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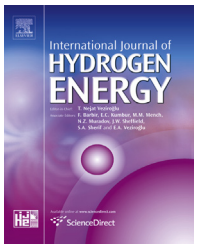

Review Article

Microalgal hydrogen production research

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ABSTRACT

Microorganisms can produce hydrogen biologically, with species ranging from photosynthetic and fermentative bacteria to green microalgae and cyanobacteria. In comparison with the conventional chemical or physical hydrogen production methods, biological processes demonstrate several advantages by operating at ambient pressure and temperature conditions, without a requirement for the use of precious metals to catalyze the reactions. In addition to using cellular endogenous substrate from which to extract electrons for H_2-production, a number of green microalgae are also endowed with the photosynthetic machinery needed to extract electrons from water, the potential energy of which is elevated by two photochemical reactions prior to reducing protons (H^+) for the generation of molecular hydrogen (H_2). Sunlight provides the energy for the microalgal overall strongly endergonic reaction of H_2O-oxidation, electron-transport, and H_2-production. Thanks to a substantial amount of research, a number of diverse experimental approaches have been developed and applied to establish and improve sustainable production of hydrogen. This review summarizes and updates recent developments in microalgal hydrogen production research with emphasis on new trends and novel ideas practiced in this field.

Contents

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Nomenclature

AcDH	acetaldehyde dehydrogenase
ACK	acetate kinase
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
Chl	chlorophyll
CSTR	continuously stirred reactor
Cyt b_6-f	cytochrome b_6-f complex
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
dcw	dry cell weight
FCCP	carbonyl cyanide p-trifluoro-methoxyphenylhydrazone
Fd	ferredoxin
Fd_{red}	reduced ferredoxin
FQR	ferredoxin-plastoquinone reductase
HUP	hexose uptake protein
Hyd	hydrogenese
LSPR	localized surface plasmon resonances
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OMW	olive mill wastewater
PAR	photosynthetically active radiation
PAT	phosphate acetyl transferase
PBR	photobioreactor
PC	plastocyanin
PDC	pyruvate decarboxylase
PEMFC	proton exchange membrane fuel cell
PFL	pyruvate-formate-lyase
PFOR	pyruvate-ferredoxin oxidoreductase
PQ	plastoquinone
PQH_2	plastoquinol
PSI	photosystem I
PSII	photosystem II
RNA	ribonucleic acid
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
S-deprived	sulfur depleted
S/V	surface to volume ratio
TAP	tris-acetate-phosphate medium
TAP-S	sulfur-deprived tris-acetate-phosphate medium
TLA	truncated light-harvesting antenna
VS	volatile solids
η	light conversion efficiency

Introduction

Many microorganisms are capable of hydrogen metabolism, whereby the molecule (H_2) is either the reactant or end product of various metabolic processes and pathways in the cell. Processes such as direct biophotolysis of water (algae and cyanobacteria), photofermentative nitrogen fixation (photosynthetic bacteria and heterocysts of filamentous cyanobacteria), non-photosynthetic hydrogen production from the fermentation of organic compounds (obligate anaerobic bacteria), and fermentative nitrogen fixation (fermentative bacteria) [1,2] contribute to H_2-production. While several reviews are available in the literature to cover individual process [1–12], the current article focuses on the topic of microalgal biohydrogen generation with highlights on evaluating new trends, challenges, and evolving new ideas.

Algae are a highly diverse mostly phototrophic group of organisms ranging from unicellular to multicellular. Algae are eukaryotes, while blue-green algae (cyanobacteria) are excluded from this eukaryotic group [13]. Many algae have the ability to uptake and assimilate organic carbon compounds, while some of them can only uptake inorganic carbon in the form of CO_2 or -HCO_3, requiring reductant and ATP energy from photosynthesis for the active uptake process and in order to internally convert the inorganic carbon to organic substrate [14,15]. Algae occupy a wide range of ecotypes, from the hypersaline to marine and freshwater, including soil and permafrost, while some of them can even exist and grow in aerial settings [14].

Unicellular green algae are the dominant group in photobiological hydrogen production, while certain diatoms and a few multicellular or microcolonial algal strains are also capable of H_2 generation [16]. Among the various tested microalgae, *Chlamydomonas reinhardtii* occupies a unique place and is of special interest, as it is most promising in H_2 production research [12,15–19]. Although about three-quarters of a century have passed from the pioneering discovery by Gaffron and co-workers of the photosynthetic hydrogen metabolism by green microalgae [20,21], only recently this field received impetus and is actively evolving via further exploration.

Recent approaches and current challenges in microalgal hydrogen research are reviewed in this article, with highlights on advancements in research areas such as bioreactor design; cellular immobilization; utilization of wastewater and associated compounds as a substrate; integrated microbial systems; metabolic and genetic engineering approaches; and other multidisciplinary techniques that aim to improve

sustainability of H_2-production. In an attempt to compare the activity and productivity of each sub-research area, publications prior to year 2010 and during the 2010–2016 period of time are evaluated.

Microalgal hydrogen production

Two light-dependent photosynthetic electron transport pathways and one light-independent fermentative pathway have been identified as capable of contributing electrons to microalgal H_2-production [4,22–25]. The water oxidation reaction in photosystem-II (PSII) is the source of electrons in the first light-dependent photosynthetic pathway, which involves both photosystems (Fig. 1a, **pathway 1)**. Electrons for the second photosynthetic pathway derive from the oxidation of cellular endogenous substrate and are fed directly into the plastoquinone pool. In this case, light absorption by photosystem-I (PSI) is required to elevate the potential energy of the electrons to ferredoxin prior to H^+ reduction by the [FeFe]-hydrogenase (Fig. 1a, **pathway 2)**. The main reaction steps of these pathways are schematically shown in Fig. 1a. Electrons for the light-independent fermentative pathway also derive from the oxidation of cellular endogenous substrate in a process whereby H^+ serve as the sink of excess metabolic electrons with H_2 as the end (waste) product (Fig. 1b). These are explored in some greater detail below.

Chemical energy for the reduction of CO_2 into initially triose-phosphate derives from the "light reactions of photosynthesis", which occur in two steps in all photoautotrophic organisms [14,22]. The first step entails absorption of sunlight by light harvesting pigments in the two photosystems (PS), which is efficiently transferred to the respective (PSII and PSI) photochemical reaction centers. In these reaction centers, excitation energy generates a strongly endergonic charge separation reaction, literally pushing electrons to a much higher potential-energy level, followed by electron transport through several intermediates in the electron transport chain of the thylakoid membrane. This overall endergonic electron transport process leads electrons from H_2O to ferredoxin and also generates adenosine triphosphate (ATP). Thus, the light reactions of photosynthesis are responsible for converting sunlight energy and storing it as chemical energy in the form of reduced ferredoxin (Fd_{red}) and ATP. These forms of chemical energy are necessary and sufficient to drive the reactions of the Calvin–Benson cycle, leading to reduction of CO_2 into triose-phosphate and eventually carbohydrate synthesis [22].

Green microalgae can perform photosynthesis under either oxygenic or anoxic conditions. Oxygenic photosynthetic organisms (i.e., plants, algae, cyanobacteria) utilize H_2O as the source of both electrons and protons and "evolve" oxygen as a byproduct. Microalgae and cyanobacteria are further capable of producing hydrogen with electrons and protons from the oxidation of the water molecules [17,26]. Under anaerobic conditions, driven by the energy of sunlight, unicellular green algae can shift their endogenous photosynthetic electron flow in the thylakoid membranes toward H_2-production, in a terminal reaction catalyzed by the [FeFe]-hydrogenase enzyme, which is nuclear-encoded and chloroplast-localized [3,15,17,22].

In the first light-dependent H_2 photoproduction pathway, also called as the PSII-dependent pathway, H_2 is generated with electrons from the oxidation of water and requires participation of both photosystems. Following the oxidation of H_2O, electrons are transferred from PSII to PSI through several electron transport steps. Electrons are first passed from PSII to the plastoquinone (PQ) pool, then through the cytochrome b_6-f complex and plastocyanin (PC) before reaching PSI. In PSI, the potential energy of these electrons is elevated upon utilization of light energy so that they can reduce ferredoxin (Fd) [12,15,22]. High potential energy electrons in Fd_{red} are reconstituted with protons to yield molecular H_2 in a reversible reaction catalyzed by the [FeFe]-hydrogenase, according to chemical equation (1) (see also Fig. 1a, **pathway #1**).

$$2H^+ + 2Fd_{(reduced)} \leftrightarrow H_2 + 2Fd_{(oxidized)} \quad (1)$$

In addition to the PSII-dependent pathway, electrons for H_2-production can be generated from the oxidation of cellular endogenous substrate, e.g. carbohydrates and other metabolites [21,27]. This alternative pathway is also light dependent, but it does not involve PSII and is known as the PSII-independent pathway. Electrons from the glycolytic breakdown of endogenous substrate are transferred via the plastoquinone pool to PSI and the [FeFe]-hydrogenase (Fig. 1a, **pathway #2)** [27–29].

The enzyme [FeFe] hydrogenase is nuclear-encoded but localized in the chloroplast of green microalgae [30]. It has a $k_{cat} = 2000\ s^{-1}$, i.e., relatively higher by 10–100-fold, catalytic activity compare to the [NiFe]-hydrogenases of bacteria and cyanobacteria [31,32]. It is worth noting that the microalgal [FeFe]-hydrogenase is different from the so-called [Fe] hydrogenase, which was previously known as *iron sulfur cluster free hydrogenase* of the hydrogenotrophic methanogenic archaea, due to its lacking the [Fe-S] clusters that are characteristic of the [FeFe]-hydrogenase, possessing only a mononuclear Fe active site [33]. In contrast, the active site of a [FeFe] hydrogenase enzyme - referred to as the *H-cluster* – operates at a fairly negative redox potential, and is uniquely defined by a [4Fe-4S] iron-sulfur cubane structure that is covalently linked to the [2Fe-2S] catalytic subcluster [22,33–35], thereby comprising a unique in biology [6Fe-6S] functional complex. However, the delicate structure and catalytic activity of the *H-cluster*, which also involves a unique in this enzyme non-ligand cysteine and its sulfhydryl group, makes this enzyme prone to damage by oxygen and, thereby, very oxygen sensitive, as O_2 acts to modify irreversibly and deactivate the catalytic structure of the *H-cluster* [36,37].

The [FeFe]-hydrogenase and related hydrogen metabolism genes have been cloned from several strains of green microalgae including *C. reinhardtii*, *Chlorella fusca*, *Chlorococcum littorale*, *Platymonas subcordiformis,* and *Scenedesmus obliquus* [6,11]. The model green microalga *C. reinhardtii* encodes two [FeFe]-hydrogenases, named *HydA1* and *HydA2*, which are expressed only under anaerobic conditions [38,39]. It is believed that *HydA1* is the main gene responsible for hydrogen production [40], while *HydA2* has a 68% amino acid identity with the *HydA1* [38] and may play a secondary role in this

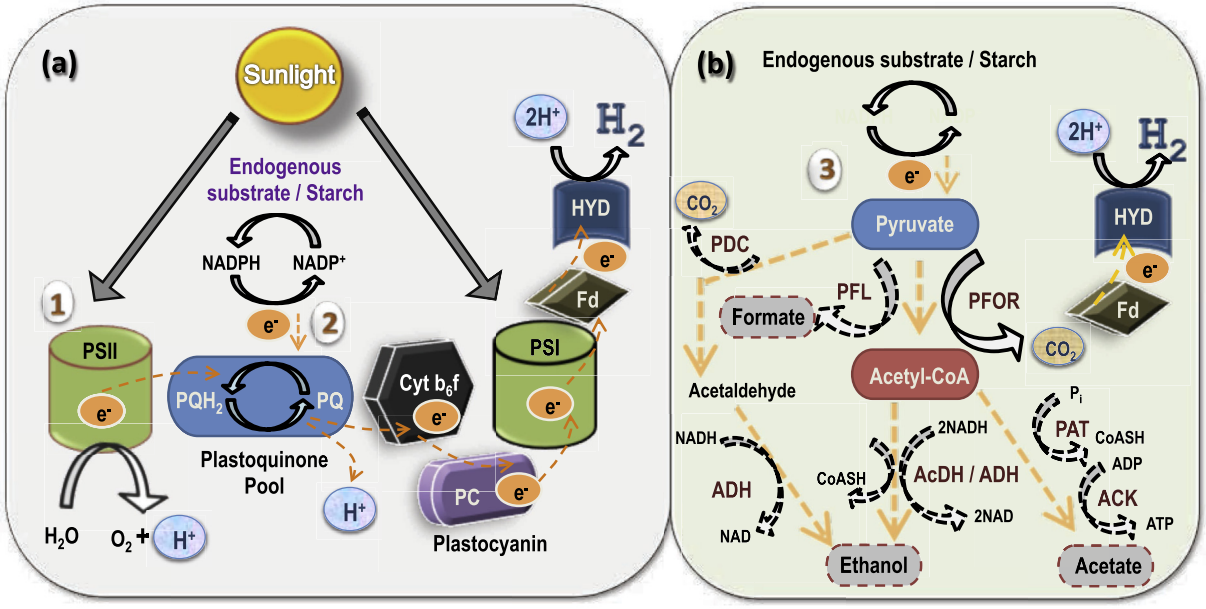

Fig. 1 – Schematic of H_2-production pathways in microalgae. (a) Light-dependent alternatives (#1 and #2) of electron transport pathways to H_2. Water oxidation is the electron source of the PSII-dependent pathway (#1), whereas the electron supply of the PSII-independent pathway (#2) is oxidation of endogenous or exogenous substrate. (b) The light-independent fermentative pathway in green microalgae (pathway #3), in which endogenous or exogenous substrate is the exclusive source of electrons for H_2-production. Although other routes exist, selected fermentation pathways for the major metabolites (i.e., pyruvate, acetate and ethanol) are also given. Electron transport steps are shown with dashed arrows in orange color. Illustrations have been adapted from Subramanian et al. [23], Doebbe et al. [78], Eroglu et al. [15], and Atteia et al. [214]. Abbreviations used are AcDH: acetaldehyde dehydrogenase; ACK: acetate kinase; ADH: alcohol dehydrogenase; Cyt b_6-f: Cytochrome b_6-f complex; Fd: Ferredoxin; Hyd: Hydrogenese; NADPH: Reduced nicotinamide adenine dinucleotide phosphate; PAT: phosphate acetyl transferase; PC: plastocyanin; PDC: pyruvate decarboxylase; PFL: pyruvate-formate-lyase; PFOR: pyruvate-ferredoxin oxidoreductase; PQ: Plastoquinone; PQH_2: Plastoquinol; PSI: Photosystem-I; PSII: Photosystem-II. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

process. It was also reported that *HydEF* and *HydG* accessory genes encode proteins necessary for the assembly and activation of the [FeFe]-hydrogenase enzyme [38,41]. Several hypotheses aimed to explain the evolutionary origin of the genes for hydrogen production in green microalgae, including the possibility of lateral gene transfer from obligate anaerobic bacteria, as some of these bacteria (e.g. *Clostridium pasteurianum*) were found to encode highly similar [FeFe]-hydrogenases and related genes with those encountered in green microalgae [10,34].

It should be emphasized that the H_2 production reaction of equation (1) can occur only under hypoxic conditions due to the extreme O_2 sensitivity of the hydrogenase enzyme [9,17,31]. Inactivation of all [FeFe]-hydrogenases by O_2 is known to be irreversible [42]. However, a recent study reported that short term exposure of the [FeFe]-hydrogenase to O_2 could be partially reversible [43], a finding that merits further investigation. Removal of O_2, which is generated as a byproduct of PSII and H_2O-oxidation, is necessary and sufficient to induce *HydA* gene expression, thereby enabling a light-driven sustainable hydrogen production [44,45]. Some well-known and successful approaches by which to alleviate the O_2-inhibition include purging the microalgal culture medium with inert gasses, which actively remove oxygen from the suspension [46,47], and the consumption of oxygen by the cell's own mitochondrial respiration, aided by experimental manipulation of the balance between photosynthesis and respiration upon the use of metabolic tools such as an imposed sulfur-deprivation [9,17]. An alternative approach is to engineer an O_2-tolerant [FeFe]-hydrogenase enzyme for microalgal H_2-production [48,49], changing the protein configuration to shrink the opening of channels in the [FeFe]-hydrogenase tertiary structure that permit molecular O_2 to diffuse toward the H-cluster [50], or changing the protein settings around the [FeFe]-hydrogenase active site [42] to prevent the oxidative inhibition. These efforts have so-far proven to be difficult due to challenges in genetically engineering the enzyme to block diffusion of O_2 to the HC catalytic site, while maintaining the enzymatic activity. In a H_2O-to-H_2 process, this approach is further encumbered by the difficulty of separating H_2 from the simultaneously-produced O_2.

A third approach to microalgal H_2 production encompasses the light-independent fermentative pathway that can generate various small organic molecules such as formate, acetate, and ethanol along with H_2 [51]. This relies on the pyruvate-ferredoxin oxidoreductase (PFOR) enzyme in a process similar to the one observed in obligate anaerobic microorganisms (Fig. 1b, **pathway #3**). In this pathway, cellular

starch reserves in microalgae that accumulated during the process of oxygenic photosynthesis are biochemically degraded by the cells into pyruvate, followed by the oxidation of pyruvate through the PFOR enzyme to generate acetyl-CoA and CO_2, along with the attendant reduction of Fd, which then transfers electrons to the hydrogenase for the catalytic production of H_2 [4,23,51–54]. It was also reported that the pyruvate-formate-lyase (PFL) enzyme in *C. reinhardtii* could catalyze a reaction that oxidizes pyruvate to formate, while generating acetyl-CoA [55,56]. Although other metabolic routes exist, ethanol can be generated via the acetaldehyde dehydrogenase (AcDH)/alcohol dehydrogenase (ADH) pathway [51,57], while phosphate acetyl transferase (PAT) together with acetate kinase (ACK) contribute to acetate production from acetyl-CoA [51,58]. In comparison with the aforementioned light-dependent pathways, the light-independent fermentative pathway generates relatively smaller amounts (traces) of H_2 in the microalgal cells [18,27].

Although the specific contribution and percentages of the electrons derived either from water oxidation or endogenous substrate catabolism are not precisely known, it is understood that the physiological significance of electron-transport leading to H_2-production in the algal chloroplast is to enable ATP synthesis, which is needed for housekeeping functions and survival of the cells under prolonged and adverse anaerobic conditions [15]. In this respect, survival under anaerobic conditions depends on the amount of starch stored in the cells and coincides with the duration of starch breakdown and H_2-production [25,59,60]. It is of interest that sulfur-deprivation, as a tool of inducing the H_2-production process, triggers a temporal sequence of events beginning with the accumulation of starch (0–36 h), concomitant with a slow-down of oxygenic photosynthesis, arriving at a balanced photosynthesis–respiration activity, after which H_2-production starts (36–120 h) [17,61]. It is very telling that inactivation of PSII in sulfur-deprived and hydrogen producing cells by a PSII inhibitor treatment, such as 3-3,4-dichlorophenyl-1,1-dimethylurea (DCMU), right after transferring cells to a sulfur-free media resulted in no starch accumulation and also in no H_2 generation [62,63]. On the other hand, cells that were induced to accumulate starch prior to the DCMU treatment were able to generate H_2, showing the significance of both PSII and starch accumulation in the H_2 production process [62,63].

Approaches for enhancing the yield of hydrogen production

Current approaches that focus on elevating hydrogen production efficiencies and yields are briefly discussed below. The literature uses diverse units for reporting hydrogen production rates. The most commonly used rate units are: (i) volume of H_2 per time per either volume of liquid culture (i.e., mL H_2 L^{-1} culture h^{-1}) or per volume of the photobioreactor used (i.e., mL H_2 L_{PBR}^{-1} h^{-1}); (ii) volume of H_2 per culture mass per time (i.e., mL H_2 g^{-1} dcw h^{-1}), where culture mass is typically measured as dry cell weight (dcw); or (iii) moles of H_2 per total chlorophyll (Chl) present within the algal cells per time (i.e., μmol H_2 mg^{-1} Chl h^{-1}). Since hydrogen production rates depend on several parameters including (i) photobioreactor geometry; (ii) construction material and size of the reactor; (iii) source of electrons; (iv) algal strain used; (v) initial concentration of the cells; (vi) mixing and cultivation conditions; and (vii) illumination settings, it is not always straightforward to make a direct comparison and evaluation between individual studies based on rates reported in various publications.

Metabolic manipulations

In this section, attention is focused on the metabolic alterations caused by nutrient deprivation(s). Deprivation of sulfur, phosphorus, nitrogen or potassium induce severe metabolic changes in microalgal cells, ranging from inhibition of the photosynthetic process to altered amino acid biosynthesis and metabolism, accumulation of starch, and interruption of cell growth and division [26,64–71].

In year 2000, an important scientific innovation was reported by Melis and co-workers upon the application of sulfur deprivation as an effective tool for the partial inactivation of PSII, lowering the activity of PSII and balancing the capacities of photosynthesis and respiration in the cell, thus allowing the establishment of anoxic conditions in the culture, triggering sustainable rates of H_2-production [17]. The duration of photosynthetic H_2-production was extended by this process from 2 min to about 5 days, while at the same time enabling substantially greater yields than that of the earlier approaches [17]. Sulfur-deprivation induced a down-regulation in the number of functional PSII units that gradually lowered the amount of photosynthetic oxygen production to a point where all photosynthetically-generated oxygen was consumed internally by the cell's own respiration [17,72]. As the rate of photosynthetic oxygen production became the same, or lower than the respiratory oxygen consumption, any remaining oxygen present in the sealed culture was consumed, reaching anoxia and thereby lifting the O_2-dependent suppression of *HydA* gene expression, alleviating the O_2-induced enzyme inhibition, and enabling H_2-production by directing the residual photosynthetic electron transport towards the [FeFe]-hydrogenase enzyme [17].

Sulfur deprivation triggered consecutive stages of culture transition from aerobic growth and oxygen evolution to oxygen consumption, to the establishment of anoxia, followed by H_2 production [4,73]. During the aerobic growth stage, microalgal cells do normal photosynthesis and O_2 evolution, while accumulating starch [9,17,73] and potentially triacylglycerides as energy reserves [74]. During S-deprivation, protein biosynthesis and cell divisions stop, and cells accumulate starch and triacylglycerides, as the biosynthesis and accumulation of these compounds does not depend on sulfur nutrients (0–36 h). A persisting S-deprivation, however, causes the cells to undergo internal protein degradation, as the means by which to recover needed S-nutrients for survival. According to detailed proteomic analysis of S-deprived *C. reinhardtii* cells, alterations in protein composition affected the photosynthetic apparatus, molecular chaperones, and the protein-biosynthetic machinery; together with changes in proteins involved in antioxidant reactions, metabolism of cell wall and flagella, and the assimilation of sulfate, nitrogen and acetate [75]. The primary targets are the Ribulose-1,5-

bisphosphate carboxylase/oxygenase enzyme (RuBisCO) and the D1/32 kD reaction center protein of PSII [61,67]. This hierarchy results in a steep decline in RuBisCO content and CO_2 fixation activity, as well as a slow-down in PSII activity due to loss of D1 protein [76]. These conditions favor H_2-production activity, as the unaffected by the S-deprivation cellular respiration is then sufficient to consume photosynthetic O_2 resulting in medium anaerobiosis [9,17]. Hydrogen production and release permits a lower-level but sustained photosynthetic electron transport, coupled with a sustained oxidative phosphorylation in mitochondria, thus facilitating photophosphorylation and ATP production, under conditions when CO_2 fixation is absent [10,11]. The continuous production of ATP enables cells to perform housekeeping and maintenance functions and to survive for prolonged periods of time (up to 180 h) in complete S-deprivation and anaerobiosis [61,77]. Timmins et al. [74] suggested that anaerobic fermentation and glycolysis gain importance in the modified cellular biochemistry for the generation of ATP, as they are needed to supplement oxidative phosphorylation under diminishing levels of internal O_2. Doebbe et al. [78] reported that H_2-production activity competes for pyruvate emanating as a product of glycolysis, consistent with the observed starch degradation during H_2-production.

C. reinhardtii accumulated large amount of starch within the first 24 h of sulfur-deprivation [17,61,76,79]. Although the haploid *C. reinhardtii* cells are around 10 μm in length with an ellipsoidal shape, sulfur-deprived cells changed, within 24–36 h, their shape from small and ellipsoidal to large and spherical, a swelling that occurred in parallel with the starch accumulation [61]. The reasons for starch accumulation was reported to be inability of the cells to channel newly assimilated carbon toward amino acid and protein biosynthesis, coupled with the gradual degradation of RuBisCO and the conversion of the resulting substrate into storage carbohydrate [11,17,61]. It was also reported that sulfur-deprived *C. reinhardtii* accumulated lipids in the form of triacylglycerides along with starch, to serve as the carbon and energy reserves of the cells [74].

Sulfur-deprivation proved to be a successful method for the sustainable production of H_2 by green microalgae. However, the process cannot last forever, as cells eventually run out of starch. Alternation of the stages was successfully applied, whereby normal oxygenic photosynthesis alternated with S-deprivation and H_2-production in repeated cycles [9]. This method is easy to do in the lab, but difficult to apply to scale, where large volumes of growth media are concerned. Several investigators focused efforts on easing the alternation of the stages by enhancing the cell density while at the same time minimizing the volume of the culture media [17,73]. As it will be discussed in Section Cell Immobilization Technologies below, immobilization of microalgal cells on solid matrices provided an alternative solution for an easier switch between sulfur-replete (S+) and sulfur-deprived (S-) stages of the experiment, without any further requirement for labor-intensive and costly harvesting of the cells, such as by centrifugation, prior to nutrient supply change [70,80,81].

Other than S-deprivation, phosphorous [82]; nitrogen [69]; or potassium depleted media [83] were also effective in achieving a sustainable hydrogen photoproduction. The P-deprivation method was reported to be a suitable alternative to the S-deprivation, when a high sulfate concentration would be difficult to avoid from natural growth media, as the case would be with brackish and seawater cultures. However, unlike the S-deprivation method, P-deprivation is not as effective in balancing the activities of photosynthesis and respiration, owing to the high amounts of phosphate reserves within the microalgal cells, translating into a very long time for the onset of H_2-production, a consequence of which is limited yields [82]. The nitrogen-deprivation method was applied by Philipps and coworkers [69], who showed that *C. reinhardtii* responds to this stress by accumulating significant amounts of starch (nearly two-fold higher than in the S-deprived counterparts). However, they detected a delay in the onset of hypoxic conditions in the culture due to a prolonged strong PSII activity, yielding only about 50% of H_2 accumulation compare with their sulfur-deprived counterparts. Papazi et al. [83] reported that potassium depletion is an alternative approach to induce H_2 production, also occurring upon down-regulation of the activity of PSII, establishment of anoxic conditions, and the concomitant up-regulation of PSI and the associated [FeFe]-hydrogenase activity. They supplied wild type *Scenedesmus obliguus* with a glucose supplemented mixotrophic culture medium, as the catabolism of glucose generates endogenous substrate that serves as the source of electrons to the PQ pool, a metabolic process that leads to up-regulation of PSI activity (see Fig. 1a, **pathway #2**). Papazi et al. observed that the potassium depleted media yielded a two-fold increase in H_2-production compared with the control [83]. Another recent study examined the efficacy of ascorbic acid, cysteine, and hydroquinone as electron donors for H_2-production. Only cysteine was shown to induce anaerobic conditions and H_2-production, with yields exceeding those of the S-deprivation approach. Higher yields were attained, under these conditions, upon the consumption of the photosynthetically-generated O_2 in cultures of *Chlamydomonas gloeopara* and *S. obliquus,* without an apparent inhibitory effect on the activity of PSII, which was then available to support greater rates of electron flow in the thylakoid membrane [84].

Cell immobilization technologies

Immobilization of microorganisms within or on the surface of solid matrices has recently received attention due to the advantages that it affords, such as high cell density in relatively small areas and volumes; ease of culture manipulation and cell harvesting; requirement of smaller amounts of growth media; protection of the cells from unfavorable environmental conditions such as rapid pH changes, high salinity or toxic effluents with heavy metal content [85]. Immobilization processes can occur either naturally such as in the form of biofilms, or artificially by the attachment of the cells on solid surfaces or entrapment of the cells within matrices [86]. One of the most common ways for the artificial microalgal cell entrapment is the immobilization of the cells within spherical gel lattices, made either of natural (i.e., agar, alginate, carrageenan) or synthetic polymers (i.e., polyacrylamide, polyvinyl alcohol, and polyurethane) [87]. Various immobilization processes have been used to improve photosynthetic product generation, including H_2, biodiesel, bioethanol, and

photosynthetic pigments production [70,88,89], or simply for wastewater treatment [87,90]. The immobilization matrices used for biohydrogen production by various photosynthetic microorganisms ranged from agar [91] to carrageenan gels [92], porous glass [93], alginate films [94] and clay surfaces [95]. Fig. 2 offers examples of immobilization matrices used for microalgal H_2-production. Optimal immobilization matrices should allow for ease of nutrient delivery to the cells, efficient sunlight penetration through the cell biomass, while also permitting the removal of cellular by-product from the immobilized biomass [89,90].

Drawbacks of the immobilization processes include slower rates of nutrient infusion and product effusion from the biomass and steep sunlight gradients within the immobilized biomass due to the very high cell density [96]. Entrapment of cells within spherical gel-lattices also showed diffusion limitations due to their lower surface to volume ratios compared to thin films. Some of these drawbacks can be addressed by the attachment of cells on solid matrices by natural biofilm formation, on thin films such as alginate films [94], glass surfaces [80], chitosan nanofibers [97], or thin nanoporous latex coatings [98].

An important benefit from the application of immobilization technologies in the field of microalgal H_2-production is the ease of removal of the cells from their growth medium, a feature that allows for shifts in sulfur supply, i.e., from sulfur replete to sulfur deprived media. As stated in Section Metabolic Manipulations, washing of cells in S-deprived media upon centrifugation may be necessary for the effective application of the S-deprivation method [17], and H_2 generation by the cycling of the S-replete and S-depleted stages can be optimized with immobilized cells [9]. Various immobilization processes on solid supports provided evidence of minimizing the energy- and labor-intensive aspects of this process [70,80,99].

Laurinavichene et al. [70,80] employed a non-motile mutant of *C. reinhardtii* attached on glass fiber matrix under S-deprivation conditions, a cell immobilization process that served to facilitate easy switch between the S(+) and S(−) stages of the experiment (see Fig. 2b). They observed a significant extension in the duration of the H_2-production stage that reportedly could reach up to 3 months, when these immobilized cells were used [70]. Once the sulfur-deprived cells were immobilized on porous aluminum-borosilicate glass sheets, the average hydrogen production rates were nearly doubled from 2.5 to 4.3 mL H_2 L^{-1} h^{-1}, and reached a maximum rate of 9.2 mL H_2 L^{-1} h^{-1} [80]. They reported that the system required extra mixing and gas removal support for reaching higher H_2 yields, owing to the inhomogeneous colonization of the cells onto the glass fibers. This apparently resulted in an uneven sunlight penetration and inhomogeneous nutrient delivery, which caused some regions of the immobilization matrix to generate oxygen rather than hydrogen [70].

Hahn et al. [99] used immobilized cells of *C. reinhardtii* on fumed-silica particles to facilitate the shift of the S-stages, i.e., the transition from oxygenic photosynthesis to H_2-production. Although these immobilized cells generated similar yields of hydrogen with their suspended counterparts, the immobilization process provided for an easier shift between the two S-supply modes. *Chlorella* sp. cells were also immobilized in square pieces of agar for an easier shift from oxygenic photosynthesis to S-deprivation and the ensuing anoxic conditions, a cyclic process that yielded multiple rounds of H_2-production [100]. In the presence of photoheterotrophic conditions (exogenous glucose added to the medium), immobilized *Chlorella* cells achieved H_2-production during 10 consecutive cycles, and each cycle generated on the average 470 mL H_2 per liter of reactor volume.

Entrapment of *C. reinhardtii* within Ca^{+2}-alginate thin films resulted in higher cell densities (2000 μg Chl mL^{-1} of immobilization matrix) and better light utilization efficiencies per matrix area than with their suspended counterparts [94] (see Fig. 2a for the experimental set-up). Immobilized cells also achieved higher specific hydrogen production rates (12.5 μmol H_2 mg^{-1} Chl h^{-1}) than cell suspensions, as the alginate polymer offered an effective protection of the [FeFe]-hydrogenase enzyme from O_2 that was present in the culture medium or the nearby microenvironment. The solar energy conversion to H_2 efficiency of the immobilized cultures was increased to about 1%, compared with a value of about 0.2% for their free-suspended counterparts, an improvement attributed to optimized irradiance distribution with a better control of the thickness and the concentration of the cells within the entrapment matrix [94]. Entrapment of *C. reinhardtii* in alginate films was proposed to be a useful method in the screening of transformants for the H_2-production properties of the different lines [86]. In this vein, Das et al. [101] used immobilized *C. reinhardtii* cells on polyester fabric and sodium alginate hydrogel composites for the development of a horizontal and rectangular microfluidic bioreactor, a so-called artificial leaf device, that allowed for ease of nutrient delivery from the bottom channels of the microfluidic bioreactor and H_2 collection from the headspace. They observed significantly higher H_2-production rates for the immobilized cells grown in this bioreactor (0.58 mL H_2 h^{-1} g^{-1} dcw) in comparison with their suspended batch reactor counterparts (0.08 H_2 h^{-1} g^{-1} dcw). This approach also provided an easier cell harvesting step in the course of the experiment, and easier transitions from the S-replete to the S-depleted stage [101].

Separation and removal of oxygen from the cell microenvironment is an essential requirement for H_2-production. This is not always achieved with ease in alginate matrices. Such matrices often retard the diffusion of O_2 from the cells to the surrounding medium. Insufficient release and removal of O_2 that was generated during normal PSII activity, eventually lowered the rate and yield of H_2 production, especially under high light conditions [102]. In order to minimize this problem, mutant *C. reinhardtii* cells with a Truncated Light-harvesting Antenna size (TLA strains, see Section Genetic Engineering Approaches for more details) were immobilized within a Ca^{+2}-alginate film. One of these TLA mutants, the truncated light-harvesting antenna 1 (*tla1*) mutant (Chlamydomonas Culture CC-4169 strain) showed improvement in sunlight penetration, but was still affected by the photo-oxidative inhibition of PSII under high irradiance conditions. In spite of the gas exchange problems and the persistence of photoinhibition, the *tla1* strain produced considerably greater amounts of H_2 compared to the parental (CC-425) control.

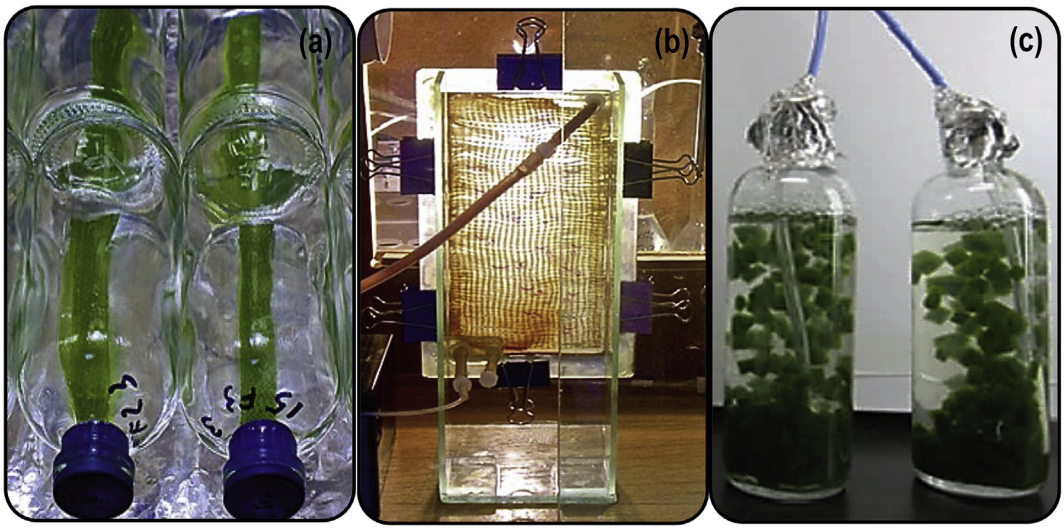

Fig. 2 – Examples of selected experimental setups used for H_2-production by immobilized microalgae. (a) *Chlamydomonas reinhardtii* are entrapped in thin Ca^{+2}-alginate films. A plastic window/insect-screen is used as the template material for the preparation of algae immobilized alginate films, which was later cut in the form of 1 cm wide strips and then placed within gas-tight sterile glass vials (75 mL) containing 10 mL of growth media [94] (image is modified from Tsygankov and Kosourov [86]-reproduced with permission from Wiley); (b) Photosynthetic cells are immobilized on the surface of a glass-fiber matrix, made of Al-borosilicate glass. A similar setup has been used for the immobilization of either *Rhodobacter sphaeroides* photosynthetic bacteria (as seen on the current image) [215], or *C. reinhardtii* microalgal cells [70,80]. The glass plates of the rectangular photobioreactor are held together with binder clips and sealed by applying vacuum grease (image is modified from Tsygankov and Kosourov [86]-reproduced with permission from Wiley); (c) *Chlamydomonas reinhardtii* are entrapped within square pieces of agar gels with a surface area of about 1 cm^2. Optical fibers are applied inside these photobioreactors, to serve as an internal light source [216] (image modified from Rashid et al. (2013) [216]-reproduced with permission from Springer).

Stojkovic et al. [103] encapsulated *C. reinhardtii* cells within TiO_2 nanocrystallites, producing a 2-fold greater yield of H_2-production under S-deprived conditions, as compared with their suspended counterparts. This approach required dark-adaptation of the cells for 48 h before the light-dependent H_2-production phase could be tested. The necessity of a dark-adaptation was attributed to the need to diminish the cell-toxic effect of the catalytic peptides, which were used as a component of the encapsulation material by selectively binding TiO_2 [103].

These promising developments in nanotechnology research constitute advances in the field, afford new insights, confer know-how on the properties of different immobilization matrices, and further suggest innovative designs to better prime the microalgal cells for the functions and productivity intended.

Optimization of photobioreactor conditions

Commercial production of algal biomass has been successfully applied in outdoor ponds, such as natural or raceway ponds, preferred due to the lower cost of such facilities [104]. In terms of biohydrogen, the open-air ponds are not suitable for the generation and collection of a diffusible gaseous product. As such, fully closed photobioreactor systems offer a needed alternative, as they provide a greater process control [4,105].

In general, design criteria for optimally operating a microalgal photobioreactor, for generating and collecting volatile and gaseous products include: (i) sufficient amount of sunlight penetration and delivery with a high proportion of illuminated surface to volume ratio; (ii) adequate mixing; (iii) control of the pH and temperature at about the optimal value for the culture, which typically ranges between pH 7.0–8.5 and temperatures of 20–30 °C; (iv) sufficient CO_2 mass transfer to the cell biomass; (v) efficient removal of O_2 from the reactor; (vi) efficient removal of the H_2 gas or other volatile chemical and recovery of the product; (vii) maintenance of a low H_2 partial pressure in the reactor headspace; (viii) efficient delivery of essential nutrients to the cells; (ix) resistance to contamination of the culture by invading microorganisms; (x) prolonged operational stability; (xi) easy maintenance of the facility; and (xii) construction and operation of the facility on land that does not compete with food crops [3,4,105–108].

The geometry and construction material of the photobioreactor are highly important for reaching the above criteria. Different types of photobioreactors with various shapes have been tested for the cultivation of microalgal cells, including vertical-column; stirred-tank; horizontal/conical/alpha/helical/torus shaped tubular; flat-plate; or alveolar panels [104,106–111]. A high surface-to-volume ratio (S/V) is one of the prerequisites for an efficient photobioreactor [104]. Although vertical column reactors are cost effective, they do not fulfill the requirement of a high S/V ratio; while flat-plate type reactors have the highest S/V ratio needed for an efficient

H_2 production [112] but are more expensive and difficult to maintain. Airlift systems combined with flat plate or helical bioreactors are cited as most efficient designs for photobiological H_2 production and CO_2 sequestration [109].

Provision of sufficient light intensity, penetration, and efficient utilization of absorbed irradiance through the microalgal culture remains one of the major challenges in the design of innovative reactors that would satisfy this requirement. Scenarios that have been tested in this respect include reactors that operate under limited light intensities, such as during overcast or inclement weather. Ogbonna et al. [113] proposed the collection of solar irradiance by Fresnel lenses equipped with light sensors, connected to fiber optics that transmit natural and artificial sunlight to the culture interior in microalgal photobioreactors. Hoshino et al. [114] reported that H_2-production from *C. reinhardtii* under long-wavelength red light (680–700 nm) was greater than under a white light source. They found that such long-wavelength red light results in lower PSII activity, which is below the level of cellular respiration, thus creating anaerobic conditions in the culture, suitable for the physiological expression and function of the {FeFe]-hydrogenase. They also established conditions for interchanging the culture between oxygenic photosynthesis and H_2-production by simply switching the red light on and off [114].

Helical or torus-shaped designs with a central-illumination column offer the advantage of allowing light to reach larger surface areas [4]. In such a study, a vertical torus-shaped photobioreactor was enclosed within a rectangle that had three-walls made of transparent polymethyl-methacrylate. The backside of the vertical torus was placed against a sheet of stainless steel for cooling via an external fan. A bank of fluorescent tubes was placed at the front surface of the reactor together with a reflecting mirror on the rear of the fluorescent light tubes to reflect light in the opposite direction and toward the photobioreactor [111]. Use of conical or pyramid designs was also advised, as these can give the reactors optimum angles of inclination toward the sunlight [4], a design that mimics the conical shape of trees in the natural environment.

Solar energy conversion efficiency (η) is the ratio of the energy stored as hydrogen gas over the photobioreactor's total sunlight energy input. The efficiency (η) depends on several parameters including the sunlight intensity, irradiated area, absorbed versus utilized irradiance, and duration and amount of hydrogen production [8,115]. Although sufficient sunlight is necessary for efficient photosynthesis, excessive adsorption of irradiance beyond the point of saturation of photosynthesis results in the wasteful dissipation of the extra-absorbed irradiance, a pitfall that should be avoided [104,116,117]. Lower then theoretical sunlight energy conversion efficiencies to hydrogen gas were observed at higher light intensities, showing an inverse relationship between the intensity of the incident sunlight and the solar energy conversion efficiency of the photosynthetic system [80,118].

Estimated theoretical maximum for the solar-to-biomass energy conversion efficiency and solar-to-hydrogen energy conversion efficiency of the microalgal photosynthesis was reported to be about 8–10% [117] and 12–14% [117,119], respectively. Kruse and coworkers estimated that a 5% solar-to-hydrogen energy conversion efficiency is needed in microalgae, as the minimum for a cost-effective photobiological H_2-production, provided that the process can be integrated with the production of additional biomass-related high-value product(s) [71]. However, the maximum values of η reached in laboratory experiments under ambient conditions do not exceed 3% for green microalgae, and are substantially lower for other plant systems, showing a requirement for further improvement in this arena [117,119]. A photosynthetic antenna engineering solution to this problem (TLA-technology) has been achieved and briefly reviewed in Section Genetic Engineering Approaches. Other approaches and normalizations have been used in the literature for measuring sunlight energy conversion efficiencies. For instance, based on the theoretically maximum quantum yield of 0.25 (molar ratio of H_2 generated per quanta absorbed) and a wavelength threshold at 680 nm, Boichenko et al. [120] reported that the maximum monochromatic energy conversion efficiency for algae could reach a value of 34%. Greenbaum [47] measured solar energy conversion efficiencies for several microalgal cells based on photosynthetically active radiation (PAR = 400–700 nm only), and reported values for *Scenedesmus* D_3 (=16–23%), *Chlamydomonas moewusii* (=13–24%), *C. reinhardtii* (sup) (=13–21%), and *C. reinhardtii* (UTEX 90) (=6–8%).

Adequate mixing of the culture media is another important prerequisite for achieving more efficient H_2-production, as it is useful for attaining: (i) homogenous distribution of both nutrients and algal biomass within the liquid media, thus facilitating better nutrient uptake and sunlight delivery to the cells, (ii) a lower oxygen concentration in the culture, thus minimizing the sensitivity of the photosystems and of the [FeFe]-hydrogenase by assisting the removal of oxygen from the growth media, (iii) enhanced removal of hydrogen gas produced by the culture for an effective collection of the target product, (iv) minimizing temperature gradients that can be formed within the photobioreactor, and (v) more equitable sunlight distribution to the cells [1,105]. Either mechanical mixing with propellers/impellers or sparging of gases through the reactors is typically used for mixing purposes. For larger scale applications, gaseous sparging is preferred as being more gentle in cell agitation, as this method avoids the generation of large shearing forces that could cause cell damage and death, while avoiding the expense of mechanical stirring. In terms of mechanical mixing, plug-flow type reactors are preferred over continuously stirred reactors (CSTR) as CSTRs tend to have lower product yields and high residence-time distribution of the cells within the reactors [4,121].

Sustained H_2-production for long periods of time is important for commercial applications, and has been achieved through continuous cultivation processes by periodically removing and replacing portions of the culture media with fresh nutrients. As stated in Section Metabolic Manipulations, sulfur-deprivation is currently the most used and critical tool for achieving a sustained H_2-production that can be well supported with the proper photobioreactor design. For example, Fedorov et al. [81] observed a prolonged hydrogen production for more than 4000 h (with a maximum H_2 production rate of 0.58 mL H_2 L^{-1} h^{-1}) by initially growing *C. reinhardtii* CC124 under aerobic and sulfate-limited conditions in a chemostat, and then subjecting the cells to anaerobiosis for the H_2-production stage.

In typical indoor-laboratory experiments, H_2 gas collection is achieved with a simple set-up including an inverted glass burette or volumetric cylinder standing inside a beaker and filled with water. The rate and yield of H_2-production is measured by the rate and amount of water displacement from the inverted burette or volumetric cylinder. The majority of such lab-scale and indoor microalgal H_2-production studies employed *Roux* type (flat) culture bottles with a short optical pathlength for efficient illumination of the culture, as the laboratory photobioreactor [17,122–124]. The *Roux* bottle with its offset neck is easy to plug with a stopper that can be used to insert probes for culture and headspace sampling, nutrient replacement or addition, gas collection, and bubbling with inert gases. Giannelli et al. [122] used a Roux-type reactor for H_2-production in *C. reinhardtii*, after equipping the reactor with multiple impellers for a more homogenous culture mixing. They tested this system under different light conditions, and observed significantly greater light energy conversion efficiencies (up to 1.7%) and H_2-production rates (max rate of 5.7 mL H_2 L^{-1} h^{-1}) in comparison with reactors having a single magnetic stir bar (max rate of 3.7 mL H_2 L^{-1} h^{-1}). Oncel et al. [123] further examined the effect of light intensity and the light:dark cycles on the long term hydrogen production of *C. reinhardtii* in batch cultures.

Scale-up studies under natural sunlight conditions need to overcome additional challenges, as process control should mainly focus on the temperature and diurnal sunlight and also maintain continuous H_2-production, while sustaining an anaerobic environment within the photobioreactors. Scoma et al. [125] used a 50 L horizontal-tubular photobioreactor, made of 10 parallel Pyrex glass tubes, for H_2 production by the S-deprivation method in *C. reinhardtii* CC124, and observed a lower level of hydrogen production compare to experiments conducted indoors in the lab. It was reported that wild type cells with the full complement of their light-harvesting antenna over-absorbed incident sunlight, forcing the photosystems to dissipate most of it the form of heat. This excessive dissipation of photons resulted in photodamage to PSII and photoinhibition of photosynthesis. The adverse photoinhibition was accentuated due to the combined effect of high sunlight intensity and sulfur-deprivation. They recorded an incident solar irradiance intensity reaching a maximum of about 1850 μmol photons m^{-2} s^{-1} in the course of the day, compared to less than 10% of that in the lab, thus explaining the enhanced photoinhibition in these microalgae.

Table 1 provides a comparison of process conditions and maximum hydrogen production rates for sulfur-deprived microalgal cultures from a number of studies, where rate of hydrogen production is given as volume of H_2 per time per either volume of liquid culture (mL H_2 L^{-1} h^{-1}) or per volume of the photobioreactor used (mL H_2 L_{PBR}^{-1} h^{-1}). Although a direct comparison of values is not easy or straightforward between different publications due to variations in multiple process parameters, it can still be observed that when more than one H_2-production parameter was improved, e.g. use of a TLA microalgal mutants with S-deprivation conditions [124], or immobilizing TLA mutants on solid matrices together with the S-deprivation method [80], relatively greater H_2-production rates and yields were achieved.

A challenge encountered with large-scale photobioreactors is the potentially high construction and operating costs that reduce practicality. There is a need to identify and manufacture low-cost durable materials suitable for photobioreactors, while also ensuring a leak-free environment for the H_2 gas generated, specifications that may be difficult to achieve. Thus, recent developments in the field of material science are keys to opening-up new avenues and possibilities in the H_2-production endeavors.

Wastewater compounds as H_2-production feedstock

Use of waste chemicals as feedstock for H_2-production was suggested through a wide range of studies, serving to improve the economics and efficiency of the process. Suitability of the waste source is judged by cost, availability, and biodegradability [126]. The consumption of selected waste compounds for biohydrogen production also brings an advantage from an environmental perspective, as it entails degradation and removal of pollutants. On the other hand, some of the main drawbacks of this approach include generally observed lower H_2-production efficiencies and possible toxic effects from the waste compounds, adversely affecting cell fitness. In order to overcome these difficulties, several approaches were employed, either by gradually acclimating cells to the waste feedstock, or by pretreatment(s) of the feedstock prior to using it for H_2-production [126–129].

Heterotrophic H_2-production by microalgae has received attention, as it holds the potential of waste chemicals biodegradation [127,130,131], generating, in addition to H_2, a variety of end-products such as acetate, formate, lactate, ethanol, glycerol, butanediol, and carbon dioxide from the intracellular anaerobic waste compound catabolism [21,132–134]. Sources of potentially useful wastewater compounds in the literature are olive mill wastewater [127]; urban wastewater [135]; starch wastewater [136]; corn stalk [137]; sweet sorghum stalks [131]; dark fermentation effluent [130]; and landfill-leachate [138]. It was reported that *Chlorella vulgaris* MSU 01, isolated from a pond sediment, could generate H_2 from corn stalk (1–5 g/L) via anaerobic fermentation, and also elevate the butyrate concentrations of the fermentation effluent [137]. *C. reinhardtii* was also reported to be capable of producing H_2 from wastewater samples derived after the pressing stage of fermented sweet sorghum stalks during an "advanced solid state fermentation" process [131]. The major carbon compounds of this wastewater included sucrose, glucose, fructose, acetic acid and butyric acid. H_2-production yield increased by about 8.5-fold compared with the yield of *C. reinhardtii* cells grown in Tris-Acetate-Phosphate (TAP) medium. Hwang and coworkers [130] used *Micractinium reisseri* YSW05, microalgae isolated from the effluent of a municipal wastewater treatment plant, and showed photoheterotrophic H_2-production from acetate- and butyrate-rich dark fermentation effluent. The highest amount of H_2-production (191.2 mL H_2 L^{-1}) was achieved with the undiluted effluent, and continuous illumination conditions resulted in better yields of H_2 than under light/dark cycles.

Faraloni et al. [127] observed enhanced H_2-production, when sulfur-deprived cultures of *C. reinhardtii* were grown in organic acid and sugar rich media supplemented with

Table 1 – Process conditions and maximum hydrogen production rates for sulfur-deprived algal cultures grown in various laboratory photobioreactors. The references are listed in an ascending order based on the reported maximum H_2 production rates.

Microalgal strain	Photobioreactor	Reactor volume (L)	Light intensity (μmol photons. m^{-2} s^{-1})	Initial pH	Temp. (°C)	Maximum H_2 production rate (mL H_2 L^{-1} h^{-1})	Ref.
Platymonas subcordiformis	- Serum bottle - mixing at 150 rpm by an orbital shaker	0.3	160	8.0	25	0.04[a]	[205]
C. reinhardtii CC-124	- Two-stage glass chemostats, - mixing at 350 rpm by vertical glass rods & magnetic bar	1.1*	100 × 2 (PBR1, both sides) 50 × 2 (PBR2, both sides)	7.5 (PBR1) 7.8 (PBR2)	28	0.6[b,c]	[81]
C. reinhardtii CC124	- Roux type flat glass PBR - magnetically mixed at 450 rpm - culture was sparged with CO_2 (3% v/v)	1.1	70 × 2 (both sides) (L:D = 18/6) 70 × 2 (both sides) (continuous illumination)	7.2	27	0.5[a] 1.1[a]	[123]
Chlorella sorokiniana Ce 1.4[c]	- Glass flasks			0.5	120	7.2	30 [206]
C. reinhardtii 137c	- Torus shaped photobioreactor - mixing at 300 rpm by the loop configuration of the reactor and an agitator-shaft	n.r.	110	7.2	25	2.5	[207]
C. reinhardtii CC124	- Roux type flat glass PBR - magnetic stirring			0.9	200	7.0	25
2.8[a,b]	[76]						
C. reinhardtii CC-124	Roux type flat glass PBR (OMW supplemented media)	1.1*	70 × 2 (both sides)	7.2	28	3.3	[127]
C. reinhardtii CC-124	- Roux type flat glass PBR - mixed by stir bars - Roux type flat glass PBR - mixed by multiple impellers	1.1	70 × 2 (both sides) 140 × 2 (both sides) 70 × 2 (both sides) 140 × 2 (both sides)	7.2	28	1.9 3.7 2.9 5.7	[122]
C. reinhardtii CC-1036 (non-motile mutant)	- Glass plates - Argon bubbling - Cells were immobilized on Al-borosilicate glass-fiber matrix	0.2	120	7.0	27–29	9.2[b]	[80]
C. reinhardtii CC-124 *C. reinhardtii* L159I-N230Y (D1 protein mutant)	- Roux type flat glass PBR - Culture was sparged with CO_2 (3% v/v)	1.1	140 × 2 (both sides)	7.2	28	7.5 11.1	[124]

(*): culture volume; n.r: Not reported.

[a] Approximate calculation from the data presented in its corresponding reference.

[b] Rate is based on photobioreactor volume rather than the culture volume (mL L_{PBR}^{-1} h^{-1}).

[c] Average hydrogen production rate based on the overall amount of hydrogen production per culture volume over the total duration of the hydrogen production process.

pretreated olive mill wastewater (OMW). A bio-filtration process using activated carbon and *Azolla caroliniana* plants initially removed the phenolic compounds of the OMW. Under these photochemotrophic conditions, electrons from both water oxidation and the catabolism of endogenous substrate were used for H_2-production. Their S-deprivation control cells, previously cultured only in TAP media, had a H_2 production yield of 100 mL H_2 L^{-1} with an average H_2 production rate of 1.03 mL H_2 L^{-1} h^{-1}, both of which were enhanced in the S-deprived cultures grown in OMW and TAP mixed-media, yielding 150 mL H_2 L^{-1} and 1.29 mL H_2 L^{-1} h^{-1}, respectively [127]. White et al. [138] reported that S-deprived *C. reinhardtii* cells initially cultured in TAP media supplemented with landfill leachate (optimum dilution ratio: 16% v/v) showed about 35% greater hydrogen and biomass production compared with the control cultures pre-grown only in TAP media.

In summary, a significant portion of recent investigations focused on the utilization of compounds from wastewater sources to support microalgal cell growth and H_2-production, followed by a subsequent biomass fermentation in the dark for further biohydrogen production. Such integrated processes are discussed in greater detail in the following section.

Integration of diverse H_2-production bioprocesses

Green microalgae can use a limited range of the solar spectrum, mainly in the 400–700 nm visible region, which contains only about 45% of the energy of solar irradiance reaching the surface of the earth [139]. However, there are microorganisms capable of using longer-than-visible wavelengths of sunlight. For example, anoxygenic photosynthetic bacteria can use the near-infrared region of the solar spectrum, including a wavelength range between 700 and 900 nm [140]. This property invites the possibility of increasing the photochemically-useful range of solar irradiance upon integration of the photosynthesis of microalgal cells with anoxygenic photosynthetic bacteria. Integration of more than one bioprocess is also advantageous for enhancing the yield of H_2 production on the basis of substrate, and of lowering the total energy demands of the process, while creating various synergistic effects among the microorganisms in the integrated system [5,140–143].

Integration of *C. reinhardtii* (green microalgae) with *Rhodospirillum rubrum* (purple anoxygenic photosynthetic bacteria) cultures was first tested as a means of expanding the absorption range of solar irradiance from 400-700 nm to 400–900 nm [140,142]. Other mutual benefits upon integration of the function of these two microorganisms in a unitary culture include potential metabolite exchange between the two cell types, as photosynthetic bacteria can utilize small organic compounds secreted by the microalgae, while the latter could benefit from nitrogen-rich compounds secreted by the N_2-fixing photosynthetic bacteria, under conditions when both systems generate H_2. It was proposed that a dual microalgal-photosynthetic bacterial biosystem could further be integrated with dark fermentative bacteria, a process that could potentially create a more comprehensive biosystem, recycling the biomass generated and further using wastewater as a source of nutrients (Fig. 3) [140]. In such a comprehensive integrated bioprocess, non-photosynthetic and anaerobic fermentations would serve to consume carbohydrates and other organic substrate derived from either spent biomass or wastewater, by metabolizing these compounds into newly-formed microbial biomass, while releasing H_2, CO_2, and small organic molecules [1,6,140]. The dark fermentation process can therefore act in the regeneration of small organic nutrients, as these can be utilized by photosynthetic bacteria for the production of hydrogen under anaerobic conditions (Fig. 3).

Application of co-cultures of various microorganisms in unitary media, or following a biorefinery approach towards subsequent bioprocesses (i.e., combining biophotolysis with photofermentation, biophotolysis with dark fermentation, or dark-fermentation with photofermentation) has been shown to enhance the overall hydrogen production rate, while at the same time affording synergy between the components of the system, such as balancing the pH of the media, enhancing the biodegradability of the substrate source, and removing oxygen from the culture media to prevent inactivation of the photobiological H_2-production process in microalgae [144]. Conversely, microalgal cells have been used to capture and convert the CO_2 produced by the dark and photofermentation processes (Fig. 3). Lo et al. [143] reported that a dark fermentation stage could yield feedstock for the subsequent autotrophic microalgal growth in a process generating CO_2-free hydrogen.

Other than using microalgae for direct phototrophic hydrogen production, the residual algal biomass was considered as a substrate for a subsequent anaerobic fermentation and production of hydrogen, methane, ethanol, or butanol [145–149]. Although residual microalgal biomass can be used in agriculture, such as animal feedstock, it would be more valuable to recover stored energy in biomass via an efficient fermentative H_2- or other biofuel-production process.

Lignocellulosic biomass and plant-related products from corn, sugar cane, canola, sugar beet, can be used as feedstock for the production of fuel and chemicals. However, these plant-derived feedstocks invariably grow on arable agricultural land, which raises the issue of arable land use for food versus fuel [150]. Microalgae are not subject to this pitfall, and algal biomass has been proposed as a suitable alternative to plant biomass and products, offering the advantage of faster rates of growth, the ability to be cultivated in non-arable land, and the ability to use fresh, brackish, or seawater [148,151]. The high content of protein (50% of the biomass), lipids (up to 20% of the biomass), carbohydrate (variable content depending on growth conditions), and the absence of difficult-to-deconstruct lignin make microalgal biomass an attractive candidate for anaerobic digestion processes [147,148].

Table 2 offers a comparative evaluation of integrated biosystems in published research. It is shown that only a few studies focused on the direct photosynthetic generation of hydrogen by mixed cultures. The majority of the research focused on the utilization of microalgal biomass as a substrate for a subsequent fermentation, leading to a second stage H_2 production.

Several obligate anaerobic bacteria (e.g. *Clostridium*), and facultative aerobic bacteria (e.g. *Bacillus*, *Citrobacter*, *Escherichia*, *Enterobacter*) can produce H_2 during dark fermentation

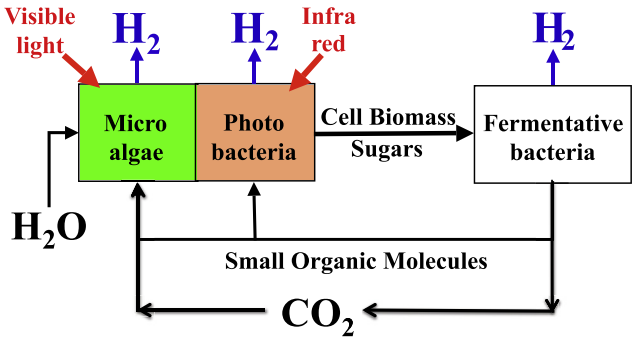

Fig. 3 – Integrated biological H_2-production system. Green algae, cyanobacteria, and photosynthetic bacteria are co-cultured anoxically in a photobioreactor, and dark anaerobic bacteria in a fermentor. Feedstock for the dark anaerobic bacteria is derived from the residual cell biomass/sugars of the algae, cyanobacteria and photosynthetic bacteria. Additional feedstock for the dark anaerobic bacteria is derived from wastewater and lignocellulosic degradation products. Carbon dioxide (CO_2) and the small organic molecule by-products of the dark, anaerobic, bacterial fermentation are subsequently utilized as feedstock for the growth of the microalgae, cyanobacteria and photosynthetic bacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[128]. A drawback of the anaerobic fermentation process is the potential for rapid consumption of H_2 by hydrogen-consuming microorganisms and methanogens. For this reason, the process requires fortification of H_2 production over CH_4 generation, including: (i) inhibition of H_2 consuming microorganisms within mixed microbial consortia either by heat or chemical treatment (e.g. methanogens of the mixed consortia can be suppressed by the addition of inhibitors such as 2-bromoethanesulfonic acid); (ii) setting the pH of the growth media at slightly-acidic values (pH = ~5); (iii) making provision for shorter H_2-retention times in the mixed microbial cultures by frequent removal of the generated hydrogen from the system; and (iv) application of suitable pretreatment of the biomass to increase the fermentative value of the feedstock [128,148,152,153].

Microalgal biomass with a high carbohydrate content is a feedstock of choice, since it yields higher fermentative H_2 production than biomass with a low-carbohydrate content [149]. The efficiency of the algal biomass fermentation depends on the microalgal strain chosen as a feedstock, mostly because of the protein-carbohydrate-lipid ratio of the cells, the presence and composition of the cell wall, and the pretreatment that needs to be applied to the biomass prior to use [150,154,155]. For instance, when no particular pretreatment was applied, cell-wall-lacking *Dunaliella salina* and *C. reinhardtii* with a protein-based cell wall that lacks lignocellulosic components had a higher digestion and cellular deconstruction rates during anaerobic fermentation, compared with microalgal strains that possessed a more rigid cell walls, such as *Chlorella kessleri* and *S. obliquus* cells [150].

Integration of bioprocesses for H_2-production can support self-sustaining cyclic systems, as the fermentative bacteria eventually convert the microalgal biomass into hydrogen and small organic compounds, which can be reverse-utilized by microalgal and photosynthetic bacterial cells for growth and biomass accumulation (Fig. 3) [26]. Mixing the microalgal and photobacterial biomass with organic waste, or applying a pretreatment of the biomass are tested approaches for enhancing the bioavailability and assimilation of residual photosynthetic microorganism biomass. Various pretreatment techniques, including mechanical, chemical, thermal, or combinations thereof, were applied to microalgal biomass in efforts to increase the efficacy of the substrate toward fermentation and H_2 production [154,155]. The possible presence of toxic compounds should also be closely monitored and assessed prior to using the microalgal biomass fermentation residue as a reverse-feedstock for microalgal and photosynthetic bacterial growth. This is a requirement in order to avoid inhibition of cell growth and productivity of the microorganisms in the subsequent stage of such cyclic growth and productivity process (i.e., inhibition of H_2 generation by photosynthetic bacteria in the presence of ammonia). To avoid such pitfalls, post-treatments or adequate dilution of the fermentate has been applied [156].

Genetic engineering approaches

A limited number of microalgal strains only have been used in genetic engineering studies, such as *C. reinhardtii* and *Nannochloropsis gaditana* [157,158]. The limitation is due to the lack of transformation technologies that could be applied to microalgae, and the lack of suitable molecular tools for microalgal genetics. *C. reinhardtii* is unique in this respect, as it can be transformed and has a well-characterized genetics system. It has been widely used as a model microorganism for H_2 production studies. Additionally, its genome has been sequenced, and nuclear, plastid, and mitochondrial genetic transformation protocols are available for this microalga [157,159,160].

Several genetic engineering approaches have been applied to enhance H_2-production, such as truncating the size of light-harvesting antenna of the photosynthetic apparatus to improve efficiency, disrupting the thylakoid-proton gradient to enhance H^+ supply to the [FeFe]-hydrogenase, down-regulating the expression of the chloroplast envelope sulfate transporter(s), and other approaches as discussed below [68,102,124,161,162]. Interesting techniques have been developed for screening for H_2-production mutant strains, including the use of chemochromic sensors for the selection of DNA insertional transformants with aberrant H_2-production properties, or for monitoring the O_2-tolerance of mutant strains on grid agar plates with the colonies in contact with the chemochromic sensor to test for H_2-production under illumination and partial O_2 conditions [19,60,163].

Sulfur-deprivation impairs cellular protein biosynthesis. One of the proteins immediately affected by a slow-down in protein biosynthesis is the D1/32 kD reaction center protein of PSII. Because of the frequently-occurring PSII photo-oxidative damage, and the S-deprivation introduced limitation in *de novo* biosynthesis of D1, the PSII activity is gradually lowered,

along with the attendant rate of water-oxidation and oxygen evolution, reaching a steady state level whereby oxygen production by PSII is lower than that of the cell's own mitochondrial respiration [17]. In sealed cultures, this condition generates a hypoxic environment, which is necessary and sufficient for the cell to activate the genes and commence the process of H_2-production.

However, the S-deprivation method entails a labor-intensive step that is of concern in scale-up H_2-production, as it requires periodic alternation between the sulfur-replete and sulfur-depleted media during cultivation. For this reason, genetic engineering approaches were pursued to identify alternative solutions. Efforts aimed to create conditions for continuous hypoxic hydrogen generation, upon the design of sulfate transporter mutants that are exclusively

Table 2 – Integrated bioprocesses for hydrogen production that include microalgal strains. Please note that the research on integrated bioprocesses is mostly dominated by the fermentation of microalgal biomass by other microorganisms (i.e., 4th raw and onwards).

Microalgal strain	Overall process	Process remarks	Ref.
Chlamydomonas reinhardtii C238	Mixed-cultivation of microalgae with *Rhodospirillum rubrum* NCIB8255 photosynthetic bacteria for a combined H_2 production in a membrane reactor, where each cells were cultured in separate sections. *C. reinhardtii* cells generated acetate, ethanol, formate, glycerol, CO_2 and H_2 as the fermentation products.	- Sustained H_2 production was achieved for one week under the light/dark cycle - Within other fermentation products of *C. reinhardtii*, *R. rubrum* preferably consumed formate for the generation of H_2 through the formate hydrogen-lyase pathway during dark/anaerobic phase - Around **2.5 mol H_2 mol^{-1} glucose** was produced from mixed culture under dark periods of the L/D cycles, which is 5-folds higher than the amount generated by *C. reinhardtii* itself	[142]
Chlorella vulgaris C-C	Dark fermentation process by acid-forming bacterium (*Clostridium butyricum* CGS5) was sequentially followed by a photofermentation stage by *Rhodopseudomonas palutris* WP3-5. These dark→photo fermentation steps were later integrated with an algal stage by directly feeding the generated biogas for the autotrophic microalgal growth process to obtain a CO_2-free hydrogen production	- Of all the carbon sources examined (sucrose, glucose, fructose, xylose); sucrose fed sequential dark and photo fermentation stages yielded the most effective H_2 production; reached a maximum yield of **11.6 mol H_2 mol^{-1} sucrose** and a rate of **673.9 mL H_2 L^{-1} h^{-1}** on a continuous mode for ~ 80 days - The CO_2 generated by the dark and photo fermentation stages (~40% CO_2 in biogas), were almost entirely consumed by the autotrophic microalgal growth, leading to a total algal biomass production of around **2 g L^{-1}** within ~30 days	[143]
Chlamydomonas MGA 161	Microalgal cells were used for the photosynthetic accumulation of starch throughout the day followed by the fermentative production of organic compounds (acetic acid, ethanol, glycerol) during the night, which is then used for the photoproduction of H_2 by photosynthetic bacteria, *Rhodovulum sulfidophilum* W-1S	- The conversion yield of organic compounds from microalgal starch and molar yield of H_2 production by *R. sulfidophilum* was 80–100% and 40% of the theoretical yield, respectively. - PHB accumulation, which is competitive with H_2 production, was repressed by enhancing the nitrogenase activity of *R. sulfidophilum* W-1S through adding sodium succinate to promote H_2 generation	[208]
C. reinhardtii; *Dunaliella tertiolecta*	Photoautotrophically grown algal biomass was fermented using a starch-hydrolyzing lactic acid bacterium (*Lactobacillus amylovorus*), and then fermentate was used for H_2 production by different photosynthetic bacteria (*Rhodobacter sphaeroides* RV; *Rhodobacter capsulata*; *Rhodospirillum rubrum*; *Rhodovulum sulfidophilus*; *Rhodobium marinum*)	- During the first stage, the amount of starch accumulated within *C. reinhardtii and D. tertiolecta* biomass were **0.6 g g^{-1} dcw** and **0.2 g g^{-1} dcw**, respectively - In comparison with the other photosynthetic bacteria, *R. marinum* showed the highest H_2 production abilities, as the lactic acid fermentate of *C. reinhardtii* biomass yielded **7.9 mol H_2 mol^{-1} starch-glucose**, while **6.2 mol H_2 mol^{-1} starch-glucose** was obtained from the *D. tertiolecta* biomass counterpart	[156]

(*continued on next page*)

Table 2 – (continued)

Microalgal strain	Overall process	Process remarks	Ref.
C. reinhardtii; *Dunaliella tertiolecta*	As an initial stage, algal biomass was photoautotrophically grown for the accumulation of starch, followed by its fermentation by *Lactobacillus amylovorous* lactic acid bacterium that converts starch into lactic acid. Then this lactic acid fermentate was used as a substrate source for H_2 production by *Rhodobium marinum* A-501 photosynthetic bacterium. H_2 production by the mixed cultures of *L. amylovorus* and *R. marinum* were also investigated at one single stage.	- Starch reserves of the microalgal biomass was more suitable than the commercial starch for the lactic acid fermentation stage - H_2 production yields by mixed cultures of *L. amylovorus* and *R. marinum* A-501 were **1.6 mmol h^{-1} L^{-1}** and **0.9 mmol h^{-1} L^{-1}** when *D. tertiolecta* and *C. reinhardtii* biomass was used as a substrate source, respectively - One-step H_2 production by mixed cultures of *L. amylovorus* and *R. marinum* had an advantage of sustaining a stable pH value (~7–7.5) throughout the experiment, while two-stage process required extra pH adjustments by NaOH addition to the lactic acid fermentate, which had the disadvantage of inhibiting the H_2 production stage by *R. marinum* due to high salt concentrations	[144]
Nannochloropsis sp.; *Dunaliella tertiolecta*; *Dunaliella salina*; *Chlorella vulgaris*; *Cosmarium* sp.	Pretreated biomass of various microalgal cultures was used as a substrate source for the production of H_2, acetone, ethanol, butanol by anaerobic fermentation of *Clostridium acetobutylicum* bacterial cells, immobilized in polyvinyl alcohol cryogels	- Based on the yields of fermentation products, thermal decomposition (108 °C, at an excess pressure of 0.5 atm for 30 min) was chosen to be the most effective pretreatment technique for microalgal biomass - Maximum H_2 production of **8.5 mmol L^{-1} d^{-1}**, was achieved from *Nannochloropsis* sp. biomass - Immobilized bacterial cells could be recycled and reused for the same purpose at least for five cycles	[155]
Scenedesmus spp.	Pretreatment of lipid-extracted microalgal biomass was utilized as a substrate source for fermentative H_2 production by anaerobic digested sludge. During the fermentation stage; H_2 production was accompanied by acetate, propionate, and butyrate production	- The most effective solubilization of the lipid-extracted microalgal biomass was achieved by thermo-alkaline pretreatment (NaOH & 100 °C for 8 h), which led to higher fermentative H_2 production yields during the next stage (**45.5 mL g^{-1} volatile solids (VS)**; **2.6 mL H_2 h^{-1}**) than their untreated counterparts (**17 mL g^{-1} VS**; **1.2 mL h^{-1}**)	[154]
Chlorella vulgaris; *Dunaliella tertiolecta*	Microalgal biomass mixed with cellulose was used as a substrate source for anaerobic digestion by a thermophilic and cellulolytic microbial consortium (TC60) to generate H_2. In addition to H_2 and CO_2; lactate, acetate, and butyrate were also generated by the anaerobic digestion of TC60	- Both of the microalgal cultures contained satellite heterotrophs, showing anaerobic activities - Anaerobic digestion of *D. tertiolecta* biomass & cellulose mixture by TC60 generated about **8 mmol H_2 g^{-1} VS** as a maximum yield; while it was around **4 mmol H_2 g^{-1} VS** for the *C. vulgaris* biomass & cellulose mixture - *D. tertiolecta* biomass led to higher H_2 results due to the easier lysis of the cells without any rigid cell wall, which allowed a better release of the nutrients from the cell to its environment compare to the *C.vulgaris* cells with cell wall	[209]

Table 2 – (*continued*)

Microalgal strain	Overall process	Process remarks	Ref.
Chlorella sp.	H_2 production from anaerobic fermentation of microalgae (substrate) by anaerobic digested sludge (inoculum). In addition to H_2, acetate and butyrate were given as the other main fermentation products	- The ratio of inoculum to substrate (I/S), and NADH concentration had an inverse correlation with H_2 production, while volatile fatty acids were positively correlated - The maximum H_2 production, **7.1 mL g^{-1} VS**, was obtained from a low I/S ratio of around 0.3	[210]
Chlorella vulgaris ESP6	Microalgal biomass was initially grown under photoautotrophic conditions, and the resulting carbohydrate-rich biomass was pretreated for the hydrolysis of the biomass. The hydrolysate of algal biomass was later used as a substrate source for the fermentative H_2 production by *Clostridium butyricum* CGS5	- Acid pretreatment (1.5% HCl) was preferred to the alkaline/enzymatic pretreatment (NaOH & endo-glucanase) for hydrolyzing the algal biomass, based on its conversion efficiency into reducing sugars as glucose and xylose - Acid-treated, carbohydrate-rich (~57% of dcw) microalgal biomass was efficiently utilized by *C. butyricum* cells for the production of H_2, without any requirement for an extra carbon source - Under optimal conditions, fermentative process yielded a H_2 production of **1.15 mol mol^{-1} reduced sugars**, with a production rate of **246 mL H_2 h^{-1} L^{-1}**	[211]
C. reinhardtii; *Chlorella kessleri*; *Dunaliella salina*; *Euglena gracilis*; *Scenedesmus obliquus*	Utilization of various microalgal strains for biogas generation through anaerobic fermentation by sewage sludge. They also explored a two-step bio-refinery approach by integrating the microalgal hydrogen production as the initial stage (only for *C. reinhardtii*) with the anaerobic fermentation of the residual algal biomass	- The highest biogas production was achieved by the fermentation of *C. reinhardtii* biomass (**587 mL g^{-1} VS**), whereas *S. obliquus* was the least efficient biomass (**287 mL g^{-1} VS**), possibly due to its very rigid and carbohydrate-based cell walls - Drying of microalgal biomass was found to be highly unfavorable for the biogas production, thus using fresh biomass was advised for an efficient fermentation stage - Integration of H_2 production by *C. reinhardtii* with the anaerobic fermentation of the residual biomass led to enhanced biogas production (**~720 mL g^{-1} VS**), mostly due to the enhanced starch and lipid content of the cells during H_2 production stage	[150]
Nannochloropsis sp.	Initial extraction of lipids and pigments from microalgal biomass by supercritical CO_2 extraction process, followed by H_2 generation from the remaining biomass via dark fermentation by *Enterobacter aerogenes*	- Lipid and pigment extracted biomass yielded a maximum H_2 production of **61 mL g^{-1} dcw**, which was slightly higher than the amount obtained by the whole algal biomass (**48 mL g^{-1} dcw**). - Enhanced H_2 production after lipid extraction stage was related with the high concentrations of the fermentable sugar compounds present in the remaining biomass - Inoculation of microalgal biomass at higher concentrations (10 mg dcw L^{-1}) showed almost 45% lower fermentative H_2 production yields than their lower biomass concentration (2 mg dcw L^{-1}) counterparts	[212]

(*continued on next page*)

Table 2 – (continued)

Microalgal strain	Overall process	Process remarks	Ref.
Thalassiosira weissflogii	Marine diatom cells were photosynthetically grown at the initial stage for the accumulation of intracellular carbohydrates, followed by the utilization of their biomass by a thermophilic bacterium (*Thermotoga neapolitana*) for H_2 production through dark fermentation	- Extract of the *T. weissflogii* biomass alone was sufficient as a substrate source for the growth and H_2 production by *T. neapolitana*, without any requirement for extra nutrient supplementations - Fermentative H_2 production from microalgal extract yielded **1.9 mol H_2 mol^{-1} glucose**, and a maximum rate of **36.2 mL L^{-1} h^{-1}**. - After the dark fermentation stage, the residue of microalgal biomass still had a remaining lipid-rich fraction, showing a potential to integrate the process with a bio-diesel production stage	[213]
Chlorella vulgaris; *Dunaliella tertiolecta*	Microalgal biomass was used for either hydrogen or methane production using anaerobic digested sludge	- Both of the algal biomass contained algae-associated satellite bacteria –2-bromoethanesulfonic acid was added to suppress methanogens within H_2 fermenting cultures - Cell-wall-lacking *D. tertiolectra* (**12.6 mL H_2 g^{-1} VS**) had slightly higher H_2 production yields than *C. vulgaris* biomass (**10.8 mL H_2 g^{-1} VS**) - Conversely, CH_4 production was considerably higher for *C. vulgaris* (286 mL CH_4 g^{-1} VS) than *D. tertiolecta* biomass (24 mL CH_4 g^{-1} VS). This was explained by the high salinity of *D. tertiolecta* marine microalgal cells, which would inhibit the methanogens in the anaerobic digested sludge	[148]
S. obliquus YSW15	Microalgal biomass, cultivated in swine wastewater effluent, was initially pretreated by ultrasonication. Then this pretreated biomass was used as a feedstock for H_2 and ethanol production by anaerobic bacteria, which were obtained from the anaerobic digesters of a municipal wastewater treatment plant	- Ultrasonication stage was effective for the lysis of the cell walls of *S. obliquus*, while increasing the bioavailability of the microalgal carbohydrates to fermentative bacteria through increasing the amount of dissolved carbohydrates - The optimal time for the sonication treatment was 15 min, leading to a maximum hydrogen production rate of around **116 mL H_2 h^{-1} L^{-1}**, and a yield **of 1.9 mol H_2 mol^{-1} glucose**.	[153]

endowed with a slow rate/low level of PSII activity, by limiting the sulfate supply to the chloroplast [68]. A *C. reinhardtii* transformant with a diminished chloroplast envelope sulfate permease activity, attained upon down-regulation of the cognizant chloroplast envelope sulfate permease *SulP* gene, was applied in tests of H_2-production, as an alternative to the physical sulfur-deprivation process. This chloroplast envelope enzyme plays a key role in the active transport of sulfate anions from the cytosol to the chloroplast [164]. This approach was promising, as it permitted slow rates of oxygenic photosynthesis and cell growth, when the cultures were exposed to atmospheric air, and H_2-producing photosynthesis, when the cultures were sealed, upon which they became anoxic.

Along these lines, Torzillo et al. [165] employed a *C. reinhardtii* D1 protein mutant in combination with cells having a small chlorophyll antenna size, and further applied the sulfur-deprivation method, leading to higher photosynthetic efficiency and higher hydrogen production. In a different study by the same group, they observed enhanced carbohydrate storage in addition to increased hydrogen generation with the D1 protein mutant of *C. reinhardtii* [124], underscoring the importance of the starch reserves in supporting a sustainable H_2-production. Kruse et al. [77] also showed that the *stm6* mutant strain of *C. reinhardtii*, which is aberrant in cyclic electron transport, had an increased hydrogen production rate under sulfur-deprivation conditions, with a maximum H_2 production rate of 4 mL L^{-1} h^{-1}. This mutant strain had an altered respiratory metabolism causing a significant increase in the amount of cellular starch reserves. A variant of the *stm6* strain, the *C. reinhardtii stm6Glc4* with an inserted *HUP1* hexose symporter gene, encoding a hexose uptake protein, showed H_2-production rates at about 6 mL L^{-1} h^{-1}, thanks to its ability

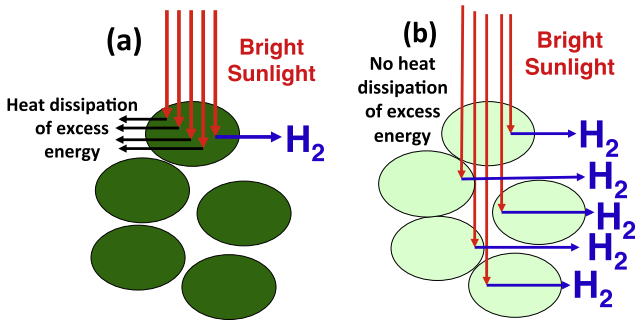

Fig. 4 – Schematic illustration depicts the pathway of incident sunlight absorption and conversion (photochemical utilization or heat dissipation) by high cell-density microalgal cultures. (a) Fully pigmented (dark green) microalgal cells at the surface of the culture over-absorb incoming sunlight (more than can be utilized by photosynthesis), and wastefully dissipate most of it in the form of heat, thereby compromising productivity and hydrogen (H_2) yield. (b) Truncated chlorophyll antenna size microalgae (light green TLA stains) in a high-density mass culture. Individual TLA cells have a diminished probability of absorbing sunlight, thereby permitting greater penetration and a more uniform distribution and utilization of irradiance through the depth of the culture. Such optical properties alleviate heat dissipation of the absorbed sunlight and enhance photosynthetic productivity (H_2) by the culture as a whole. Adapted from Melis 2009 [117]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to uptake and consume organic carbon from the exogenous glucose supply [166].

As an alternative to sulfur-deprivation, Surzycki and co-workers [167] developed an inducible chloroplast gene expression system in *C. reinhardtii* to repress the activity of PS II by combining the coding sequence of the nucleus-encoded Nac2 chloroplast protein to a copper-sensitive cytochrome c6 promoter. Once copper was introduced to the cells suspended in a Cu-depleted medium, PSII activity declined to levels lower than that of respiration leading to anaerobic conditions, as the rate of mitochondrial O_2-consumption exceeded the O_2 evolution rate of PS II, enabling the H_2-production process [167].

The elaborate interplay of electron sources and sinks was recognized to be of import in the process of H_2-production. Studies with *C. reinhardtii* mutants focused on the reversible inactivation of PSII in combination with approaches that would regulate the flux of electrons in the photosynthetic apparatus [63,168]. Regulation of the activity of the Calvin–Benson cycle, as the dominant sink of photosynthetic electrons, or hindering the activity of RuBisCO as the main enzyme of the Calvin–Benson cycle, have been proposed for this purpose [4,26]. Hemschemeier et al. [63] observed hydrogen production in S-replete growth media by the RuBisCO-deficient *C. reinhardtii* strain CC-2803. The latter shows a lower-than-wild type PSII activity, whereby the cells attain anoxic conditions followed by H_2 generation in sealed cultures, albeit in the presence of sulfur-replete media. Absence or inactivation of RuBisCO was shown to be an effective approach via which to direct photosynthetic electrons to the [FeFe]-hydrogenase instead of to the Calvin–Benson cycle, resulting in a nutrient-independent hydrogen production process [26]. Rühle et al. [168] isolated a *C. reinhardtii* strain termed **a**ttenuated **p**hotosynthesis/**r**espiration 1 (*apr1*), having a low photosynthesis-to-respiration ratio that generated hydrogen, when the Calvin–Benson cycle was inhibited by the addition of glycolaldehyde. Inhibition of the Calvin–Benson cycle was necessary to let the hydrogenase perform as the alternative electron sink, otherwise photosynthetic electrons were preferentially consumed in the Calvin–Benson cycle for triose ~ P synthesis [168].

Wu et al. [169] observed a 4-fold higher H_2 production after the expression of codon-optimized *hemH* (ferrochelatase gene from *Bradyrhizobium japonicum*) and *lba* (leghemoglobin gene from *Glycine max*) in the chloroplast of *C. reinhardtii*. Leghemoglobin is an O_2 scavenging protein, which binds oxygen within the root-nodules of leguminous plants as they perform nitrogen fixation. The improvement in H_2 production was underscored by the higher transcript levels of *hydA1* and *hydA2* genes than encode the [FeFe]-hydrogenase, attributed to lower levels of internal oxygen concentrations attained thanks to the expression of the *hemH* and *lba* genes [169].

A large light-harvesting chlorophyll antenna size in the photosynthetic apparatus, containing up to 250 chlorophyll *a and b* molecules for PSII and 210 chlorophyll *a and b* molecules for PSI, is an evolutionary trait that confers a survival advantage to photosynthetic organisms [1]. This is because in most ecotypes occupied by photosynthetic organisms, sunlight is the prime ingredient for growth, and one that is often in limited supply. Cyanobacteria, microalgae, and all plants need to compete for the absorption of sunlight, and this is achieved by having a large light-harvesting chlorophyll antenna size for efficient light absorption. On the other hand, a large antenna can cause excessive-absorption under direct and bright sunlight, where a large portion of excess absorbed photons would be dissipated as fluorescence or heat. This phenomenon is pronounced in high density cultures under bright sunlight conditions (Fig. 4a), and it is counterproductive as it causes losses in sunlight utilization and culture productivity, when the commercial application seeks exactly the opposite [109,117]. Under bright sunlight, *C. reinhardtii* dissipate more than 80% of absorbed photons (Fig. 4a). The dissipation process has the capacity to cause photodamage to PSII, which further lowers the culture's sunlight energy conversion efficiency [170]. Diminishing the pigment content of the photosystems was proposed to be an approach that would alleviate the over-absorption and wasteful dissipation of energy, and increase the overall productivity and H_2-production by enhancing the penetration and productivity of sunlight through the high-density culture (Fig. 4b).

An inverse relationship exists between the size of the light-harvesting antenna and sunlight energy conversion efficiencies in photosynthesis, which prompted several groups to undertake efforts leading to the regulation of the light-harvesting chlorophyll antenna size in the photosynthetic apparatus [102,116,118,162,171–174]. It was already known that a **T**runcated **L**ight-harvesting **A**ntenna (TLA) in the

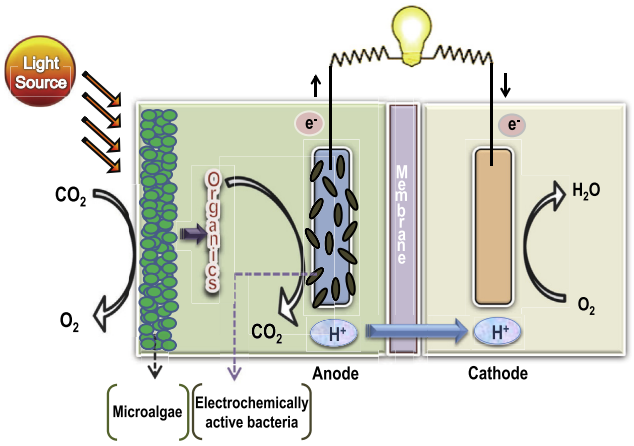

Fig. 5 – Outline of a microbial solar cell using mixed microorganisms. (i) Microalgal photosynthesis generates organic matter from CO_2 and sunlight; (ii) Transfer of organic metabolites from the microalgae towards the anode section; (iii) Oxidation of the organics by electrochemically active bacteria at the anode surface; (iv) Cathodic reduction of O_2 to water. The image is adapted from Strik et al. [193], and Wang and Ren [188].

photosynthetic apparatus will minimize excessive absorption and wasteful dissipation of sunlight (Fig. 4b), thereby leading to higher productivities on a per chlorophyll basis in green and other microalgal and cyanobacterial cultures [118,175]. Tetali et al. [172] and Mitra and Melis [176] employed DNA insertional mutagenesis and screening, upon which they isolated strains of *C. reinhardtii* with smaller PSII and PSI antenna size (TLA-strains). They further cloned and analyzed the function of the genes that lead to this TLA property. Polle et al. [118] showed that the ***tla1*** mutant of *C. reinhardtii* with truncated light-harvesting antenna size (50% of the wild type control) had a 2-fold higher rates and yields of both oxygen evolution and biomass accumulation compare to the wild type, when grown in dense cultures under direct sunlight conditions. In further work, Kirst et al. [173] observed a lighter-green phenotype with lower chlorophyll content per cell, and higher Chl *a*/Chl *b* ratio in the ***tla2*** and ***tla3*** DNA insertional mutagenesis strains of *C. reinhardtii*, compared to their wild-type counterparts. The ***tla2*** mutant was shown to have a deletion of the *CpFTSY* gene [173], while the ***tla3*** mutant had a deletion in the *CpSRP43* gene [177]. The corresponding encoded proteins (CpFTSY and CpSRP43) are components of the so-called "signal recognition particle" which, in chloroplasts, is responsible for the assembly of the peripheral Chl *a/b* light harvesting proteins in the thylakoid membrane [178]. Their work showed that deletion of either of the two proteins had an adverse effect in the ability of the *C. reinhardtii* chloroplast to assemble the peripheral Chl *a/b* light harvesting antenna of the photosystems, resulting in a small (truncated) light-harvesting antenna. It was concluded that interference with the CpSRP mechanism is a useful approach for the generation of ***TLA*** mutants.

TLA mutants were also isolated by other genetic methods, such as overexpression of the *nab1* RNA binding protein [179], or down regulation in the expression of the genes encoding the light harvesting complex proteins by RNA interference (RNAi) [180]. Immobilization of TLA mutants on solid substrate was shown to improve the overall solar energy conversion efficiency and H_2-production of the system. Kosourov et al. [102], working with cells immobilized in alginate films, showed an 8-fold greater H_2-production with a *TLA* mutant of *C. reinhardtii* under sulfur-deprivation and sufficient irradiance conditions, as compared to the wild type.

In yet another approach toward improvements in H_2-production, it was proposed that release of the cross-thylakoid proton gradient would free protons (H^+) that, in the process of electron transport, are sequestered in the thylakoid lumen, and make them available to the chloroplast stroma, where the [FeFe]-hydrogenase functions. Such regulated "uncoupling" of the cross-thylakoid proton gradient from the lateral electron transport process would have the extra benefit of accelerating electron transport from PSII through the cytochrome b_6-f complex (Cyt b_6-f) and, therefore, toward more H_2-production [4,181]. For this reason, research aimed to dissipate thylakoid-proton gradients by uncoupling it with carbonyl cyanide p-trifluoro-methoxyphenylhydrazone (FCCP) [181,182]. It was also observed that *C. reinhardtii* mutants lacking the ferredoxin-plastoquinone oxidoreductase (FQR) had an enhanced rate of H_2-production under sulfur-deprivation conditions, due to the absence of competition between hydrogenase and FQR for electrons from the reduced ferredoxin pool [181]. Lastly, heterologous host strains such as *Escherichia coli*, *Shewanella oneidensis*, and *Clostridium acetobutylicum*, were used to study the regulation of expression of the [FeFe] hydrogenase, in an effort to gain insight into the [FeFe]-hydrogenase expression, folding, and function, in the hope that such understanding would lead to improved yields of H_2 production [48,183,184].

Innovative interdisciplinary approaches

Bio-inspired technologies have the potential to fast-forward renewable H_2-production. In the recent literature, artificial processes have already used various components of the natural photosynthetic H_2-production. Some of these R&D efforts include hybridization of the hydrogenase enzyme with Ru dye-synthesized TiO_2 nanoparticles to generate H_2 with a sustained electrocatalytic activity [185]. Immobilization of the [FeFe]-hydrogenase and PSII complexes on separate electrodes served to disconnect and separate the O_2 production from the H_2 generation step [7]. Tethering Pt nanoparticles to the reducing side of PSI complexes resulted in the generation of H_2 in a process that bypasses the [FeFe]-hydrogenase [186]. Finally, proton reduction catalysts were designed in a biomimetic approach based on the structure of the [FeFe]-hydrogenase active site [187].

Microalgal cells were also applied in a variety of emerging hybrid technologies such as microbial electrochemical systems that can transform the stored chemical energy of biodegradable substrates into electrical current or chemicals such as hydrogen [188,189]. Microbial electrochemical technologies were used to support growth of microalgae by providing efficient recycling of CO_2 into algal biomass (Fig. 5, see also [190,191]). Strik et al. [192] used algal cells and

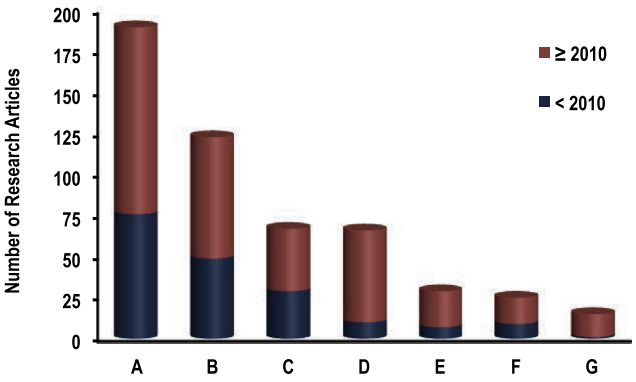

Fig. 6 – Number of published research articles on microalgal hydrogen production with emphasis on (A) metabolic alterations (Section Metabolic Manipulations); (B) genetic engineering approaches (Section Genetic Engineering Approaches); (C) photobioreactor optimization (Section Optimization of Photobioreactor Conditions); (D) integration of diverse biosystems (Section Integration of Diverse H_2-production Bioprocesses); (E) interdisciplinary approaches (Section Innovative Interdisciplinary Approaches); (F) cell immobilization studies (Section Cell Immobilization Technologies); (G) utilization of waste resources as feedstock (Section Wastewater Compounds as H_2-production Feedstock). The A-to-G columns are divided into two time periods, with papers published before year 2010 (<2010) and after 2010 through the present time (≥2010). Source of the results: Scopus & Google Scholar; Latest update: March 26, 2016. Recurrence in different categories and modeling papers were counted in this analysis, while review papers were not included.

electrochemically active bacteria as electron generators and donors for the anode-oxidation, while potassium ferricyanide was used as an electron acceptor for the cathode-reduction to generate electricity by a photosynthetic microbial fuel cell. The same group provided an overview of the basic stages of a microbial solar cell with mixed microorganisms. The process is initiated upon the absorption of irradiance by the microalgal photosynthesis stage, which generates organic metabolites. This is followed by the transfer of the organic effluent towards the anode section; and the anodic oxidation of the organics by electrochemically active bacteria, while oxygen is reduced to water at the cathode section (Fig. 5) [193]. Along these lines, Rosenbaum et al. [194] investigated the concept of direct electricity generation from microalgal cells by the *in situ* oxidation of biohydrogen gas, which was produced by S-deprived *C. reinhardtii*, using a conductive polymer-coated platinum electrode.

Direct integration of microalgal H_2-production with fuel cells was reported as a concept for the direct conversion of H_2 into electricity [195,196]. Chader et al. [195] reported that hydrogen produced by S-deprived *Chlorella sorokiniana* cells was coupled directly with a Proton Exchange Membrane Fuel Cell (PEMFC), which generated electricity with a similar conversion efficiency as a counterpart operated with chemically pure hydrogen. Their system was based on a discontinuous gas injection mode and generated a maximum voltage output ranging between 20.1 and 89.1 mV [195]. This output was comparatively higher than the continuously generated voltage outputs (around 10 mV maximum) of a system that coupled a PEMFC with microalgal H_2 production by *S. obliquus* in a continuous gas flow tubular photobioreactor [196]. Oncel and Sukan [197] reported on a relatively efficient PEM fuel cell application for the direct utilization of H_2 generated by S-deprived *C. reinhardtii*, which could reach a maximum open-circuit voltage of 800 mV only for a short time interval, whereas about 140 mV could be maintained for much longer durations under an open-exhaust valve and dead-end configurations, respectively. Based on 100 m^3 volume of a microalgal culture, their system yielded an average power of about 72 W for 50 h under a 10 Ω load at a stable voltage of 110 mV [197], which compared favorably with the theoretical maximum of 240 W for 100 h of a PEM fuel cell operated on the basis of the same volume of a *C. reinhardtii* culture [198]. Oughli et al. [199] used a viologen-modified hydrogel for the protection of the *C. reinhardtii* [FeFe]-hydrogenase from O_2 by quenching it before reaching the hydrogenase. This approach was successfully utilized in a single-compartment fuel cell with an inlet of a H_2 and O_2 gas mixture. It generated a maximum power density of 225 mW based on 1 cm^2 of anode area with an open-circuit voltages of 1080 mV.

Introduction of nanotechnology to both microalgae and biohydrogen research has the potential to improve the overall process by creating alternative and improved solutions for cell harvesting, biomass pretreatment, enzyme immobilization, reactor design, hydrogen storage, and end-product applications. Other than its utilization as a fuel source, biogenic hydrogen has the potential to be used directly as an cost-effective reducing agent for chemical applications such as the reduction of palladium [200] and gold ions [201] into their corresponding nanoparticles. Using metal nanoparticles to target desirable wavelengths of irradiance toward the microalgal cell culture also offers the promise of enhancing microalgal growth [202–204]. Eroglu et al. [202] reported that localized surface plasmon resonances (LSPR) of spheroidal silver nanoparticles and gold nanorods could be successfully used to filter certain wavelengths of sunlight, which enhanced photosynthetic pigment formation in *C. vulgaris* cells after placing these metal nanoparticle solutions around the microalgal culture flasks. The maximum efficiency of pigment formation was achieved upon using a dual-mixture of silver nanoparticle and gold nanorod solutions, showing dually scattered light intensities in both violet-blue and red regions of the spectrum [202]. Such a bioreactor system affords a shielding to limit direct irradiance of the culture by the full solar spectrum, while targeting preferred specific wavelengths towards the system by applying a surface plasmon backscattering effect of selected nanoparticle solutions located within a separate container around the culture flasks. Giannelli and Torzillo [204] reported on *C. reinhardtii* grown in a novel tubular photobioreactor, where the tubes were immersed in a suspension of silica nanoparticles. Silica nanoparticles were used to scatter the light delivered from an artificial light source in order to achieve a more homogenous light distribution and light dilution through the culture. They observed a higher chlorophyll content and hydrogen production from the silica nanoparticle submerged photobioreactor,

as compared with the corresponding control [204]. These nanoparticle-assisted approaches have the potential to avoid the ineffective dissipation of the absorbed irradiance that would otherwise lower sunlight energy conversion efficiencies and H_2-production by the microalgal systems [80,104,116–118].

Outlook of the microalgal hydrogen production research

The ability of microalgae to metabolize hydrogen was initially reported by Hans Gaffron three-quarters of a century ago [20,21]. After a long hiatus with limited or no progress in the field, research interests in the concept of photobiological H_2-production gained significant acceleration after the turn of the millennium, concomitant with pioneering breakthroughs and the availability of diverse tools that could be applied in this field. Fig. 6 shows the number of published research articles on microalgal hydrogen production, divided into two time-periods, i.e., before year 2010 (<2010) and after 2010 through the present time (≥2010). Literature was also divided into 7 main categories (A-G) of research tools that proved useful at enhancing H_2 production efficiency. It should be noted that the sum of the two components in each column does not necessarily reflect the total number of papers on algal hydrogen production, since manuscripts that used more than one tool were counted more than once. In order to have a more specific target, we only considered publications that have a direct relationship with hydrogen production. Review papers were not counted, whereas modeling papers were taken into consideration.

Results in Fig. 6 show that metabolic alterations (**column A**) and genetic engineering approaches (**column B**) generated by far the most research publications, with a total number of 190 (≥2010: 114, column A) and 123 papers (≥2010: 74, column B), respectively, reflecting efforts to improve rate and yield of microalgal H_2-production. A substantial number of investigators used a combination of these two approaches. Therefore, 68 papers were counted in both columns A and B. Attempts at metabolic alterations were predominantly concentrated on S-deprivation studies, as this breakthrough method was applied in 154 papers out of a total of 190, which is ~81% of the papers referred to in column A. Next best are studies based on photobioreactor design (Fig. 6, **column C**) with a total number of 67 papers (≥2010: 38). In that section, papers with novel reactor designs and papers that investigated the effect of various experimental conditions (such as irradiance, reactor geometry, and mixing) were summed up, instead of considering every-single study that employed a photobioreactor for H_2 production. It can be clearly seen from Fig. 6, **column D**, that trends of the past five years are in the direction of biorefinery concept applications upon integrating diverse biosystems with 66 papers (≥2010: 56, column D). Interdisciplinary approaches comprising various fields like nanotechnology and electrochemistry (**column E**) accounted for 29 papers (≥2010: 22). Immobilization of cells (**column F**) contributed 25 papers (≥2010: 16), and utilization of wastewater resources as the renewable feedstock (**column G**) with 15 papers (≥2010: 14). Given the development of these new research areas and interdisciplinary R&D approaches, one can reasonably well anticipate continued progress in biohydrogen research. Evolution of this research field shows a clear shift from the "basic science" perspective to more "applied research" with efforts to adapt this technology to daily domestic and industrial applications upon improving the efficiency and yield of the process, as well as the practicality and sustainability of the overall operation.

Conclusions

Development and application of biological hydrogen production to advanced bioenergy strategies and commercialization is a long-term objective. Nevertheless, the product (H_2) has distinct advantages over alternative carriers of energy, notably energy density (145 MJ kg^{-1}), which is 2.6-fold greater than that of methane (55 MJ kg^{-1}). Procedurally, photobiological generation of H_2 affords the benefit of spontaneous product separation from the microalgal biomass, a feature that substantially improves the economics of the process. Thanks to the effort of numerous dedicated investigators, several innovative developments in this field, ranging from cell harvesting to photobioreactor design, metabolic alterations via nutrient-deprivation, and genetic modifications are poised to move the biohydrogen production and associated microalgal research field forward. Currently, there is a growing interest in new research areas including integrated biosystems for H_2 production in mixed-cultures, or direct utilization of residual algal biomass as a feedstock for H_2 production by a consecutive anaerobic digestion stage; interdisciplinary approaches such as nanotechnology and electrochemistry; immobilization of cells on novel materials; and utilization of wastewater resources as a convenient and renewable source of nutrients for H_2 production. Combining the advances in the aforementioned fields promises to increase the yield of microalgal H_2-production, to the point where it will reach commercially viable levels of a carbon-free energy carrier, derived from abundant natural resources, such as sunlight and water. Application of integrated bioprocesses encompassing wastewater treatment can also increase the viability of the microalgal process by contributing to the removal of contaminants from the environment, while at the same time improving the economics and practicality of biohydrogen production.

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