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ARTICLE

Recent advances in microalgae-based biohydrogen production: A review of innovative strategies, pretreatment, genetic engineering, and bioelectrochemical technologies

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ABSTRACT

Microalgae are promising platforms for sustainable biohydrogen (H₂) production, which couples both the production of H₂ and CO₂ sequestration and wastewater treatment. Limiting oxygen sensitivity of [FeFe]-hydrogenases and the limitations of reactors are the main implementation issues. This review discusses advances in the biohydrogen production from green microalgae and cyanobacteria. We discuss the photobiological H₂ pathway, dark fermentative H₂ from algal biomass and bioelectrochemical H₂-hybrids. Process strategy involving nutrient-starvation methods, algae and bacteria consortia as well as photobioreactor designs, is analyzed for better H₂ yield. We review pretreatment approaches, i.e., mechanical, thermal, chemical, enzymatic, and bio-nanoparticle approaches that increase fermentative hydrogen production by solubilising solids. Genetic engineering advances are also featured, which include hydrogenase engineering, pathway modifications, and starch accumulation in species. We discuss some bio-electrochemical systems with a particular focus on the microalgae integration and microbial electrodes cells coupled with photosynthesis for hydrogen evolution. Pilot demonstrations are evaluated, where research priorities are genetic modifications, biorefinery concepts, reactor engineering and techno-economic analysis.

1. Introduction

1.1 Background and rationale

Hydrogen is increasingly considered to be a central plank of future low-carbon energy systems, though most of the hydrogen produced worldwide is still dependent on fossil-based production

processes, which rely on steam methane reforming (SMR) or coal gasification processes with significant carbon dioxide (CO₂) emissions (Chu et al., 2021; Sharma et al., 2023). Green hydrogen produced by water electrolysis using renewable electricity is still rather costly, with associated electrolyser and renewable capacity limitations. In this regard, biological hydrogen ("biohydrogen") presents a complementary route, which is able to couple the

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production of hydrogen with the capture of CO₂, wastewater treatment, and biomass valorisation (Balakrishnan et al., 2023).

Microalgae, specifically unicellular green microalgae, have become promising systems for the production of biohydrogen because of the following features: (i) microalgae grow rapidly with high areal productivity (Tsai et al., 2012), (ii) microalgae can achieve even better CO₂ fixation relative to terrestrial crops (Tsai et al., 2023), (iii) microalgae can be grown on non-arable land using saline or wastewater (Unpaprom et al., 2017) and (iv) microalgae do not contain lignin, making downstream processing easy (Saengsawang et al., 2020). Over the past five years, several reviews have discussed microalgae as 'third-generation' biofuel feedstock and a potential 'future supply house' of biohydrogen, especially in conjunction with biorefinery-based ideas.

1.2 Microalgal biohydrogen production pathways

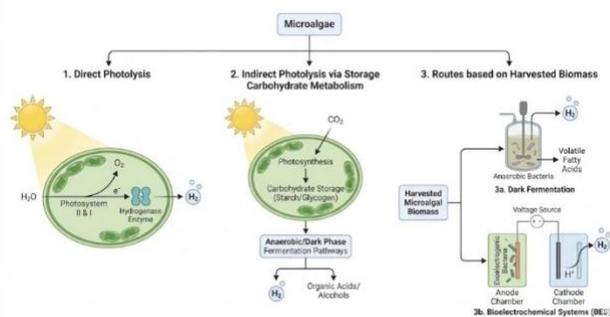


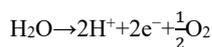
Figure 1. Overview of microalgal hydrogen production pathways.

This schematic (Figure 1) focuses on three major paths in which microalgae can play a role in the generation of hydrogen (H₂):

- (i) direct photolysis,
- (ii) indirect photolysis, and
- (iii) routes based on even harvested biomass, e.g., dark fermentation and bioelectrochemical systems.

(i). Direct photolysis (Light-driven pathway)

In the direct photolysis pathway, photosynthetic microalgae convert incident solar energy directly into chemical energy stored as molecular hydrogen. Light is absorbed predominantly by Photosystem II (PSII), where water splitting occurs according to the reaction:



The liberated electrons of water are passed on through the photosynthetic electron transport chain to Photosystem I (PSI), which is followed by ferredoxin. During anaerobic conditions, the reduced ferredoxin donates electrons to [FeFe]-hydrogenase that reduces protons (H⁺), thus generating H₂. This pathway is conceptually appealing since it is the most direct interaction of sunlight, water, and algal metabolism to hydrogen evolution. Nevertheless, direct photolysis is self-inflicted. Molecular oxygen generated at PSII is an effective inhibitor of [FeFe]-hydrogenase; the inactivation of the enzyme happens at oxygen concentrations as low as 2%. Consequently, Hydrogenase action is restricted to

short intervals of anaerobic or micro-oxic cultures, and it is technically difficult to retain such conditions in actively photosynthesizing cultures. Theoretically, direct photolysis has an idealised performance of solar-to-hydrogen (STH) efficiency of about 10-13%. In practice, microalgal strains of the wild-type tend to give STH efficiencies that are much less than 1% mainly due to O₂ inhibition and alternative metabolic sinks. In line with this, the volumetric hydrogen production rates that have been reported are relatively low, typically ranging between 0.015-1.0 mmol H₂ L⁻¹ h⁻¹ when operating in a laboratory.

(ii) Indirect Photolysis (Two-Stage process)

To overcome the oxygen sensitivity of hydrogenase, the indirect photolysis was invented as a method of separating the space or time between the oxygen evolution and hydrogen generation process. It takes place in two phases. Phase I (aerobic growth) consists of the growth of the microalgae under normal photosynthetic conditions and with adequate nutrients. At this point, cells are concerned with growth and energy stores, primarily in the form of storage carbohydrates (e.g., starch in green algal chloroplasts, and glycogen in cyanobacteria) or energy stores (e.g., ATP). Oxygen evolution occurs just like it does in normal photosynthesis, but hydrogenase is not an active major sink yet.

Phase II (anaerobic or nutrient-stressed conditions). This is the transfer of culture to conditions that prevent the evolution of oxygen and activate hydrogenase. This is usually done by the deprivation of sulfur, the variation of oxygen and/or darkness. In these circumstances, the stored carbohydrates are catabolised by the cell via the glycolysis pathway in which ATP and reduced cofactors are produced (e.g., NADH). The electrons obtained in these reserves are then channelled to hydrogenase, which reduces protons into H₂. By so doing, the oxygen-evolving step and the hydrogen-producing step are time-decoupled.

The amount of stored carbohydrate is important in the determination of the theoretical yield of hydrogen in the process of indirect photolysis. Provided that carbohydrate catabolism is coupled to the production of acetate, as many as 2 mol H₂ per mol of glucose equivalent could theoretically be produced. Techno-economically, indirect photolysis cost has been estimated to be around 1.42 USD kg⁻¹H₂, which may be even more favourable as compared to 2.13-7.24 USD kg⁻¹H₂ effective range for direct photolysis in part because of reusability of stored biomass for subsequent H₂ production cycles, ability to operate the process semi-continuously.

(iii). Routes based on harvested biomass

In contrast to photobiological routes based on live actively photosynthesising cells, biomass-based routes use microalgae as a renewable substrate for the microbial or electrochemical conversion. Microalgae are first grown to collect biomass, which is harvested and processed in other reactors. Two major classes are recognised: dark fermentation and bioelectrochemical systems, including microbial electrolysis cells (MEC).

Dark Fermentation: In dark fermentative hydrogen production, harvested microalgal biomass (which is often subjected to physical, thermal, or chemical pretreatment to improve sugar liberation) is

inoculated with fermentative anaerobic bacteria, including species of *Clostridium*. These bacteria break down carbohydrates and digest them to volatile fatty acids (VFAs), mainly acetate and butyrate, and hydrogen gas as a by-product.

Two simplified stoichiometric routes illustrate the theoretical yield limits:

- Acetate pathway

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

Maximum theoretical yield: 4 mol H₂ per mol glucose.

- Butyrate pathway
 Theoretical maximum hydrogen yield: 2 mol H₂ per mol glucose.

In practice, the mixed acid fermentation yields are limited by the metabolic control, the hydrogen partial pressure, and the substrate composition. For pretreatment of microalgal biomass, for instance, practical H₂ yields for the microalgal biomass can often range from 200-500 mL H₂ per g VS (volatile solids). Importantly, the production rates are much higher than production rates seen for photolysis-based systems, frequently approaching 10-60 mmol H₂ L⁻¹h⁻¹, which makes dark fermentation attractive as a means of high-throughput conversion of algal biomass to hydrogen.

Bioelectrochemical Systems (BES, including MEC): Bioelectrochemical systems, especially microbial electrodiagnosis cells (MECs), maximize the conversion of energy of algal biomass and fermentation residues. In MECs, the exoelectrogenic bacteria located on the anode oxidise organic matter (such as sugars from algae or VFAs from dark fermentation), transferring electrons to the electrode. These electrons flow through an external circuit to the cathode, where, in the presence of a small externally applied voltage, they reduce protons to produce H₂.

This arrangement has several advantages. MECs can use residual organic acids from dark fermentation, in effect "polishing" fermentation effluents, and extract further energy (which would otherwise be lost). Electrical input requirements are not high - anyway, only around 0.6-1.0 V, significantly lower than the 1.8-2.0 V required for conventional water electrolysis. Under

optimised conditions, electrical energy recovery efficiencies are reported to be greater than 90% and volumetric rates of H₂ production on the order of 1-2m³H₂m⁻³ reactor d⁻¹ have been reported. These attributes make BES's and especially MECs a promising complement to dark fermentation in the integrated biorefineries using algae for the production of hydrogen. These different pathways differ according to their major energy source (sunlight vs. stored biomass vs. biomass + electricity), dependence on the metabolism of live algae, and their potential yields and production of further hydrogen rate and yield.

2. Microalgal Biohydrogen Production: Mechanisms and Challenges

Microalgae-Based Hydrogen Pathways: Green microalgae and cyanobacteria are capable of producing hydrogen via photobiological processes (direct and indirect photolysis of water through the process of photosynthesis) and dark fermentative metabolism. In direct photolysis, the alga's Photosystem II breaks apart water to supply electrons to hydrogenase, but in the process O₂ is produced, and this inactivates the oxygen-sensitive hydrogenase. Indirect photolysis dissociates O₂ evolution by deriving electrons from cellular storage such as starch under nutrient-deprived conditions and the resulting electrons feed Photosystem I and hydrogenase in the absence of simultaneous also O₂ production. In addition, under dark anaerobic conditions, some algae are capable of fermenting stored substrates (starch or pyruvate) and releasing H₂ through some enzymes such as pyruvate:ferredoxin oxidoreductase coupled to hydrogenase. Each route there is, however, limitations of intrinsic capacity: Oxygen co-production, low solar-to-H₂ energy conversion efficiency, and hydrogenase with in vivo activities transient under normal operation. Indeed, the model H₂-producing alga, *Chlamydomonas*, only produces hydrogen under anaerobic stress (e.g., sulfur deprivation), and then for short periods of time before it is stopped by oxygen or other factors.

Table 1. Microalgal hydrogen pathways and key limitations

Pathway / Item	Mechanism	Main Limitation for H ₂ Production
Direct photolysis	PSII splits water; electrons via photosynthesis to hydrogenase → H ₂ in live cells.	O ₂ from PSII inactivates [FeFe]-hydrogenase; very low STH.
Indirect photolysis	Two-stage: grow and store starch → stress/anaerobiosis; stored carbon drives H ₂ .	Needs tight nutrient/anaerobic control; hydrogenase still O ₂ -sensitive.
Dark fermentative (algal route)	Under dark anaerobic stress, algae ferment internal substrates; H ₂ via PFOR + hydrogenase.	Limited by intracellular carbon pool; short, low-yield phases.
O ₂ sensitivity of hydrogenase	Trace O ₂ blocks hydrogenase expression and activity.	Requires special cultivation (e.g. sulfur starvation) to work.
Competing electron sinks	Electrons diverted to CO ₂ fixation, nitrate reduction, biosynthesis, respiration.	Less reductant reaches hydrogenase → low H ₂ yield.
Energy dissipation	Absorbed light lost as heat or stored as ΔpH instead of going to H ₂ .	Further depresses solar-to-H ₂ efficiency.
Low hydrogenase expression	Hydrogenase expressed weakly and transiently under stress/anaerobiosis.	H ₂ production is episodic and not sustainable in normal growth.

Major Bottlenecks: The oxygen sensitivity of algal [FeFe]-hydrogenases is the most important bottleneck - the presence of

even traces of O₂ inhibits production of H₂ at both the gene expression and enzyme activity levels. This requires special culturing techniques (such as sulfur/nutrient-starvation to stop O₂ evolution) in order to obtain any photohydrogen production. Others include competition for electrons by other metabolic pathways (e.g., CO₂ fixation, nitrate reduction), dissipation of photosynthetic energy in the form of proton gradients, and typically low levels of expression of hydrogenase in algal cells. As a result, yields of raw H₂ from microalgae are very low - one review cites that in spite of the theoretical advantages of algae (fast growth, no requirement for arable land, direct use of water and sunlight), this has yet to be commercialized due to yield limitations. More recent studies therefore, focus on increasing the production of hydrogen through clever workarounds, but taking advantage of the peculiarities of microalgae (e.g., high carbohydrate content, CO₂-fixation capacity, etc.). In the following sections, we review how scientists have enhanced the microalgal hydrogen production related to the novel process strategies, pretreatment techniques, genetic modifications, and the bioelectrochemical system integration, with the focus on studies from ~2020 onwards. Microalgal hydrogen pathways and key limitations are stated in Table 1.

3. Algal-Bacterial Consortia & Symbiotic Systems

3.1 Oxygen-Scavenging Bacteria

Co-cultivation of microalgae with bacteria is an effective way to reduce O₂ inhibition (Ramaraj et al., 2013). Heterotrophic bacteria use O₂ and algal exudates, which form micro-anaerobic niches that preserve hydrogenase and increase net H₂ production (Fakhimi et al., 2020).

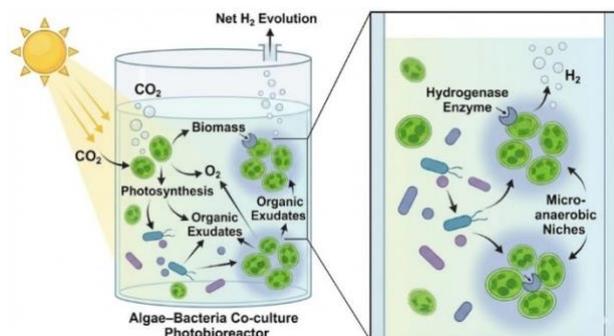


Figure 2. Algae-Bacteria co-culture photobioreactor for improved H₂ production
An algae-bacteria co-culture photobioreactor for improved H₂

production shown in Figure 2.

3.2 Co-benefits associated with Wastewater Treatment

Algae-bacteria consortia can be cultivated in nutrient-rich wastewaters as the nutrients and CO₂ are assimilated by the microalgae and the organics are degraded by bacteria (Ramaraj et al., 2014). This synergy is the basis for the development of integrated systems for simultaneous wastewater treatment and H₂ production (Bhuyar et al., 2021; Malode et al., 2025; Pathy et al., 2022). Conceptual configuration of algal-bacterial co-culture photobioreactor depicting the microalgae conversion of light and CO₂ to biomass (Ramaraj et al., 2015a) and release of O₂ while heterotrophic bacteria consume O₂ and organic exudates to create micro-anaerobic conditions, which enhances H₂ase activity and the net evolution of H₂.

3.3 Cell immobilisation and advanced photobioreactors designs

3.3.1 Encapsulation in alginate and alternative matrices

Microalgae encapsulation with calcium alginate beads can enhance stability maintenance and provide favourable microenvironments. Khedr et al. (2023) found 2-3-fold increased rates of H₂ production and improved operational stability of immobilised cells in comparison to free suspension under visible light. Immobilisation also makes biomass retention and separation easier (Khosravitarbar et al., 2024).

3.3.2 Flat panel reactors and thin layer reactors

Flat-panel and thin-layer photobioreactors minimise light path length and increase light distribution and gas handling which are important for H₂ production. When used in combination with immobilised biofilms or beads they support high cell densities and efficient H₂ collection (Goswami et al., 2021).

3.4 Modifiers of metabolism and chemical aids

Metabolic modulators, such as uncouplers and ATP synthase inhibitors, could be used to re-route electrons to be generated in hydrogenase temporarily by partly collapsing the proton motive force, though they may not be suitable for large scale usage (King et al., 2022). More practical ones are pH buffers and redox mediators.

Table 2. Recent strategies to enhance microalgal photobiological H₂

Strategy / Intervention	Microalgal species	Brief description	Scale	H ₂ improvement	Main mechanism
Algae-bacteria co-culture	<i>Chlorella</i> spp., <i>C. reinhardtii</i>	Mixed consortia in (waste)water	Lab PBR	Up to several-fold ↑	Bacterial O ₂ removal; use of exudates
Sulfur-deprivation (two-stage)	<i>C. reinhardtii</i>	Growth, then switch to S-free medium	Lab PBR	Longer H ₂ phase; ↑ total	Lower PSII repair and O ₂ evolution
Alginate encapsulation	Green microalgae, cyanobacteria	Cells immobilised in Ca-alginate	Lab PBR	2-3× higher rate; ↑ stability	Stress protection; micro-anaerobiosis
Optimised light regime	<i>C. reinhardtii</i>	Flashing / low-intensity LED light	Lab	Higher H ₂ per photon	Better photon use; less photodamage

Metabolic modulators	<i>C. reinhardtii</i> (lab strains)	Low-dose uncouplers / inhibitors	Lab	1.5–2× (short term)	Less ATP synthesis; more e ⁻ to H ₂
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Table 2. summarises in brief the recent tactics for increasing photobiological H₂ from microalgae. Five main strategies are highlighted, namely: Co-culturing of microalgae and bacteria for removal of O₂ and utilisation of algal exudates; Sulfur deprivation enzymes two-stage culture strategy to prolonged H₂ producing phase; Alginate encapsulation to protect cells and create microanaerobic zones; Optimisation of light regimes (flashing/low LED) to enhance photon use and minimize photodamage; Low dosage of metabolic modulators to divert electrons from ATP to the hydrogenase. Overall, it demonstrates that controlling the parameters such as O₂, light and electron flow can improve the H₂ yields from modest to several-fold improvements under lab conditions.

4. Pretreatment of microalgal biomass for dark fermentative hydrogen

4.1 Rationale for pretreatment

For dark fermentative production of hydrogen, the recalcitrance of raw microalgal biomass is generally too high to allow high yields. The thick cell walls of many species, as well as the intracellular packing of carbohydrates and proteins, make the fermentable substrates inaccessible to fermentative bacteria (Balakrishnan et al., 2023). As a result, a very small fraction of the chemical energy contained in the biomass is converted to H₂ in cases where no pretreatment is used, and most of the energy is still locked in residual solids (Nagarajan et al., 2020; Velmozhina et al., 2023).

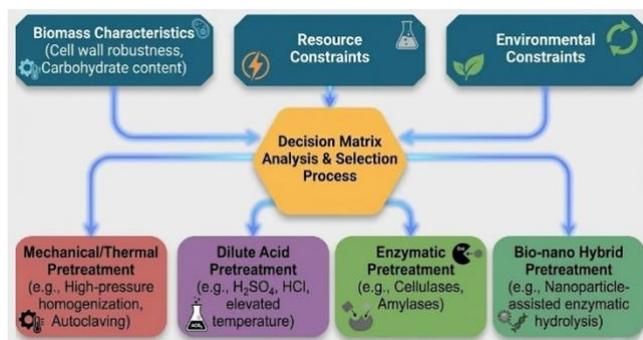


Figure 3. Decision matrix for selecting pretreatment strategies for microalgal biomass

Figure 3. shows a systematic strategy for finding the best pretreatment method for microalgal biomass. It starts by considering the main input factors, which are Biomass Characteristics (e.g., robustness of the cell wall and the content of carbohydrates), Resource Constraints (energy & chemical availability), and environmental constraints (sustainability and waste generation). These factors are then run in a Decision Matrix Analysis & Selection Process. Based upon this evaluation, one of four different pretreatment strategies is selected (Mechanical /

Thermal, Dilute Acid, Enzymatic, Bio-nano Hybrid pretreatment).

Pretreatment is therefore a critical upstream step: Cultivation strategy for oil and gas: it aims to (i) disrupt cell envelopes, (ii) solubilise volatile solids (VS), and (iii) release carbohydrates and other degradable organics into the liquid phase. Effective pretreatment can be used to increase soluble COD and sugar levels several-fold, and further, this will translate into significantly higher volumetric H₂ yield and reduction in fermentation time. However, any pretreatment requires that more energy, chemical and capital costs as well as the potential formation of inhibitory by-products are balanced.

4.2 Pretreatment methods: mechanical and thermal

Mechanical and thermal methods are the most direct methods of "opening up" microalgal biomass. Mechanical disruption techniques such as bead milling, ultrasonication, and high-pressure homogenisation physically disrupt the cell walls and decrease particle size to increase surface area to improve microbial and enzymatic access to intracellular components (Pimpimol et al., 2020). Thermal pretreatments, including autoclaving, microwaves and steam explosion, work to denature the protein and disrupt membranes and can partially hydrolyse complex biopolymers (Ramaraj & Dussadee, 2015). Both the mechanical and thermal methods, when used alone, have a significant effect on increasing solubilisation, and when used in combination as thermo-mechanical pretreatment, will routinely provide 2-3-fold higher H₂ yield than an untreated biomass (Nagarajan et al., 2020). These methods are attractive due to the fact that they are chemically simple and able to operate on a wide feedstock range. Nonetheless, they can be energy-intensive, so their incorporation into a dark fermentation process needs to be carefully balanced in terms of energy and to have opportunities for heat recovery.

4.3 Chemical Pretreatments

4.3.1 Acid hydrolysis

Dilute acid pretreatment is among the most widely researched chemical pretreatment processes for microalgal biomass. Using low concentrations of mineral acids (e.g. 1-2% H₂SO₄) at elevated temperature, algal polysaccharides are hydrolysed to soluble mono- and oligosaccharides which are readily fermented by hydrogen-producing bacteria. When optimisation of the operating conditions followed by subsequent neutralisation/detoxification are respected, acid pretreatment can boost production of fermentative H₂ to three-fold compared to untreated biomass production (Velmozhina et al., 2023). The major negatives include the requirement for pH adjustment, potential corrosion problems and possible generation of inhibitory compounds (eg. furans, phenolics) if severity is too high (Sophanodorn et al., 2022). For this reason, a balance in designing this process is required between hydrolysis efficiency, formation of inhibitors, and downstream

treatment requirements.

4.3.2 Alkali pretreatment

By contrast, pretreatment steps that use alkaline pretreatment are very effective in lignocellulosic feedstocks, still are usually less beneficial for microalgae (Ramaraj et al., 2016b,c). Because algal cell walls are rich in little or no lignin, the use of strong alkali (eg, NaOH or KOH) tends to cause saponification and solubilisation of valuable cell components without any proportional increase in the amount of fermentable sugars produced (Reansuwan et al., 2024; Ramaraj et al., 2025). As a result, the improvements in H₂ yield tend to be low and additional salt load could complicate downstream fermentation and effluent management (Nagarajan et al., 2020). For microalgal biomass, alkali is thus very well suited to mild conditioning or co-processing with lignin cellulosic residues rather than the standalone pretreatment processes.

4.4 Biological pretreatment and Hybrid pretreatment

4.4.1 Enzymatic hydrolysis

Enzymatic pretreatment involves the use of targeted enzymes i.e., cellulases, hemicellulases, and specific ATP-algal cell wall hydrolases, etc, to selectively break down structural polysaccharides under mild temperature and pH. This method provides the possibility of clean streams of sugars with low concentrations of inhibitory compounds, which is of considerable benefit when working with sensitive fermentative consortia (Nagarajan et al., 2020). The major limitations are the expense of enzymes, the need for an adequate reaction time, and reliance on enzyme-substrate specificity (Unpaprom et al., 2021; Bhuyar et al.,

2022). However, the recycling of enzymes and the production of enzymes at the point of use are indeed subjects of active research, in which improved economic feasibility could be obtained.

4.4.2 Pretreatment using bio-nanoparticle-assisted pretreatment

Emerging bio-nanoparticle assisted pretreatments: A combination of microbial and engineered nanomaterials (Van Tran et al., 2020). In these systems, microbial consortia (e.g. bacteria having in some way the ability of partial hydrolysis) are used in combination with metal oxide nanoparticles like Mg-Zn ferrite. The nanoparticles can improve cell disruption due to local heating, catalytic activities or production of reactive species, thus contributing to improving solubilisation, and hence, subsequent fermentability of the microalgal biomass (Velmozhina et al., 2023). Reported studies show a promising increase in yields of H₂, comparing conventional pretreatment, which suggests a synergy between biological and nano-enabled mechanisms. However, outstanding questions about the recovery of nanoparticles, the long-term stability and potential ecotoxicity need to be solved before such large-scale implementation can be feasible.

4.5 Comparative performance/decision criteria

In summary, pretreatment choice is typically, by nature, a trade-off: pretreatment methods that deliver the best increase in solubilisation and H₂ yield (e.g. thermo-acid or bio-nano hybrids) are also likely to be more energy-, chemical- or capital-intensive; mild methods (mechanical or enzymatic) are cleaner but less aggressive and/or slow (Table 3).

Table 3. Pretreatment methods for microalgal biomass prior to dark fermentative H₂ production

Pretreatment Type	Specific Method / Conditions	Targeted Effect	Typical Outcomes (Sugars / VS Solubilisation)	Impact on H ₂ Yield vs Untreated*	Advantages	Drawbacks / Concerns
Mechanical	Bead milling, ultrasonication, and homogenisation	Cell disruption, size reduction	Moderate increase in soluble COD	1.5–2×	No chemicals; flexible	High energy demand; equipment wear
Thermal	Autoclaving, microwave, steam explosion	Protein denaturation, membrane disruption	Significant VS solubilisation	2–3×	Simple; scalable	Heat energy input; possible Maillard products
Acid	Dilute H ₂ SO ₄ / HCl, elevated temperature	Hydrolysis of polysaccharides to sugars	High sugar concentrations; high COD	Up to 3×	Highly effective at low doses	Neutralisation cost; inhibitors if over-severe
Alkali	NaOH / KOH at moderate temperature	Deprotonation, saponification	Moderate solubilisation	Often limited	Good for mixed wastes	Less effective for algae; salt load
Biological / Enzymatic	Cellulase, hemicellulase,	Selective hydrolysis of cell	High-quality sugar stream; low	2–3×	Mild conditions;	Enzyme cost; slower kinetics

	lyases	wall components	inhibitors		low inhibitors	
Bio-nano hybrid	Microbes + metal-oxide nanoparticles	Synergistic biochemical and physical disruption	High solubilisation; improved fermentability	Often >3×	High efficiency; novel	NP cost, recovery, ecotoxicity

*Representative improvements from multiple studies; exact values depend on strain and conditions.

Rather than one "best" method, the optimal way forward is dependent on the strain of microalgae, available utilities (heat, chemicals, enzymes), inhibitor tolerance of the fermentation step, and overall process economics. Consequently, a lot of recent studies are in favour of combined/staged pretreatments, which are chosen based on multi-criteria decision frameworks (H_2 gain vs energy usage, cost, and environmental impact). Table 3 compares the principle pretreatment possibilities for converting the microalgal biomass into a more fermentable raw material for the dark H_2 production and presents a classic tradeoff between effectiveness and cost/complexity. Mechanical and thermal methods are solid, chemical-free (in case of mechanical) or easy to implement, but can be energy-consuming (Unpaprom et al., 2019). Dilute acid pretreatment has a distinction as the most consistent one for releasing sugars (up to $\sim 3x$ H_2 increase) with the condition of carefully controlling rates of formation of inhibitors and the added costs of neutralisation. Alkali is suitable for mixed or lignocellulosic wastes (Wannapokin et al., 2018), simply is not so suitable for algae and produces high salt loads. Biological/enzymatic pretreatment has the advantage of high-quality (Chuanchai et al., 2019; Taechawatchananont et al., 2024), low-inhibitor hydrolysates for low-cost mild pretreatment, against the disadvantage of high enzyme cost and relatively slow kinetics (Vu et al., 2018). Finally, bio-nano hybrid approaches seem to provide the highest levels of solubilisation and H_2 yield, nonetheless are still at the emerging stage with concerns around the cost and recovery of the nanoparticle and safety to the environment. Overall, it can be seen from the table that the near-term practical routes to this seem to be concentrated acid and thermo-mechanical,

although enzymatic and bio-nano approaches show promise but remain in development for higher efficiency (next generation) systems.

5. Genetic and metabolic engineering of microalgae

Figure 4 shows a scheme of a genetically engineered microalgae cell designed to optimise production of hydrogen by modification in photosynthetic, metabolic and regulatory networks. The design consists of the incorporation of an engineered hydrogenase with enhanced stability and the electron-accepting capacity that is controlled by synthetic promoters and regulatory elements for optimum catalytic activity (Wang et al., 2025). Competing electron-consuming pathways for example, the reduction of nitrate, the cyclic flow of electrons and fixation of carbon through the Calvin-Benson cycle are disabled and thereby the flow of reducing equivalents is enforced to H_2 formation. Photosynthetic efficiency is increased by the reduction in the antenna size, the distribution of light, and the losses of excitation. The stimulation of the hyper-accumulation of starch increases carbon reserves for sustained dark or hybrid hydrogen production. These modifications are an example of a systems approach where the supply of electrons, carbon storage, the functioning of the enzymes and transcriptional control are maximized simultaneously. This strategy represents the "designer microalgae" paradigm, which provides a recipe for the production of strains that can achieve higher levels of biohydrogen under operational conditions.

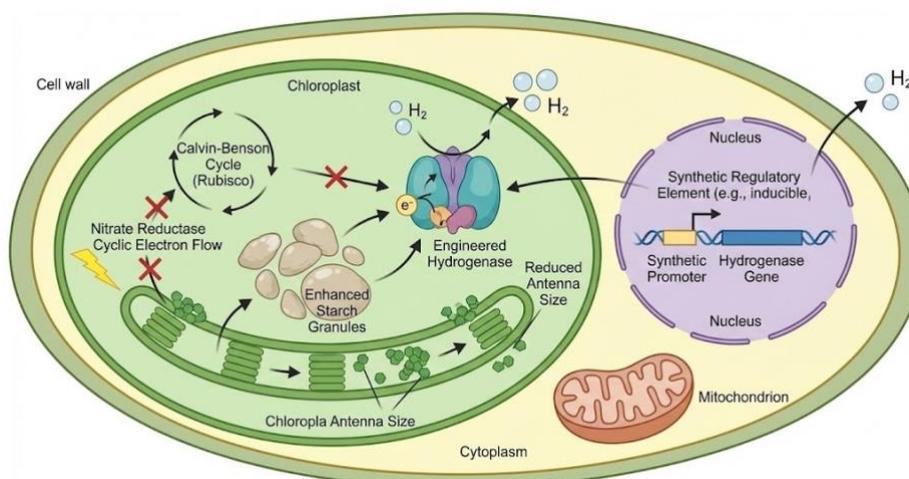


Figure 4. Genetic and metabolic engineering of microalgae for enhanced biohydrogen production

5.1 Overview of tools and model species

Genetic engineering has proven to be an important approach to overcoming the inherent biological limitations of microalgal hydrogen production. Of all the species available, *Chlamydomonas reinhardtii* is the best-developed and versatile model organism. Its fully sequenced genome, well-established nuclear, chloroplast, and mitochondrial transformation systems, and extensive omics resources provide the basis for an exceptional foundation for targeted manipulation. Importantly, *C. reinhardtii* has a naturally occurring high [FeFe]-hydrogenase activity when under anaerobic stress, making it intrinsically suitable for H₂-related metabolic engineering (Xu et al., 2019). Recent advances in gene-editing

technologies (including the popular technologies called CRISPR/Cas9 and CRISPR/Cas12a), artificial microRNAs, and modular toolkits of synthetic biology have further expanded the engineering potential of this alga. These tools now allow for precise gene knockout, knock-in and multiplex pathway re-wiring, enabling rational design of strains optimised for both photobiological and fermentative production of hydrogen (King et al., 2022). Exploratory work is also proceeding in such genera as *Chlorella*, *Scenedesmus* and some cyanobacteria; however, their transformation and regulatory toolkits are still relatively less mature (Ramaraj et al., 2015a,b). As a result, *C. reinhardtii* remains the primary world-class model of microalgae for the next generation of microalgal biohydrogen engineering (Figure 5)

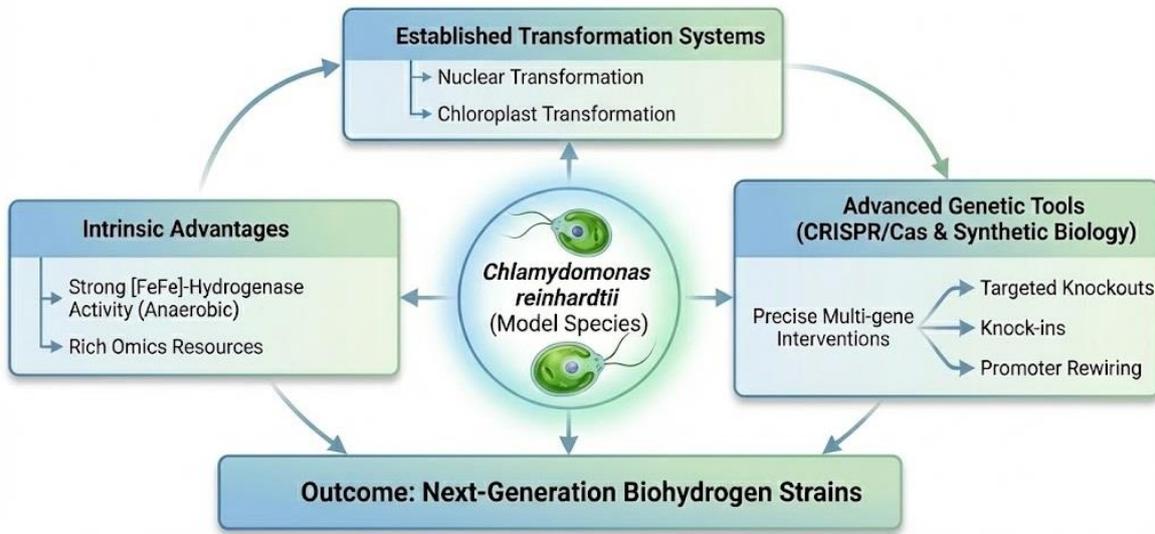


Figure 5. Integrated Genetic and Biotechnological Framework for Developing Biohydrogen Strains using *Chlamydomonas reinhardtii*

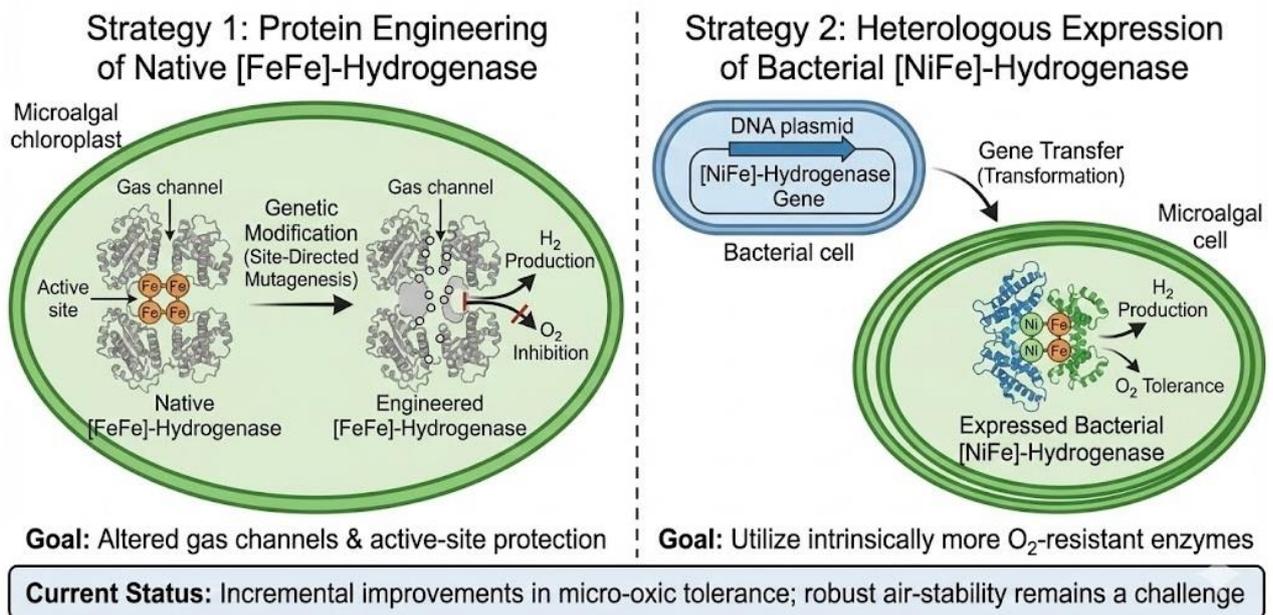


Figure 6. Strategies for engineering O₂-tolerant hydrogenases in microalgae

Figure 5. illustrates the central role of the microalga *Chlamydomonas reinhardtii* as a model species for biohydrogen research. It highlights how the organism's intrinsic advantages, such as strong anaerobic [FeFe]-hydrogenase activity and rich omics resources, are synergistically combined with established nuclear and chloroplast transformation systems. Furthermore, the application of advanced genetic tools, including CRISPR/Cas and synthetic biology for precise multi-gene interventions like targeted knockouts, knock-ins, and promoter rewiring, enables the targeted engineering of *C. reinhardtii*. The convergence of these biological properties and technological capabilities ultimately leads to the development of next-generation biohydrogen-producing strains with enhanced efficiency and yield.

5.2 Hydrogenase-based centred engineering O₂-tolerant hydrogenases

Strategies for engineering O₂-tolerant hydrogenases in microalgae are demonstrated in Figure 6. Because hydrogenase is the ultimate catalyst for H₂ production, a major thrust in the field of microalgal engineering is to enhance hydrogenase performance under physiologically realistic, microoxic conditions. There are two complementary strategies that have been explored. First, protein engineering of native [FeFe]-hydrogenases in *C. reinhardtii*, which is the attempt to modify the [FeFe]-hydrogenases in the vicinity of the active site or gas channels to improve the tolerance to O₂ without sacrificing the catalytic turnover. Second, researchers have tried to express heterologous [NiFe]-hydrogenases from bacteria that are naturally more oxygen resistant and conduct photosynthetic electrons to these alternative enzymes (King et al., 2022). To date, such approaches have produced partial improvement in stability and activity but not large distinctions of entirely O₂-insensitive hydrogenases in vivo, so highly tolerance to O₂ in vivo for the system of hydrogenase functioning in microalgae remains a major goal for long-term improvements.

In parallel, work has been in the area of making more hydrogenase available when it is needed. Promoter engineering (using strong or inducible promoters), codon optimisation and increased gene copy number have been used to increase expression of HYDA1/2 during the hydrogen-producing phase. These strategies can substantially increase auxen-housage hydrogenase species and short-term H₂ rates but, in turn, exaggerate metabolic burden and competition for cellular resources and this becomes primarily pronounced during stress conditions. Achieving a good compromise between high hydrogenase expression and viable growth and viability is therefore one of the key design problems.

5.3 Making the electrons move in a way toward hydrogen

Even if the hydrogenase is improved, however, the output of hydrogen will be low if the electrons are diverted into competing metabolic pathways. Genetic knockouts of lactate dehydrogenase, alcohol dehydrogenase and other fermentative enzymes have been used to reduce electron flow into lactate, ethanol and other reduced end-products. This diverts more reducing power in the direction of

pyruvate: ferredoxin oxidoreductase: hydrogenase axis to boost more dark H₂ yields in engineered strains (King et al., 2022). Similar approaches involve targeting of arms of the Calvin-Benson cycle or alternative electron sinks, to have a hydrogenase as a preferred enzyme over carbon fixation, or other reductive electron sinks during designated H₂ production phases.

At the photosynthetic level, optimisation in the way light is acquired and employed can have a significant impact on the availability of electrons to the H₂ase. Truncated light-harvesting antenna mutants in *C. reinhardtii* decrease excessive absorption at the surface of the culture, which allows deeper light penetration and reduces photoinhibition in dense cultures. This usually results in a higher areal H₂ productivity for high irradiance (Hippler, 2024). Complementary interventions consist of changes in cyclic electron flow as well as overexpression of ferredoxin or other electron carriers to promote linear electron flow to hydrogenase rather than ATP formation. Collectively, these strategies push the partitioning of the photosynthetic electrons that can lead to H₂ formation, causing more electrons to enter the H₂-forming pathway.

5.4 Improving carbon storage of dark hydrogen

For dark fermentative hydrogen production, the amount and quality of intracellular carbon storage are key determinants of yield. Metabolic engineering to hyper-accumulate starch (e.g., by overexpressing ADP-glucose pyrophosphorylase and down-regulating starch-degrading enzymes) provides the possibility to have bigger internal pools of carbohydrates that could then be used as a substrate for anaerobic hydrogen production (Xu et al., 2019; Lai et al., 2022). When such starch-rich strains are coupled with optimised incubated anaerobic protocols (controlled pH, redox and nutrient status), significantly greater cumulative dark H₂ output/unit of biomass can be achieved (Chu et al., 2021; 2022). This approach is effective to subtract a "growth and storage" phase from a "fermentative H₂" phase at the cell level, which is analogous to indirect photolysis, but focusing on intracellular reserves.

5.5 Systems and regulatory level engineering

Beyond single gene modifications, systems-level and regulatory engineering go for coordinating a number of traits in time and space. The use of stress-responsive or inducible promoters, logic gate circuits and dynamic regulators to control the timing of hydrogenase expression, the down-regulation of competing pathways and the activation of storage metabolism becomes impressive in the synthetic biology approaches of the past few years (Xu et al., 2019; Jaramillo et al., 2025). The aim is to build microalgal strains responsive to external cues which can switch between growth, storage and H₂ production modes on demand (in response to external signals (e.g., light regime, nutrient status) or to artificially engineered inducers).

Coupled with genome-scale metabolic descriptions and guides design, these regulatory strategies are driving the field towards "designer microalgae" for hydrogen, where hydrogenase capacity, electron supply, carbon storage and stress tolerance are

all tuned in concert, not separately. While such strains are currently at the laboratory proof-of-concept stage, they are a sign of the future where microalgal biohydrogen systems are not only more productive, but they are also more predictable and controllable on

a larger scale. Genetic engineering strategies for enhanced microalgal H₂ production in Table 4.

Table 4. Genetic engineering strategies for enhanced microalgal H₂ production

Target / Strategy	Genetic Modification	Species / Model	Expected Metabolic Effect	Observed Impact on H ₂ (qualitative)	Trade-offs / Issues
Hydrogenase engineering	Mutated native [FeFe]-hydrogenase; heterologous [NiFe]	<i>C. reinhardtii</i>	Increased activity / partial O ₂ tolerance	Higher H ₂ under micro-oxia	Complex maturation; stability
Promoter engineering	Strong / inducible promoters for HYDA1/2	<i>C. reinhardtii</i>	Higher hydrogenase expression	Increased H ₂ rate / duration	Metabolic burden; O ₂ inhibition persists
Knockout of fermentative by-products	Deletion of LDH, ADH, etc.	<i>C. reinhardtii</i>	Redirect pyruvate electrons to H ₂	Higher dark H ₂ yield	Accumulation of alternative by-products
Antenna size reduction	Truncated antenna (TLA) mutants	<i>C. reinhardtii</i>	Improved light distribution in dense cultures	Higher areal H ₂ productivity	Lower light capture at very low intensity
Starch hyper-accumulation	Upregulated starch synthesis; downregulated degradation	Green microalgae	Increased carbon reserves for dark fermentation	Improved cumulative dark H ₂	Potential slower growth in some regimes
Regulatory/systems engineering	Synthetic regulatory circuits; stress-responsive promoters	Model microalgae	Dynamic optimisation of H ₂ phase	More robust and tunable H ₂ profiles	Complexity; limited field validation

6. Bioelectrochemical technologies, hybrid microalgae-based hydrogen technologies

Bioelectrochemical systems are one of the cutting edges of actively developing research on hydrogen from microalgae (Zerveas et al., 2025). By combining the biological potential of the microalgae with electrochemical control over the reaction on engineered electrodes, such hybrid configurations help to overcome limitations experienced in purely photobiological or in fermentative routes -- notably low electron flux, oxygen inhibition and limitedness of metabolic efficiency. Three big classes of these technologies are now emerging. Figure 7. The schematic shows a photobioelectrochemical cell of dual chambers (PBEC) designed for light-induced production of hydrogen.

In the anodic chamber, there is a transparent conductive photoanode which is coated with an immobilised microalgal biofilm that, upon illumination, carries out photosynthesis and moves photosynthetically derived electrons directly to the electrode surface. A proton exchange membrane (PEM) separates the anodic and cathodic compartments, allowing different

migration of protons while the oxidative and reductive environment is kept separated (Hirsch et al., 2024). Electrons travel through a connected external circuit to the cathodic chamber, where they recombine with translocated protons at the cathode, reducing H⁺ to molecular hydrogen (H₂). This configuration is important, pointing to the potential of immobilised microalgae as living biocatalysts for solar-driven electrochemical hydrogen generation.

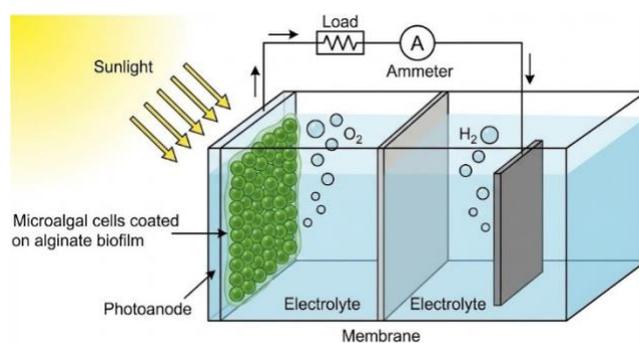


Figure 7. Photobioelectrochemical cell (PBEC) with immobilised microalgae on the photoanode

6.1 Microalgae-integrated microbial electrolysis cells (MECs)

In microbial electrolysis cells (MECs), for example, electroactive bacteria at the anode catalyse anodic oxidation of organic substrates and donate electrons via an external circuit, in which little external potential is required for efficient production of hydrogen at the cathode. Microalgae can facilitate MEC systems in two ways: first, as upstream CO₂ fixing biomass production sources to supply renewable organic feedstock for anodic oxidation and secondly, as part of algae-bacteria consortia, as the metabolites secreted by the algae enhance the electron transfer of bacteria (Goswami et al., 2021; Hirsch et al., 2024). When bioaugmentation (microalgal biomass pretreatment to enhance biodegradability) is carried out, MECs can have high coulombic efficiencies and high hydrogen yields (which often reach 70-80% of theoretical values, much higher than dark fermentation results). This kind of integration allows for achieving closed-loop bioprocessing where wastewater nutrients and CO₂ are taken up by microalgae that are then converted to hydrogen with a minimum of wastes, turning MEC-algae hybrids into a promising component of the future biohydrogen biorefineries.

6.2 Photobioelectrochemical Cell and Biophotovoltaic Systems

Photobioelectrochemical (PBE) cells make use of the fact that microalgae can direct photosynthetically-derived electrons to an electrode. In such systems, microalgae are usually immobilised on photoanodes, on which incident light causes the flow of electrons through the algal photosynthetic chain and into the electrode, allowing hydrogen to be evolved at a cathode. Recent findings reveal that alginate-immobilised mA pose superior photocurrents and better hydrogen production in comparison to the suspended

cultures because of the superior biofilm stability and mass transfer (Khedr et al., 2023). Similar benefits have been seen in types of biophotovoltaic devices where immobilised algae give higher power density and longer operational lifetime (Ng et al., 2017). These configurations provide the pathway to directly convert solar energy to hydrogen, which is free from exogenous organic substrates. Although limited performance is still imposed by the efficiency of electrode transfer, and the robustness of biofilm in long-term use there is a strong value of pursuing the use of PBE and BPV systems in the design of living photosynthetic electrodes for continuous solar energy-driven hydrogen production.

6.3 Electro-Biohydrogenation concepts

Electro-biohydrogenation includes a set of innovative hybrid systems, in which electrical energy is strategically used to help biological hydrogen production processes, from increasing the reaction rates, to increasing the operational flexibility. This category contains MEC-photobioreactor cascades, capacitive bioelectrochemical systems which store charge temporarily, charge release, as well as PBE-photosynthesis-derived advanced electrochemical stimulation devices (Hirsch et al., 2024). By providing small targeted electrical inputs, these systems provide the ability to promote increased substrate oxidation and triage redox conditions and drive H₂ generation during times of low illumination. Although current implementations are still at fairly low technology readiness levels, electro-biohydrogenation is increasingly seen to be a potentially promising link between biological CO₂ fixation and utilisation in electricity. As such, these hybrids provide a path to the future for highly scalable, flexible and grid-supplied hydrogen production systems, which are conducive towards their integration into circular bioeconomy infrastructures.

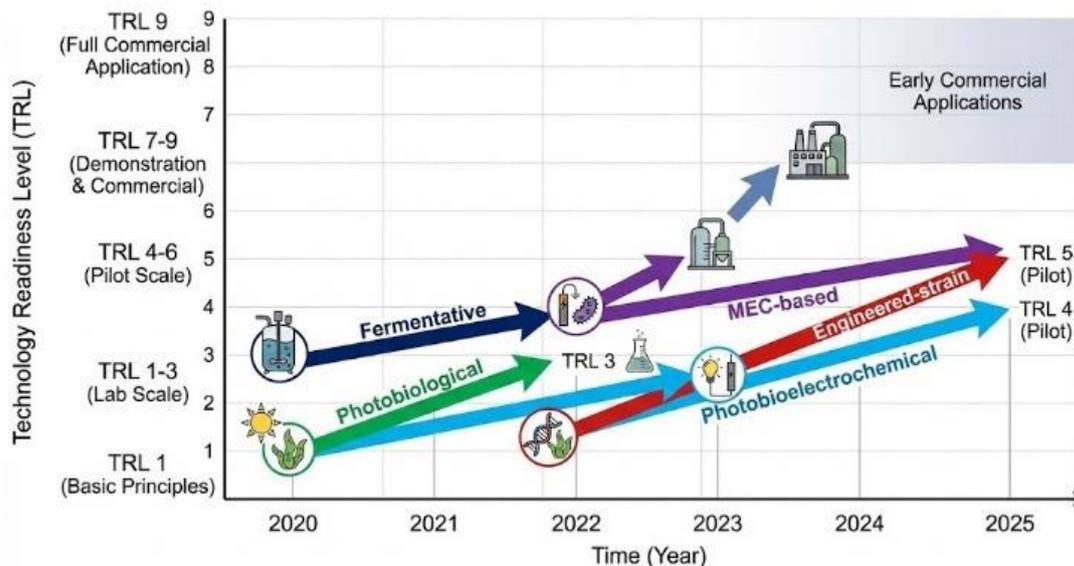


Figure 8. Research and technology readiness landscape for microalgal hydrogen production (2020–2025)

7. Technology readiness, pilot demonstrations and application nich

7.1 Readiness levels of technology

Research and technology readiness landscape for microalgal hydrogen production (2020–2025) illustrated in Figure 8. Current microalgal hydrogen technologies fall into the early stages up to emerging stages of development, with most systems falling into the range of TRL 1 to TRL 4. Photobiological hydrogen production, especially with the S-deprived *C. reinhardtii* systems is the most experimentally refined as it is supported by decades of both mechanistic and genetic studies. However, these are still in the pre-commercial stage owing to persistent limitations including the inhibition of O₂, low solar to hydrogen efficiency and instability in outdoor operation (Jiao et al., 2024; Faraloni, 2025). In contrast to this, dark fermentative hydrogen production from algal biomass has moved into bench-to-pilot-scale demonstrations with the primary impetus coming from the compatibility with existing anaerobic digestion infrastructure and for utilisation of pretreated microalgal waste biomass.

Significantly, algae-integrated MECs (combining carbon fixation by microalgae and hydrogen generation via electrolysis) have also been tested at the pilot scales, especially in wastewater treatment applications in which microalgae simultaneously remove nutrients and produce biomass for feeding MECs (Wang et al., 2021). Although photobioelectrochemical systems and electro-biohydrogenation devices are currently only at TRL 1-2, a huge leap forward is taking place in electrode materials, immobilisation matrix and photoanode design which suggests great potential in the future.

This roadmap diagram can be used for a comparative assessment of the developmental status of key microalgal hydrogen technologies (6). It plots different production pathways including photobiological systems, dark fermentation, Microbial Electrolysis Cells (MECs), and photobioelectrochemical and devices in time against the scale of Technology Readiness Level (TRL) during 2020-2025. The visual emphasizes a gap in maturity with the

fermentative and MEC-integrated systems on the left progressing to pilot-scale demonstrations (TRL 5-7) thanks to their compatibility with existing wastewater infrastructure and mainly direct photolysis and genetically engineered strain pathways in the research and validation phases (TRL 1-4) being high potential and emerging technologies that are on the transition from laboratory proof-of-concept scale to scalable validation scale.

7.2 System piloting and demonstration

Pilot projects around the world have started to investigate the integration of microalgae production with the bioprocesses of hydrogen production in real environment. Several demonstrations use industrial flue gas, agricultural effluents or municipal wastewater as nutrient-rich inputs for the growth of large amounts of algae, thus achieving a simultaneous sequestration of CO₂, nutrient removal and biomass production (Ramaraj et al., 2015c). Downstream of this biomass conversion through dark fermentation or microbial electrolysis cells (MECs) for example has successfully produced measurable hydrogen yields in addition to the co-products such as biofertiliser, organic acids or biogas, which increase the overall process economics (Velmozhina et al., 2023). In MEC based demonstrations integration with wastewater treatment reaches dual benefits; energy recovery as well as process intensification. Although these pilots are currently small, together with them the proof of concept of algae-based circular bioresource systems is confirmed, where the waste flows are transformed into energy carriers and value-added products.

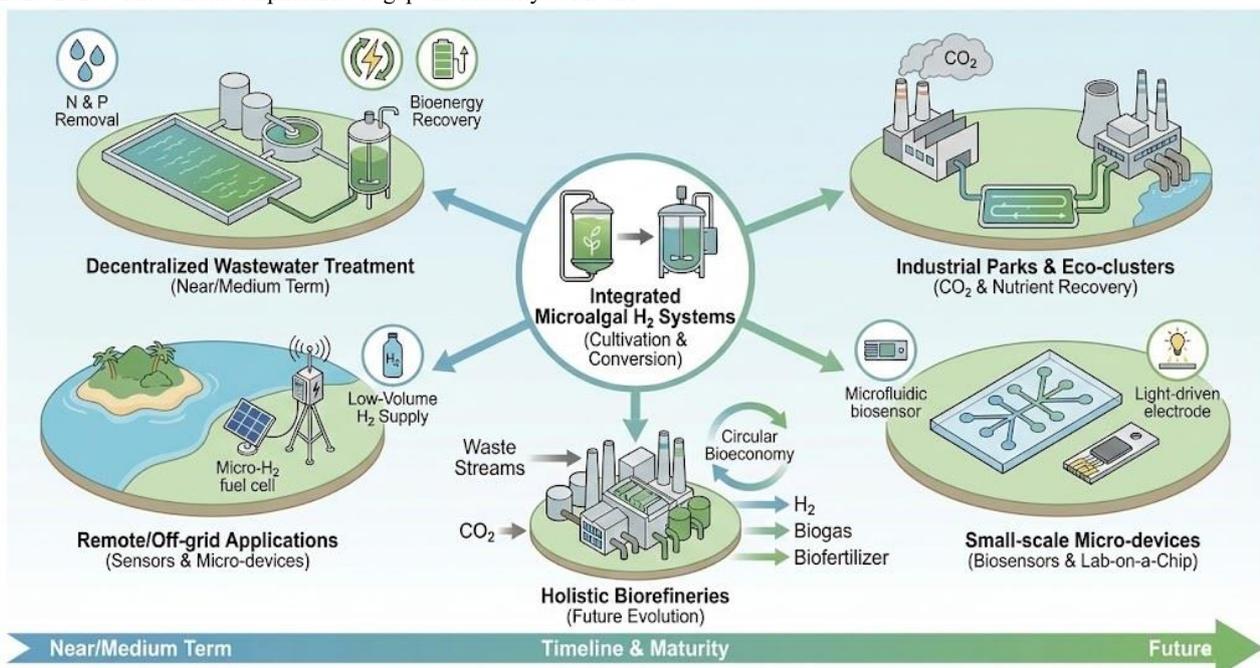


Figure 9. Potential Application Niches for Microalgal Hydrogen Technologies

7.3 Possible application niches

For the near to medium term, microalgal hydrogen technologies are likely to find their strongest foot in specialised, or hybrid, application niches where its multifunctional characteristics, concerning, for instance, CO₂ capture, nutrient removal, biomass valorisation and co-product generation, provide added value in addition to the pure hydrogen (Figure 9). Decentralised wastewater treatment installations should be a particularly interesting environment, as microalgae can be used to remove nitrogen and phosphorus while at the same time producing biomass food for dark fermentation/methyl energy capture (MEC) installation supporting energy positive and cost-effective wastewater treatment. Similarly, industrial parks and eco-industrial clusters that have concentrated emissions of CO₂ or high nutrient content effluents provide possibilities of co-locating algal cultivation systems (Ramaraj *et al.*, 2016) with hydrogen production systems for better carbon balance and favor integrated resource recovery (Balakrishnan *et al.*, 2023). In remote or off-grid environments, microalgal systems in combination with solar-powered MECs could be used to provide decentralised low-volume hydrogen for sensors, micro-power devices or niche fuel cell applications. At the small scale, emerging photobioelectrochemical devices, despite their low TRL, consider the possibility of miniaturised biosensors, self-powered microdevices and lab-on-a-chip platforms, through exploitation of immobilised algae as live photoactive catalysts. As these technologies are improved they could be developed into holistic biorefinery frameworks incorporating microalgal carbon dioxide capture, nutrient recycling, ammonia stripping and integrated hydrogen-biogas generation for the development of circular, multifunctional bioenergy systems.

8. Key Challenges and future research priorities

Despite significant improvement, there are still a number of critical issues for scaling up and making microalgal hydrogen technologies commercially viable. Foremost among them is the extreme sensitivity of [FeFe]-hydrogenases to oxygen, and hence the need for intricate strategies for O₂ management or greater robustness of designed hydrogenases. At the level of the individual reactor, providing a homogeneous light distribution and efficient gas exchange and mixing in dense cultures is a major bottleneck, especially for an outdoor or large-scale reactor. Economic and environmental concerns also remain in the area of pretreatment processes have to be optimised to minimise energy consumption, minimise chemical inputs and prevent the formation of inhibitory by-products. Additionally, the use of genetically modified microalgae outside of controlled laboratory environments presents problems with long-term stability, ecological risk and regulatory compliance.

Looking towards the future, future research activities should have an integrated systems approach including stacked genetic modification, next generation photobioreactor design, smart and energy efficient pretreatment strategies and hybridisation with MECs or photobioelectrochemical systems within circular

biorefinery frameworks. Equally important are comprehensive techno-economic analyses (TEA) and life cycle assessments (LCA) to assess the feasibility in the real world, define competitive niches of application and inform supporting regulatory/policy measures. These efforts will be essential to see the laboratory advances without scalable, sustainable and commercially viable microalgal hydrogen technologies.

6. Conclusion and future perspectives

Microalgae-based biohydrogen production has advanced significantly in recent years. Cultivation strategies like co-cultures and immobilization have reduced biological limits, while pretreatment methods have improved energy extraction. Genetic engineering and bioelectrochemical hybrids have enhanced performance by combining biology with electrical inputs. Future systems could feature engineered microalgae grown on wastewater in immobilized biofilm photobioreactors, where biomass pretreatment feeds MECs for hydrogen generation while electro-biohydrogenation produces H₂ during daytime. This integrated approach would optimize hydrogen output. Key priorities include improving algal stability outdoors, scaling up reactors, and conducting techno-economic analyses. Combining microalgal H₂ with co-products and environmental services strengthens the economic case. Microalgae-based hydrogen production is evolving through innovative strategies and bioelectrochemical integrations. While industrial implementation faces challenges, recent progress has been significant. Interdisciplinary efforts combining microbiology, engineering, and biotechnology align with sustainability goals by offering clean energy. The next decade will determine if these advances enable practical implementation and contribution to the hydrogen economy.

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