophotolysis, sulphur deprived algae	*Chlamydomonas reinhardtii*	Acetate (TAP-S medium)	2.5	Laurinavichene et al. (2006)
Biophotolysis, sulphur deprived algae, Immobilization in Al-borosilicate porous glass sheets			4.3	Laurinavichene et al. (2006)
Biophotolysis, sulphur deprived algae	*Chlorella sorokiniana* Ce	Acetate (TAP-S medium)	1.4	Chader et al. (2009)
Biophotolysis, sulphur deprivation, wild type	*C. reinhardtii*	Acetate (TAP-S medium)	0.6	Torzillo et al. (2009)
Biophotolysis, sulphur deprivation, (D1 protein mutant)	*C. reinhardtii* L159I-N230Y		5.8	
*Deletion of uptake hydrogenases (Hup^- mutants) (*Section 4.5.2*)*				
Photoferm., wild type strain	*R. sphaeroides* KD131	Malate (30 mM)	36.1	Kim et al. (2006)
Photoferm., Hup^- mutant			57.2	
Photoferm., Hup^-/PHB^- mutant strain			82.6	
Biophotolysis (heterocystous cyanobacteria), hup^- mutant	*Anabaena variabilis* ATCC29413	Na_3VO_4 instead of Na_2MoO_4, N-free, modified BG-11 (Blue Green algae medium)	20.0	Liu et al. (2006)
Photoferm., wild type strain	*Rhodobacter capsulatus* MT1131	Malate (15 mM)	14.0	Ozturk et al. (2006)
Photofermentation, Hup^- mutant			19.0	
Photoferm., wild type	*R. rubrum* K100	Lactate (50 mM)	5.7	Kern et al. (1994)
Photoferm., Hup^- mutant	*R. rubrum* K4A		19.0	
Photoferm., wild type strain	*R. sphaeroides* O.U.001	Malate (15 mM)	6.9	Kars et al. (2008)
Photoferm., Hup^- mutant			9.2	
*Deletion of the PHB synthase (PHB^- mutant) (*Section 4.5.3*)*				
Photoferm., wild type strain	*R. sphaeroides* KD131	Malate (30 mM)	36.1	Kim et al. (2006)
Photoferm., PHB^- mutant strain			43.8	
Photoferm., Hup^-/PHB^- mutant strain			82.6	

(*continued on next page*)

Table 1 (*continued*)

Process/challenges	Microorganism	Carbon source	Rate, ml H_2 L^{-1} h^{-1}	References
Optimizing bioreactor geometry (Section 4.3.1)				
Photoferm., floating type reactor, continuous culturing	*Rhodopseudomonas palustris* R-1	Acetate (800 mg/L), propionate (800 mg/L), butyrate (800 mg/L), ethanol (400 mg/L)	6.7	Otsuki et al. (1998)
Photoferm., vertical tubular reactor, continuous culturing, 12 h L/12 h dark cycles	*Rhodobacter sphaeroides* O.U.001	Malate (7.5 mM)	20.0	Eroglu et al. (1998)
Photoferm., annular – triple jacketed reactor, luminine tubular light source in the central axis	*R. sphaeroides* O.U.001	Malate (15 mM)	6.5	Basak and Das (2009)
Photoferm., tubular reactor, nearly horizontal (inclined below 10°)	*Rhodobacter capsulatus* DSM 155	Acetate (22 mM), lactate (3.8 mM)	3.3	Gebicki et al. (2009)
Photoferm., panel reactor			8.0	
Photoferm., flat plate, tilted (30°) reactor	*R. sphaeroides* O.U.001	Malate (15 mM)	10	Eroglu et al. (2008b)
Continuous culturing (Section 4.3.1)				
Photoferm., vertical tubular reactor, continuous culturing, 12 h L/12 h dark cycles	*Rhodobacter sphaeroides* O.U.001	Malate (7.5 mM)	20	Eroglu et al. (1998)
Photoferm., continuous culturing	*Rhodospirillum rubrum*	Lactate (50 mM)	180	Zurrer and Bachofen (1982)
Photoferm., floating type photobioreactor, continuous culturing	*Rhodopseudomonas palustris* R-1	Acetate (800 mg/L), propionate (800 mg/L), butyrate (800 mg/L), ethanol (400 mg/L)	6.7	Otsuki et al. (1998)
Photoferm., continuous culturing, ammonium limited conditions	*Rhodobacter capsulatus*	Lactate (32 mM)	80	Tsygankov et al. (1998a)
Optimizing illumination conditions (Section 4.3.2)				
Photoferm., 3000 lux light intensity	*Rhodobacter capsulatus* ST410 (hydrogenase deficient mutant)	Malate (30 mM)	16.0	Ooshima et al. (1998)
Photoferm., 6600 lux light intensity			100	Ooshima et al. (1998)

naturally lower in the cells (Min and Sherman, 2010). Regulation of this temporal separation is not well understood, especially as it would require a relatively rapid switching of the cellular metabolism from autotrophic oxygenic photosynthesis to anaerobic nitrogen fixation and hydrogen production.

The temporal separation of oxygen and hydrogen production by green microalgae and non-filamentous cyanobacteria contrasts with the spatial separation of oxygen and hydrogen production in filamentous cyanobacteria, where dedicated cells perform either oxygenic photosynthesis (vegetative cells) or nitrogen fixation and hydrogen production (heterocysts) (Hallenbeck and Benemann, 2002). Thus, heterocysts in filamentous cyanobacteria effectively separate in space the nitrogenase activity from oxygenic photosynthetic in the neighboring vegetative cells.

4.3. Optimization of bioreactor conditions and substrate source

4.3.1. Reactor designs and culturing

Light saturation of photosynthesis is an important parameter that impacts efficiency and yield, especially in mass culture. Critical in this case is the need to avoid over-absorption of sunlight and wasteful dissipation of the absorbed irradiance (Melis et al., 1999; Akkerman et al., 2002; Melis, 2009). An inverse relationship between the energy conversion efficiency in photosynthesis and the incident sunlight intensity was observed in recent studies (Polle et al., 2003; Nath et al., 2005; Mitra and Melis, 2008, 2010), a consequence of the early light-saturation of photosynthesis in H_2-production. Thus, light utilization in photobioreactors is a critical parameter in determining efficiency and yield. Design specifications for a hydrogen producing photobioreactor included several parameters, such as the pH and/or temperature of the media, intensity of irradiance reaching the cultures, dissolved oxygen, type of mixing, and composition of the growth medium. Several geometries of reactors were also considered for photobiological hydrogen production, such as vertical or horizontal tubular, flat-plate, or helical-tubular reactors (Table 1, see also Eroglu et al., 1998, 2008b; Dasgupta et al., 2010).

Another important photobioreactor design feature is the 'mixing' of the culture, as it enhances production by removing the hydrogen gas present in the reactor, while ensuring homogenous distribution of the nutrients and of the cells within the liquid media. These are prerequisites that facilitate enhanced substrate uptake and light exposure of the cultures (Koku et al., 2003). Mixing also helps to minimize light and temperature gradients throughout the reactor. Mixing at large scale is usually carried out by sparging gases through the reactor, and is indicated, as mechanical mixing is not economical for large-scale systems. Gaseous sparging also helps avoid large shearing forces that are inherent to mechanical mixing, which can be harmful to the fitness of the cells.

Hydrogen production generally declines once cultures reach stationary phase under batch growth conditions (Melnicki et al., 2008). Continuous culturing and production systems are an alternative way to keep the cultures at the exponential phase of growth, ensuring maximal yields of hydrogen production. Continuous culturing is carried out by periodically replacing the culture media with fresh ones at a proper culture dilution rate. Under continuous culturing of photosynthetic bacteria Tsygankov et al. (1998a) and Zurrer and Bachofen (1982) obtained rates of 80 and 180 ml H_2 L^{-1} h^{-1}, respectively (Table 1).

Working with green microalgae, Fedorov et al. (2005) demonstrated that it is possible to couple sulfate-limited *C. reinhardtii* growth to continuous H_2 photoproduction for more than 4000 h. In this approach, a two-stage chemostat physically separated photosynthetic growth from H_2 production in two automated photobioreactors. In the first reactor, the algal cultures were grown aerobically in chemostat mode under limited sulfate to obtain photosynthetically competent cells. Active cells were then

continuously delivered to the second reactor, where H_2 production was induced under hypoxic conditions. Reported rates of H_2-production under these conditions reached a maximum of 0.58 ml H_2 L^{-1} h^{-1}.

4.3.2. Effect of light source and intensity

Different organisms have different light-harvesting antenna pigments, light saturation of photosynthesis characteristics, and solar energy conversion efficiencies. The latter is calculated as the ratio of the energy stored in hydrogen over the total energy input in the photobioreactor. Obviously, solar energy conversion efficiencies depend on light intensity, irradiated area, reactor design, duration of hydrogen production, and amount of hydrogen accumulated. Using *Rhodobacter* sp. under laboratory growth conditions, Miyake and Kawamura (1987) reported solar-to-hydrogen energy conversion efficiencies reaching up to about 8%, while the theoretical maximum was reported as 10% (Akkerman et al., 2002; Kapdan and Kargi, 2006). Other studies showed that diurnal light–dark cycles help to increase the yield of hydrogen production (Koku et al., 2003; Eroglu et al., 2010), hence the solar-to-hydrogen energy conversion efficiency. The advantage of the diurnal light–dark cycles was originally reported by Meyer et al. (1978), as they achieved a more stable nitrogenase activity that translated into a greater H_2 production capacity.

In contrast to photoheterotrophic H_2-production systems, less information exists on the solar-to-H_2 energy conversion efficiencies of photoautotrophic microorganisms. Greenbaum (1988) reported light-to-H_2 energy conversion efficiencies for several green microalgae, based on photosynthetically active radiation (PAR: 400–700 nm). Accordingly, *Scenedesmus* D_3 showed PAR-to-H_2 conversion efficiencies in the range of 16–23%, *C. reinhardtii* (sup) 13–21%, *C. reinhardtii* (UTEX 90) 6–8%, and *Chlamydomonas moewusii* 13–24%. Since PAR contains only about 45% of the total solar energy reaching the surface of the earth, Greenbaum's measurements would have to be multiplied by 0.45 to convert PAR-to-H_2 into solar-to-H_2 energy conversion efficiencies. Accordingly, a measured PAR-to-H_2 conversion efficiency of 24% would translate into 10.8% solar-to-H_2 conversion efficiency in green microalgae. An independent solar-to-H_2 energy conversion analysis suggested a theoretical conversion efficiency maximum of about 12% in green microalgae (Melis, 2009). Thus, the work of Greenbaum (1988) showed that it is possible to experimentally approach the 12% theoretical maximum, when measures are taken to optimize the performance of the system.

4.3.3. Waste matter as feedstock for hydrogen production

Feedstock for the hydrogen production is an important determinant of the cost of the process. Selection criteria for suitable waste material as a feedstock for hydrogen production take into consideration cost, availability, biodegradability (Kapdan and Kargi, 2006), and the carbon to nitrogen ratio, which is especially important for PNS bacteria. A variety of waste materials or carbohydrate rich substrates have been utilized for dark fermentative hydrogen production studies. On the other hand, researchers of photobiological processes are also working to find the optimum feedstock, so as to improve the yield and economics of hydrogen production. Nutrient sources for photobiological hydrogen production are usually wastewater from (i) olive mill (Eroglu et al., 2004), (ii) sugar refinery (Yetis et al., 2000), (iii) tofu industry (Zhu et al., 1999), (iv) dairy plants (Thangaraj and Kulandaivelu, 1994), (v) brewery (Seifert et al., 2010), (vi) sewage sludge (Sunita and Mitra, 1993), and (vii) municipal solid wastes (Fascetti et al., 1998).

Use of waste matter as feedstock for hydrogen production affords the advantage of potentially alleviating human and farm animal generated pollution. Drawbacks often include low rates and yields of hydrogen production, in comparison with optimized media in the laboratory (Kapdan and Kargi, 2006). Several approaches by which to improve yields include the use of microorganisms adapted to more efficiently utilizing waste matter, or using feedstock pretreatment technologies. Feedstock pretreatment processes are known to improve the nutritional value of the substrate source, thereby enabling greater substrate conversion efficiencies by the hydrogen-producing bacteria (Eroglu et al., 2008a). Several investigators used pre-fermentation of waste matter, as an approach for the "conditioning" of the waste matter prior to its application in support of photo-fermentation (see below). Integration of dark and photo-fermentation systems allows the utilization of extended range of waste materials, such as potato wastes (Laurinavichene et al., 2010), sugar beet molasses (Ozgur et al., 2010), and food processing wastewater (Kim et al., 2001).

4.3.4. Micronutrient enhancement

The effect of various parameters on the amount of hydrogen production in association with nitrogenase activity had been investigated, with a reported decrease in the nitrogenase activity in the absence of essential metal ions such as molybdenum and iron (Kars et al., 2006; Jacobson et al., 1986). Availability of these metal ions (molybdenum and iron) was found to be critical since they are required cofactors of the FeMo-nitrogenase (Dixon and Kahn, 2004; Koku et al., 2002). Moreover, many electron carriers of the photosynthetic electron transport chain (ETS) (i.e., cytochrome b–c complex, ferredoxin) also require iron as an essential element and cofactor (Zhu et al., 2007). Hydrogen production during photo-fermentation further depends on ATP energy generated by the photosynthetic apparatus via the light-driven electron transport process, which in turn is used to drive the nitrogenase reaction toward NH_3 and H_2-production (Koku et al., 2002). Accordingly, supplementation of the growth media with Mo and Fe micronutrient salts significantly enhanced the hydrogen production of various *Rhodobacter* species (Uyar et al., 2009; Ozgur et al., 2010; Kars et al., 2006).

4.3.5. Optimization of C/N ratio of the nutrient source

Nitrogen limitations are known to alter photosynthetic bacterial metabolism and direct it more toward the release of excess energy and reducing power in the form of hydrogen (Koku et al., 2002). Accordingly, the carbon to nitrogen molar ratio in the growth medium plays a role in defining H_2-production by photosynthetic bacteria. This was investigated by Eroglu et al. (1999) with *R. sphaeroides* O.U.001. Malic acid and glutamic acid were used as the carbon and nitrogen sources, respectively. It was shown that 15 mM carbon:2 mM nitrogen ratio gave the highest amount of hydrogen production of around 12 ml L^{-1} h^{-1} (Table 1). In addition to the C to N ratio, the source of nitrogen is also an important consideration since ammonia salts tend to inhibit the nitrogenase enzyme activity (Dasgupta et al., 2010; Redwood and Macaskie, 2006). In the absence of ammonia salts, nitrogenase activity, and hydrogen production are normally enhanced.

4.4. Integrated systems

4.4.1. Dark-fermentation and photo-fermentation for H_2-production

Integration of photo-fermentative and dark-fermentative (non-photosynthetic) bacterial metabolisms has been reported as enhancing the overall hydrogen production efficiency. In the first case, anaerobic dark fermentation of sugar molecules produces biomass, H_2, CO_2, and low molecular weight organic acids. The latter are then utilized as substrate and can efficiently be converted into hydrogen by the photosynthetic bacteria via a photo-fermentation process. Photosynthetic bacteria require the energy of sunlight in photosynthesis to enable the further catabolism of small organic acids (Fig. 1a), which the fermentative anaerobic bacteria

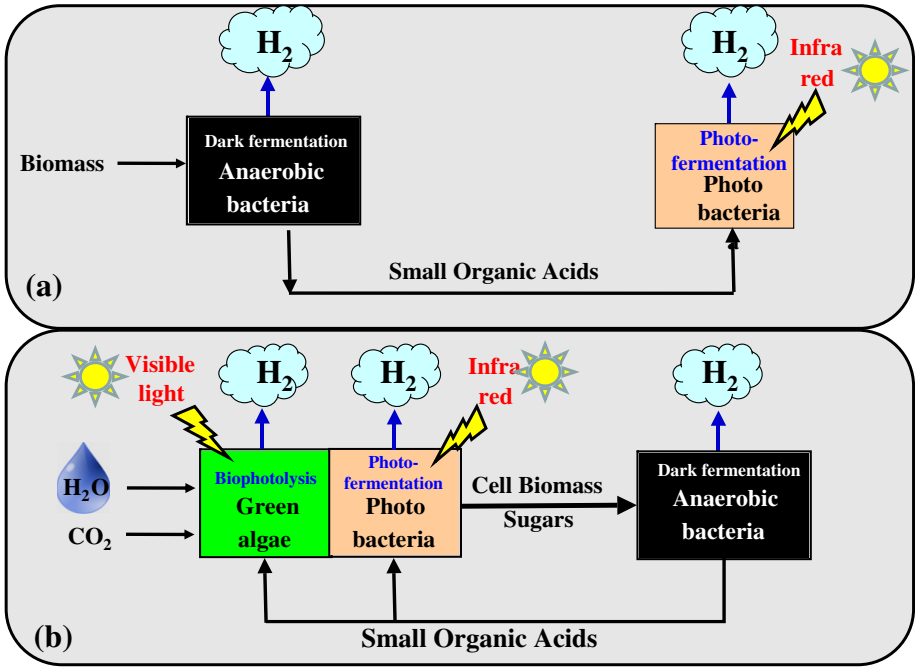

Fig. 1. Simplified schematics for integrated hydrogen production processes: (a) Dark fermentation followed by photo-fermentation process. (b) Photosynthetic process (co-cultivated green algae and photo-fermentative bacteria) followed by dark fermentation process. Adapted from Melis and Melnicki (2006).

cannot do. Integration of these two bioprocesses could theoretically yield 12 mol of hydrogen per 1 mol of glucose, where small organic acids are the intermediate metabolites linking the two processes and their products. The overall reactions in an integrated dark- and photo-fermentation system are shown below by Eqs. (6) and (7):

$$\text{Dark fermentation stage}: C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (6)$$

$$\text{Photo-fermentation stage}: 2CH_3COOH + 4H_2O \rightarrow 4CO_2 + 8H_2 \quad (7)$$

During the first stage, anaerobic bacteria (such as *Clostridium butyricum*) consume carbohydrates via dark anaerobic fermentation for their metabolism and growth. Protons serve in such obligate anaerobic bacteria as the terminal electron acceptor with CO_2 and H_2 as the byproducts of metabolism. As complete degradation of glucose into hydrogen and CO_2 is not possible by the anaerobic dark-fermentation process, small organic acids are also formed and secreted into the growth medium. Photosynthetic bacteria have the capacity to overcome the free energy barrier in the utilization of small organic acids by utilizing sunlight energy to bring organic acids into their metabolism, while fixing nitrogen and producing hydrogen under anaerobic conditions. Integration of the two processes reduces the overall energy demand of the photosynthetic bacteria and increases the amount of hydrogen production per substrate source (Das and Veziroglu, 2001).

Lee et al. (2002) employed such an integrated system for hydrogen production, while the effluents from the peptone or carbohydrate-fed anaerobic reactors were used as the substrate sources of photobioreactors. Kim et al. (2001) also combined dark-fermentation and photo-fermentation for improved hydrogen production from food processing wastewater and sewage sludge. Yang et al. (2010) obtained enhanced hydrogen production from pretreated corncob by the integration of dark and photo-fermentation processes. In the first stage (dark-fermentation of heat-shocked dairy manure) they obtained hydrogen yield of 150 ml L^{-1} h^{-1}, while a second-stage photo-fermentation by *R. sphaeroides* yielded 32 ml H_2 L^{-1} h^{-1}. Liu et al. (2010) investigated hydrogen production by combining *Clostridium butyricum* and immobilized *Rhodopseudomonas faecalis* RLD-53 in batch cultures, using defined media for both reactors. For the second-stage reactor, they reported hydrogen production rates with maxima of 38.2 ml L^{-1} h^{-1}. Nath et al. (2005) used glucose as the substrate source in the dark-fermentation reaction, and the fermentation broth was then used as the photosynthetic bacteria growth medium for hydrogen production via photo-fermentation They reported enhancement in the yield of H_2-production upon combination of the dark fermentation, which yielded 1.86 mol H_2 mol^{-1} glucose with the photo-fermentation process, which yielded an additional 1.5–1.72 mol H_2 mol^{-1} acetic acid. Similarly, Eroglu et al. (2006) observed enhancement in H_2 production rate (8 ml L^{-1} h^{-1}) when 50% (v/v) olive mill wastewater in water was used as the substrate of dark-fermentation by activated sludge, prior to photo-fermentation by *R. sphaeroides* O.U.001 (Table 1). Chen et al. (2008) also observed high rates of H_2-production (28.3 ml L^{-1} h^{-1}) by combining the dark-fermentation of *C. pasteurianum* and photo-fermentation by *R. palustris* WP3-5, while using sucrose as the source of carbon substrate for growth (Table 1). More recently, Laurinavichene et al. (2010) demonstrated H_2 production in an integrated process utilizing potato homogenate for dark, fermentative H_2 production, followed by H_2 photo-production using purple non-sulfur bacteria. They reported maximal production yields of 11.5 L gas L^{-1} culture in the dark fermentation stage and H_2 photo-production yield of 40 L gas L^{-1} of the fermentation effluent, with a total H_2 yield of 5.6 mol mol^{-1} glucose equivalent for the two-stage integrated process.

4.4.2. Green microalgae and photosynthetic bacteria for H_2-production

Melis and Melnicki (2006) employed an integrated system of two photobiological processes such as photosynthetic hydrogen production by green microalgae that used the visible region of the light spectrum (400–700 nm), coupled to H_2 production by anoxygenic photosynthetic bacteria, using the near infrared region (700–950 nm) of the solar spectrum. Integration of such photobiological hydrogen production processes holds promise in improved solar energy utilization, widening the range of the solar spectrum to include wavelengths from 400 to 950 nm. This approach also

holds promise of metabolic integration by the two organism systems, with green microalgae generating organic carbon from CO_2 and H_2O, while photosynthetic bacteria generate organic nitrogen via their nitrogenase, under conditions when both produce hydrogen. Although far from being a commercial reality, such integration promises cost-effective production of organic substrate for substantial yields of hydrogen production. Options to improve the overall efficiency are further discussed (Melis and Melnicki, 2006) by coupling these two photosynthetic systems with dark fermentative bacteria, that are known to produce smaller organic acids and can act as a regeneration step of the original source nutrients (Fig. 1b).

4.5. Genetic engineering approaches to improve efficiency

4.5.1. Truncating the light-harvesting antenna size of photosynthesis

Commercial exploitation of the photobiological hydrogen production processes, either by green microalgae or photosynthetic bacteria, entails organism growth under bright sunlight, when the productivity of the systems ought to be at a maximum (Melis et al., 1999; Melis, 2009). However, an inverse relationship exists between the photosynthetic solar energy conversion efficiency and the size of the light-harvesting antenna of the photosynthetic apparatus, raising the issue of optimization of light absorption and utilization via regulation of the size of the antenna (Tanaka and Melis, 1997; Melis et al., 1999; Mitra and Melis, 2008). Truncated antenna sizes in the photosynthetic apparatus were found to enhance the productivity of green algal mass cultures under bright sunlight, as they minimized fluorescence and heat dissipation of absorbed irradiance (Polle et al., 2003). Genes that confer a truncating Chl antenna size in green microalgae have been cloned and their function analyzed (Tetali et al., 2007; Mitra and Melis, 2010). For example, the *tla1* mutant of *C. reinhardtii*, having a 50% truncated light-harvesting antenna, was shown to perform photosynthesis and growth under mass culture conditions with a rate and yield that were twice those of the wild type (Polle et al., 2003).

In PNS bacteria, genetic approaches were also undertaken to improve solar energy conversion efficiencies and the yield of hydrogen production under mass culture conditions. Among them, efforts to truncate the bacteriochlorophyll antenna size feature prominently. Kondo et al. (2002), under mass culture conditions in a flat-plate reactor, observed significant enhancement in hydrogen production by the mutant of *R. sphaeroides* MTP4, which possessed a smaller in size light-harvesting (LH1) complex. Lower pigment concentrations in the photosynthetic apparatus minimize the early saturation of photosynthesis at the surface of the cultures, while permitting greater light penetration deeper into the reactor, thereby alleviating shading effects. Under such improved optical culture conditions, enhanced culture photosynthetic productivity and H_2 production were observed. Vasilyeva et al. (1999) worked with the P3 mutant of *R. sphaeroides*, which contained only about 37% of the LH1 core antenna and 160% of the LH2 peripheral antenna. Under simulated mass culture conditions, they reported greater hydrogen production in the P3 strain, when compared to the wild type.

Large light harvesting antennae in the photosynthetic apparatus of all classes of photosynthetic organisms (green microalgae, cyanobacteria, and photosynthetic bacteria) are useful survival strategies, conferring a competitive advantage in their respective ecotypes, where sunlight is often limited. However, this developmental feature is counterproductive in high-density cultures under bright sunlight conditions, as it leads to over-absorption of sunlight (more than is required to saturate photosynthesis) followed by loss of excess photons as fluorescence or heat (Dasgupta et al., 2010). For this reason, lowering of the pigment content in the photosynthetic apparatus improves penetration of sunlight through a high-density culture and improves overall productivity.

4.5.2. Inactivation of uptake hydrogenase

Organisms that express the nitrogenase enzyme, such as cyanobacteria and all anoxygenic photosynthesis bacteria, also contain and express the uptake *hup* hydrogenase enzymes. Since uptake hydrogenases are known to be involved in the uptake and recycling of hydrogen, there are attempts to enhance the hydrogen production capacity of nitrogenase-containing organisms upon inactivation of the attendant uptake hydrogenases. In PNS bacteria, elimination of uptake hydrogenases was achieved either upon the deletion of *hup* genes or by insertion of an antibiotic resistance gene into the *hup* operon (Kim et al., 2006; Ozturk et al., 2006; Kars et al., 2008) (Table 1). Kars et al. (2008) applied site directed mutagenesis of the uptake hydrogenase (hupSL) in *R. sphaeroides* by using a suicide vector, an alteration that yielded higher hydrogen production ($9.2\ ml\ L^{-1}\ h^{-1}$) compared to the wild type ($6.9\ ml\ L^{-1}\ h^{-1}$) under otherwise identical conditions (Table 1). Ooshima et al. (1998) observed an increase in the hydrogen conversion efficiency from 25% in the wild type to 68% in a hup-less mutant of *R. capsulatus*.

4.5.3. Elimination of alternative product accumulation

Polyhydroxybutyrate (PHB) is generated by PNS as an intracellular carbon storage material. PHB is known to preferentially accumulate under high carbon to nitrogen ratio conditions, or sulphur deprived conditions (Melnicki et al., 2009). For example, *R. sphaeroides* and *R. rubrum* showed a yield of PHB accumulation of 70% and 80% per dry cell weight, respectively (Kars and Gunduz, 2010; Melnicki et al., 2009). The PHB biosynthesis pathway competes with H_2-production; therefore, several attempts have been made to enhance the portion of substrate that is utilized for hydrogen production by repression of PHB synthesis (Kim et al., 2006). They observed significant enhancement in the hydrogen production capacity of *R. sphaeroides* KD131 after the deletion of a PHB synthase gene, with hydrogen yields increasing to $43.8\ ml\ L^{-1}\ h^{-1}$, compare to the wild type measurements of $36.1\ ml\ L^{-1}\ h^{-1}$ (Table 1). After combining *hup* and PHB gene deletions (Hup^-/PHB^- mutant) they reached a H_2 production rate of $82.6\ ml\ L^{-1}\ h^{-1}$ (Kim et al., 2006). Hustede et al. (1993) also reported increased cell growth and hydrogen production after deletion of PHB synthase gene.

4.5.4. Enhancing the ammonia tolerance of the purple non-sulphur bacteria

Qian and Tabita (1996) reported expression of the nitrogenase enzyme even under suppressive concentrations of ammonia, upon inactivation of the RegB (PrrB) gene in *R. sphaeroides*. There are several mechanisms by which to control ammonia tolerance by the nitrogenase enzyme, a feature that could be useful in hydrogen production by photosynthetic bacteria. Kars and Gunduz (2010) discussed in detail the development of ammonia-insensitive photosynthetic bacteria. Tsygankov et al. (1998a) obtained significantly higher hydrogen production rates ($80\ ml\ L^{-1}\ h^{-1}$) in the continuous culture of *R. capsulatus*, under ammonia-limited conditions (Table 1).

5. Conclusions

In the field of bioenergy generation, it could be argued that renewable H_2-production is more advanced than other biofuel-related efforts. Advantages of H_2 over other biofuels include the relatively high solar-to-product energy conversion efficiency achieved, and the spontaneous product separation from the

biomass. The latter is of critical importance in the economics of renewable energy generation, as dewatering of cultures and product extraction from within cells are laborious and expensive. Disadvantages of hydrogen, on the other hand, include the difficulty of easily and inexpensively storing and dispensing the hydrogen gas, as it would be required in widespread commercial applications.

References

Akkerman, I., Janssen, M., Rocha, J., Wijfels, R.H., 2002. Photobiological hydrogen production: photochemical efficiency and bioreactor design. Int. J. Hydrogen Energy 27, 1195–1208.

Allakhverdiev, S.I., Thavasi, V., Kreslavski, V.D., Zharmukhamedov, S.K., Klimov, V.V., Ramakrishna, S., Los, D.A., Mimuro, M., Nishihara, H., Carpentier, R., 2010. Photosynthetic hydrogen production. J. Photochem. Photobiol. C 11, 87–99.

Basak, N., Das, D., 2007. The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. World J. Microb. Biot. 23, 31–42.

Basak, N., Das, D., 2009. Photo-fermentative hydrogen production using purple non-sulfur bacteria *Rhodobacter sphaeroides* O.U.001 in an annular photobioreactor: a case study. Biomass Bioenergy 33, 911–919.

Baebprasert, W., Lindblad, P., Incharoensakdi, A., 2010. Response of H_2 production and Hox-hydrogenase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. Int. J. Hydrogen Energy 35, 6611–6616.

Barbosa, M.J., Rocha, J.M.S., Tramper, J., Wijffels, R.H., 2001. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. J. Biotechnol. 85, 25–33.

Benemann, J., 1996. Hydrogen biotechnology: progress and prospects. Nat. Biotechnol. 14, 1101–1103.

Chader, S., Haceneb, H., Agathos, S.N., 2009. Study of hydrogen production by three strains of *Chlorella* isolated from the soil in the Algerian Sahara. Int. J. Hydrogen Energy 34, 4941–4946.

Chen, C.Y., Chang, J.S., 2006. Enhancing phototropic hydrogen production by solid-carrier assisted fermentation and internal optical-fiber illumination. Process Biochem. 41, 2041–2049.

Chen, C.Y., Yang, M.H., Yeh, K.L., Liu, C.H., Chang, J.S., 2008. Biohydrogen production using sequential two-stage dark and photo fermentation processes. Int. J. Hydrogen Energy 33, 4755–4762.

Das, D., Veziroglu, T.N., 2001. Hydrogen production by biological processes: a survey of literature. Int. J. Hydrogen Energy 26, 29–37.

Das, D., Veziroglu, T.N., 2008. Advances in biological hydrogen production processes. Int. J. Hydrogen Energy 33, 6046–6057.

Dasgupta, C.N., Gilbert, J.J., Lindblad, P., Heidorn, T., Borgvang, S.A., Skjanes, K., Das, D., 2010. Recent trends on the development of photobiological processes and photobioreactors for the improvement of hydrogen production. Int. J. Hydrogen Energy 35, 10218–10238.

Dixon, R., Kahn, D., 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. 2 (8), 621–631.

Eroglu, I., Aslan, K., Gunduz, U., Yucel, M., Turker, L., 1998. Continuous hydrogen production by *Rhodobacter sphaeroides* O.U.001. In: Zaborsky, O.R. (Ed.), Biohydrogen. Plenum Press, London, pp. 143–151.

Eroglu, I., Aslan, K., Gunduz, U., Yucel, M., Turker, L., 1999. Substrate consumption rates for hydrogen production by *Rhodobacter sphaeroides* in a column photobioreactor. J. Biotechnol. 70, 103–113.

Eroglu, E., Gunduz, U., Yucel, M., Turker, L., Eroglu, I., 2004. Photobiological hydrogen production by using olive mill wastewater as a sole substrate source. Int. J. Hydrogen Energy 29 (2), 163–171.

Eroglu, E., Gunduz, U., Yucel, M., Turker, L., Eroglu, I., 2006. Biological hydrogen production from olive mill wastewater with two stage processes. Int. J. Hydrogen Energy 31 (11), 1527–1535.

Eroglu, E., Eroglu, I., Gunduz, U., Yucel, M., 2008a. Effect of clay pretreatment on photo-fermentative hydrogen production from olive mill wastewater. Bioresour. Technol. 99 (15), 6799–6808.

Eroglu, I., Tabanoglu, A., Gunduz, U., Eroglu, E., Yucel, M., 2008b. Hydrogen production by *Rhodobacter sphaeroides* O.U.001 in a flat plate solar bioreactor. Int. J. Hydrogen Energy 33, 531–541.

Eroglu, E., Gunduz, U., Yucel, M., Eroglu, I., 2010. Photosynthetic bacterial growth and productivity under continuous illumination or diurnal cycles with olive mill wastewater as feedstock. Int. J. Hydrogen Energy 35 (11), 5293–5300.

Fascetti, E., D'Addario, E., Todini, O., Robertiello, A., 1998. Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. Int. J. Hydrogen Energy 23 (9), 753–760.

Fedorov, A.S., Kosourov, S., Ghirardi, M.L., Seibert, M., 2005. Continuous H_2 photoproduction by *Chlamydomonas reinhardtii* using a novel two-stage, sulfate-limited chemostat system. Appl. Biochem. Biotechnol. 121–124, 403–412.

Fiβler, J., Kohring, G.W., Giffhorn, F., 1995. Enhanced hydrogen production from aromatic acids by immobilized cells of *Rhodopseudomonas palustris*. Appl. Microbiol. Biot. 44, 43–46.

Florin, L., Tsokoglou, A., Happe, T., 2001. A novel type of iron hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain. J. Biol. Chem. 276, 6125–6132.

Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., Ghirardi, M.L., Seibert, M., 2003. Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. Eur. J. Biochem. 270, 2750–2758.

Francou, N., Vignais, P.M., 1984. Hydrogen production by *Rhodopseudomonas capsulata* cells entrapped in carrageenan beads. Biotechnol. Lett. 6, 639–644.

Gaffron, H., 1939. Reduction of CO_2 with H_2 in green plants. Nature 143, 204–205.

Gaffron, H., Rubin, J., 1942. Fermentative and photochemical production of hydrogen in algae. J. Gen. Physiol. 26, 219–240.

Gebicki, J., Modigell, M., Schumacher, M., Burgb, J.V.D., Roebroeck, E., 2009. Development of photobioreactors for anoxygenic production of hydrogen by purple bacteria. Chem. Eng. Trans. 18, 363–366.

Gest, H., Kamen, M.D., 1949. Studies on the metabolism of photosynthetic bacteria IV. Photochemical production of molecular hydrogen by growing cultures of photosynthetic bacteria. J. Bacteriol. 58, 239–245.

Ghirardi, M.L., Zhang, L., Lee, J.W., Flynn, T., Seibert, M., Greenbaum, E., Melis, A., 2000. Microalgae: a green source of renewable H_2. Trends Biotechnol. 18, 506–511.

Greenbaum, E., 1982. Photosynthetic hydrogen and oxygen production: kinetic studies. Science 196, 879–880.

Greenbaum, E., 1988. Energetic efficiency of hydrogen photoevolution by algal water-splitting. Biophys. J. 54, 365–368.

Hahn, J.J., Ghirardi, M.L., Jacob, W.A., 2007. Immobilized algal cells used for hydrogen production. Biochem. Eng. J. 37, 75–79.

Hallenbeck, P., Benemann, J.R., 2002. Biological hydrogen production; fundamentals and limiting processes. Int. J. Hydrogen Energy 27, 1185–1193.

Happe, T., Hemschemeier, A., Winkler, M., Kaminski, A., 2002. Hydrogenases in green algae: do they save the algae's life and solve our energy problems? Trends Plant Sci. 7, 246–250.

Hatchikian, E.C., Forget, N., Fernandez, V.M., Williams, R., Cammack, R., 1992. Further characterization of the [Fe]-hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757. Eur. J. Biochem. 203, 357–366.

Hustede, E., Steinbuchel, A., Schlegel, H.G., 1993. Relationship between photoproduction of hydrogen and the accumulation of PHB in non-sulphur purple bacteria. Appl. Microbiol. Biot. 39, 87–93.

Jacobson, M.R., Premakumar, R., Bishop, P.E., 1986. Transcriptional regulation of nitrogen fixation by molybdenum in *Azotobacter vinelandii*. J. Bacteriol. 167 (2), 480–486.

Kapdan, I.K., Kargi, F., 2006. Bio-hydrogen production from waste materials. Enzyme Microb. Technol. 38, 569–582.

Kars, G., Gunduz, U., Yucel, M., Turker, L., Eroglu, I., 2006. Hydrogen production and transcriptional analysis of nifD, nifK and hupS genes in *Rhodobacter sphaeroides* O.U.001 grown in media with different concentrations of molybdenum and iron. Int. J. Hydrogen Energy 31, 1536–1544.

Kars, G., Gunduz, U., Rakhely, G., Yucel, M., Eroglu, I., Kovacs, L.K., 2008. Improved hydrogen production by hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* O.U.001. Int. J. Hydrogen Energy 33 (12), 3056–3060.

Kars, G., Gunduz, U., 2010. Towards a super H_2 producer: improvements in photo-fermentative biohydrogen production by genetic manipulations. Int. J. Hydrogen Energy 35, 6646–6746.

Kern, M., Klipp, W., Klemme, H.J., 1994. Increased nitrogenase dependent H_2 photoproduction by hup mutants of *Rhodospirillum rubrum*. Appl. Environ. Microb. 60 (6), 1768–1774.

Kim, M.S., Lee, T.J., Yoon, Y.S., Lee, I.G., Moon, K.W., 2001. Hydrogen production from food processing wastewater and sewage sludge by anaerobic dark fermentation combined with photo-fermentation. In: Miyake, J., Matsunaga, T., Pietro, A.S. (Eds.), Biohydrogen II. Elsevier Science Ltd., Amsterdam, pp. 263–272.

Kim, M.S., Baek, J.S., Lee, J.K., 2006. Comparison of H_2 accumulation by *Rhodobacter sphaeroides* KD131 and its uptake hydrogenase and PHB synthase deficient mutant. Int. J. Hydrogen Energy 31, 121–127.

Kok, B., Forbush, B., McGloin, M., 1970. Cooperation of charges in photosynthetic O_2 evolution – I. A linear four step mechanism. Photochem. Photobiol. 11, 457–475.

Koku, H., Eroglu, I., Gunduz, U., Yucel, M., Turker, L., 2002. Aspects of metabolism of hydrogen production by *Rhodobacter sphaeroides*. Int. J. Hydrogen Energy 27, 1315–1329.

Koku, H., Gunduz, U., Yucel, M., Turker, L., 2003. Kinetics of biological hydrogen production by the photosynthetic bacterium *Rhodobacter sphaeroides* O.U.001. Int. J. Hydrogen Energy 28, 381–388.

Kondo, T., Arakawa, M., Hirai, T., Wakayama, T., Hara, M., Miyake, J., 2002. Enhancement of hydrogen production by a photosynthetic bacterium mutant with reduced pigment. J. Biosci. Bioeng. 93 (2), 145–150.

Kosourov, S.N., Seibert, M., 2009. Hydrogen photoproduction by nutrient-deprived *Chlamydomonas reinhardtii* cells immobilized within thin alginate films under aerobic and anaerobic conditions. Biotechnol. Bioeng. 102, 50–58.

Kruse, O., Rupprecht, J., Mussgnug, J.H., Dismukes, G.C., Hankamer, B., 2005. Photosynthesis: a blue print for energy capture and conversion technologies. Photochem. Photobiol. Sci. 4, 957–970.

Lambertz, C., Hemschemeier, A., Happe, T., 2010. Anaerobic expression of the ferredoxin-encoding FDX5 gene of *Chlamydomonas reinhardtii* is regulated by the Crr1 transcription factor. Eukaryotic Cell 9, 1747–1754.

Laurinavichene, V., Fedorov, A.S., Ghirardi, M.L., Seibert, M., Tsygankov, A.A., 2006. Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells. Int. J. Hydrogen Energy 31, 659–667.

Laurinavichene, T.V., Belokopytov, B.F., Laurinavichius, K.S., Tekucheva, D.N., Seibert, M., Tsygankov, A.A., 2010. Towards the integration of dark and photo-fermentative waste treatment. 3. Potato as substrate for sequential dark fermentation and light-driven H_2 production. Int. J. Hydrogen Energy 35, 8536–8543.

Lee, C.M., Chen, P.C., Wang, C.C., Tung, Y.C., 2002. Photohydrogen production using purple non-sulfur bacteria with hydrogen fermentation reactor effluent. Int. J. Hydrogen Energy 27, 1308–1314.

Liu, J., Bukutin, V.E., Tsygankov, A.A., 2006. Light energy conversion into H_2 by *Anabaena variables* mutant PK84 dense culture exposed in nitrogen limitations. Int. J. Hydrogen Energy 31, 1591–1596.

Liu, B.-F., Ren, N.-Q., Xie, G.-J., Ding, J., Guo, W.-Q., Xing, D.-F., 2010. Enhanced bio-hydrogen production by the combination of dark- and photo-fermentation in batch culture. Bioresour. Technol. 101, 5325–5329.

Madigan, M.T., Martinko, J.M., Parker, J., 2000. Brock Biology of Microorganisms, ninth ed. Prentice Hall Inc., New Jersey.

Melis, A., 2002. Green alga hydrogen production: progress, challenges and prospects. Int. J. Hydrogen Energy 27, 1217–1228.

Melis, A., 2007. Photosynthetic H_2 metabolism in *Chlamydomonas reinhardtii* (unicellular green algae). Planta 226, 1075–1086.

Melis, A., 2009. Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. Plant Sci. 177, 272–280.

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

Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L., Seibert, M., 2000. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol. 122, 127–133.

Melis, A., Happe, T., 2001. Hydrogen production green algae as a source of energy. Plant Physiol. 127, 740–748.

Melis, A., Melnicki, M., 2006. Integrated biological hydrogen production. Int. J. Hydrogen Energy 31, 1563–1573.

Melnicki, M.R., Bianchi, L., De Philippis, R., Melis, A., 2008. Hydrogen production during stationary phase in purple photosynthetic bacteria. Int. J. Hydrogen Energy 33, 6525–6534.

Melnicki, M., Eroglu, E., Melis, A., 2009. Changes in hydrogen production and polymer accumulation upon sulfur-deprivation in purple photosynthetic bacteria. Int. J. Hydrogen Energy 34 (15), 6157–6170.

Meyer, J., Kelley, B.C., Vignais, P.M., 1978. Effect of light on nitrogenase function and synthesis in *Rhodopseudomonas capsulata*. J. Bacteriol. 136 (1), 201–208.

Min, H.T., Sherman, L.A., 2010. Hydrogen production by the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. strain ATCC 51142 under conditions of continuous light. Appl. Environ. Microbiol. 76, 4293–4301.

Mitra, M., Melis, A., 2008. Optical properties of microalgae for enhanced biofuels production. Opt. Express. 16, 21807–21820.

Mitra, M., Melis, A., 2010. Genetic and biochemical analysis of the *TLA1* gene in *Chlamydomonas reinhardtii*. Planta 231, 729–740.

Miyake, J., Kawamura, S., 1987. Efficiency of light energy conversion to hydrogen by photosynthetic bacteria *Rhodobacter sphaeroides*. Int. J. Hydrogen Energy 12, 147–149.

Nath, K., Kumar, A., Das, D., 2005. Hydrogen production by *Rhodobacter sphaeroides* strain O.U.001 using spent media of *Enterobacter cloacae* strain DM11. Appl. Microbiol. Biot. 68, 533–541.

Ooshima, H., Takakuwa, S., Katsuda, T., Okuda, M., Shirasawa, T., Azuma, M., Kato, J., 1998. Production of hydrogen by a hydrogenase-deficient mutant of *Rhodobacter capsulatus*. J. Ferment. Bioeng. 85 (5), 470–475.

Otsuki, T., Uchiyama, S., Fujiki, K., Fukunaga, S., 1998. Hydrogen production by a floating-type photobioreactor. In: Zaborsky, O.R. (Ed.), Biohydrogen. Plenum Press, London, pp. 369–374.

Ozgur, E., Mars, A.E., Paksel, B., Louwerse, A., Yucel, M., Gunduz, U., Claassen, P.A.M., Eroglu, I., 2010. Biohydrogen production from beet molasses by sequential dark and photo-fermentation. Int. J. Hydrogen Energy 35, 511–517.

Ozturk, Y., Yucel, M., Daldal, F., Mandaci, S., Gunduz, U., Turker, L., Eroglu, I., 2006. Hydrogen production by using *Rhodobacter capsulatas* mutants with genetically modified electron transfer chains. Int. J. Hydrogen Energy 31, 1545–1552.

Peters, J.W., Lanzilotta, W.N., Lemon, B.J., 1998. X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 Angstrom resolution. Science 282, 1853–1858.

Polle, J.E.W., Kanakagiri, S., Melis, A., 2003. Tla1, a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size. Planta 217, 49–59.

Posewitz, M.C., King, P.W., Smolinski, S.L., Zhang, L., Seibert, M., Ghirardi, M.L., 2004. Discovery of two novel radical *S*-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. J. Biol. Chem. 279, 25711–25720.

Qian, Y., Tabita, F.R., 1996. A global signal transduction system regulates aerobic and anaerobic CO_2 fixation in *Rhodobacter sphaeroides*. J. Bacteriol. 178, 12–18.

Redwood, M.D., Macaskie, L.E., 2006. A two-stage, two-organism process for biohydrogen from glucose. Int. J. Hydrogen Energy 31, 1514–1521.

Sasikala, K., Ramana, C.V., Rao, P.R., Subrahmanyam, M., 1990. Effect of gas phase on the photoproduction of hydrogen and substrate conversion efficiency in the photosynthetic bacterium *Rhodobacter sphaeroides* O.U.001. Int. J. Hydrogen Energy 15 (11), 795–797.

Seifert, K., Waligorska, M., Laniecki, M., 2010. Brewery wastewaters in photobiological hydrogen generation in presence of *Rhodobacter sphaeroides* O.U.001. Int. J. Hydrogen Energy 35, 4085–4091.

Shima, S., Pilak, O., Vogt, S., Schick, M., Stagni, M.S., Meyer-Klaucke, W., Warkentin, E., Thauer, R.K., Ermler, U., 2008. The crystal structure of [Fe]-hydrogenase reveals the geometry of the active site. Science 321, 572–575.

Sunita, M., Mitra, C.K., 1993. Photoproduction of hydrogen by photosynthetic bacteria from sewage and wastewater. J. Biosci. 18, 155–160.

Tamagnini, P., Axelsson, R., Lindberg, P., Oxelfelt, F., Wunschiers, R., Lindblad, P., 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiol. Mol. Biol. R 66, 1–20.

Tamagnini, P., Leitao, E., Oliveira, P., Ferreira, D., Pinto, F., Harris, D.J., Heidorn, T., Lindblad, P., 2007. Cyanobacterial hydrogenases: diversity, regulation and applications. FEMS Microbiol. Rev. 31, 692–720.

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

Tetali, S.D., Mitra, M., Melis, A., 2007. Development of the light-harvesting chlorophyll antenna in the green alga *Chlamydomonas reinhardtii* is regulated by the novel Tla1 gene. Planta 225, 813–829.

Thangaraj, A., Kulandaivelu, G., 1994. Biological hydrogen photoproduction using dairy and sugarcane waste waters. Bioresour. Technol. 48, 9–12.

Tomitani, A., Okada, K., Miyashita, H., Matthijsk, H.C.P., Ohno, T., Tanaka, A., 1999. Chlorophyll *b* and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature 400, 159–162.

Torzillo, G., Scoma, A., Faraloni, C., Ena, A., Johanningmeier, U., 2009. Increased hydrogen photoproduction by means of a sulfur-deprived *Chlamydomonas reinhardtii* D1 protein mutant. Int. J. Hydrogen Energy 34, 4529–4536.

Tsygankov, A., 2001. Hydrogen production by purple bacteria: immobilized versus suspension cultures. In: Miyake, J., Matsunaga, T., Pietro, A.S. (Eds.), Biohydrogen II. Elsevier Science Ltd., Amsterdam, pp. 229–243.

Tsygankov, A.A., Hirata, Y., Miyake, M., Asada, Y., Miyake, J., 1994. Photobioreactor with photosynthetic bacteria immobilized on porous glass for hydrogen photoproduction. J. Ferment. Bioeng. 77, 575–578.

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

Tsygankov, A.A., Hall, D.O., Liu, J., Rao, K.K., 1998b. An automated helical photobioreactor incorporating cyanobacteria for continuous hydrogen production. In: Zaborsky, O.R. (Ed.), Biohydrogen. Plenum Press, London, pp. 431–440.

Uyar, B., Schumacher, M., Gebicki, J., Modigell, M., 2009. Photoproduction of hydrogen by *Rhodobacter capsulatus* from thermophilic fermentation effluent. Bioproc. Biosys. Eng. 32, 603–606.

Vasilyeva, L., Miyake, M., Khatipov, E., Wakayama, T., Sekine, M., Hara, M., Nakada, E., Asada, Y., Miyake, J., 1999. Enhanced hydrogen production by a mutant of *Rhodobacter sphaeroides* having an altered light-harvesting system. J. Biosci. Bioeng. 87, 619–624.

Wall, J.D., 2004. Rain or shine – a phototroph that delivers. Nat. Biotechnol. 22 (1), 40–41.

Winkler, M., Hemsehemeier, A., Gotor, C., Melis, A., Happe, T., 2002. [Fe]-hydrogenase in green algae: photo-fermentation and hydrogen evolution under sulfur deprivation. Int. J. Hydrogen Energy 27, 1431–1439.

Yang, H., Guo, L., Liu, F., 2010. Enhanced bio-hydrogen production from corncob by a two-step process: dark- and photo-fermentation. Bioresour. Technol. 101, 2049–2052.

Yetis, M., Gunduz, U., Eroglu, I., Yucel, M., Turker, L., 2000. Photoproduction of hydrogen from sugar refinery wastewater by *Rhodobacter sphaeroides* O.U.001. Int. J. Hydrogen Energy 25, 1035–1041.

Zhang, L., Happe, T., Melis, A., 2002. Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta 214, 552–561.

Zhu, H., Suzuki, T., Tsygankov, A.A., Asada, Y., Miyake, J., 1999. Hydrogen production from tofu wastewater by *Rhodobacter sphaeroides* immobilized in agar gels. Int. J. Hydrogen Energy 24, 305–310.

Zhu, H., Fang, H.H.P., Zhang, T., Beaudette, L.A., 2007. Effect of ferrous ion on photo heterotrophic hydrogen production by *Rhodobacter sphaeroides*. Int. J. Hydrogen Energy 32, 4112–4118.

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