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Review

Genetic engineering for biohydrogen production from microalgae

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SUMMARY

The development of biohydrogen as an alternative energy source has had great economic and environmental benefits. Hydrogen production from microalgae is considered a clean and sustainable energy production method that can both alleviate fuel shortages and recycle waste. Although algal hydrogen production has low energy consumption and requires only simple pretreatment, it has not been commercialized because of low product yields. To increase microalgal biohydrogen production several technologies have been developed, although they struggle with the oxygen sensitivity of the hydrogenases responsible for hydrogen production and the complexity of the metabolic network. In this review, several genetic and metabolic engineering studies on enhancing microalgal biohydrogen production are discussed, and the economic feasibility and future direction of microalgal biohydrogen commercialization are also proposed.

INTRODUCTION

Fossil fuels are the most important energy source globally and have brought great economic benefits.[1] Their excessive use, however, has negative environmental and human health impacts. At present, more than 80% of global energy use originates from fossil fuels.[1] With continuous population growth, fossil fuel demand will only increase, and the output of limited fossil fuel resources will inevitably decline in this century.[2,3] Excessive exploitation of fossil fuels has also caused deforestation, loss of farmland, and damage to ecosystems because of acid rain.[4] Global pollution caused by fossil fuels has multiple negative consequences[5]; because the burning of fossil fuels contributes to about 75% of global carbon-dioxide emissions, it is estimated that carbon emissions from fossil fuels will increase to 39 billion tons by 2030.[6] Global warming caused by carbon emissions from human activities has resulted in a global temperature 1°C higher than that before industrialization. Global warming has also led to increased frequency and severity of ice melt, leading to rising sea levels and extreme weather.[7] The combustion of fossil fuels also negatively impacts human health, causing and exacerbating respiratory and nervous system diseases.[8] Reducing reliance on fossil fuels and finding alternative energy sources are therefore crucial for the long-term sustainability of human development.[9]

Hydrogen has the highest energy content per unit of mass of all known fuels. It is the most abundant element in the universe and is a clean energy source because water is the only by-product of its combustion.[10] The high energy efficiency, high energy density, and safety of solid hydrogen demonstrate promise for scaling hydrogen energy.[11] As hydrogen is considered the most promising alternative energy source, hydrogen produced from renewable resources could be a sustainable and clean fuel.[12,13] Biomass-derived hydrogen can be generated through thermochemical and microbial processes.[14,15] The low concentration of hydrogen produced by thermochemical methods and the need for high-temperature complex operating conditions increase production costs and energy consumption, making them unsustainable.[16,17] Compared with chemical hydrogen production, biological hydrogen production by microbes can be carried out under lower temperatures and pressures.[18,19] Microorganisms can easily become hydrogen production sources.[20]

Microalgae are a kind of biomass with great potential for hydrogen production.[21] Owing to their highly adaptable nature, they are widely distributed globally.[22] Microalgae are the basis of the global carbon cycle and absorb 50% of global carbon dioxide.[23] They also have rapid growth rates as well as high yields and carbohydrate contents.[24] They can be cultured in a variety of substrates including fresh water, seawater, domestic sewage, and wastewater.[25,26] As microalgae convert light into chemical energy and produce

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hydrogen by cracking water, they are considered a green production source. In addition, algal biomass can be cultured at a large scale, and could be used as a substrate fuel source for hydrogen production.[27,28,29] Novel strategies for microalgal hydrogen production have been explored to improve competitiveness.[30] Various pretreatments could also improve hydrogen recovery.[31] Despite these successes, there are some obstacles to developing large-scale hydrogen production using algae.[24] The production of hydrogen in these systems depends on interactions in the microalgal metabolic network, including photosynthesis, respiration, and fermentation; these natural processes are not enough to support large-scale industrial production.[32,33] With the application of genetic engineering, microalgae are becoming the most promising microorganisms for hydrogen production.[19] Directed transformation has been shown to improve hydrogen production capacity.[34]

This review provides an overview of pretreatment techniques for microalgal biomass and discusses methods to improve biohydrogen production using genetic and metabolic engineering. Many strategies, including adjusting environmental tolerance, metabolic remodeling, and the expression of synthetic pathways from high-yield strains have been adopted to improve hydrogen production on an industrial scale.[35]

MICROALGAL BIOMASS AS A RAW MATERIAL FOR BIOHYDROGEN PRODUCTION

Introduction of algal substrate

The selection of the right substrate for industrial-scale fuel production is important because it accounts for 60–70% of the cost of production.[36] Traditional hydrogen production methods involving lignocellulose consumption lead to competition with farmland and utilization of land that could be used for other purposes.[18] The crystalline structure of lignocellulose limits its utilization by microorganisms and enzymes. The scarcity of land also hampers energy crops.[37] In contrast, microalgae have almost no lignin and low hemicellulose content, and the required pretreatment is not energy-intensive and does not require the use of ecologically toxic solvents that can cause secondary pollution.[38] Biofuel production has shifted from using sugar and starch to lignocellulosic materials, and finally to the third generation of algal biomass in recent years.[36] The transformation of microalgal biomass is an efficient and environmentally friendly strategy to produce hydrogen.[39] It can be converted into hydrogen by microbial fermentation.[40] 'Microalgae' is a general term for algae whose morphology can be distinguished under a microscope.[41] They are extremely diverse single-cell populations that can be as small as 0.2 μm.[42] Microalgae can be composed of one or a few cells, and there can also be structures formed by the aggregation of many cells.[35,24] The composition of algal cells also varies with species and the surrounding environment, with carbohydrate content reaching as high as 70%, making them ideal substrates for biohydrogen fermentation.[43,44]

Pretreatment of algal biomass

Pretreatment increases biohydrogen production and is a key step used to extract the main components from cells for conversion into biofuels.[25] Pretreatment of algal biomass to degrade cell walls enhances fermentative H_2 productivity by 50–70%.[45] Because microalgae lack lignin and hemicellulose, only simple pretreatment is required greatly reducing costs.[46] Biohydrogen is mainly obtained from the conversion of carbohydrates, so the best pretreatment strategies aim to release more carbohydrates.[43] The destruction of a cell wall composed of cellulose, pectin, and algaenan is the main obstacle.[47]

The properties of microalgae are varied and pretreatment conditions vary from strain to strain.[36] Usually, pretreatments are classified into four categories: physical, chemical, biological, and combinatorial (a combination of several treatment methods).[48] Common pretreatment methods and their advantages are shown in Table 1. Physical pretreatment uses external machinery or electricity to destroy cells, changing the surface area and biomass crystallinity; hydrothermal pretreatment, milling, ultrasonic, and microwave irradiation are the main methods used.[49] Chemical pretreatment uses chemicals to extract biomolecules.[49] The most commonly used acid pretreatments are HCl, H_2SO_4, and HNO_3 with concentrations ranging between 0.1 and 6.0 M. The most commonly used alkali pretreatments are NaOH, KOH, and $Ca(OH)_2$ at concentrations ranging between 1.0 and 8.0 M.[50] Most studies have shown that acidic pretreatment is more effective.[51] Physical and chemical pretreatments and their combinations generally require high-energy or corrosion-resistant high-pressure reactors. These traditional pretreatment methods produce various inhibitors, affecting enzymatic hydrolysis and yield. Biological pretreatment is low energy and low cost, involves no chemicals, generates no pollutants, and involves easy genetic manipulation.[44] Biological pretreatment involves the lysis of microalgal cells with enzymes or microorganisms.[36] Because this process can be carried out under mild conditions, the power requirements are low. The application of a variety

Table 1. Comparison of different hydrogen production methods

	Specific method	Time	Effect	Advantage	Disadvantage	Reference
Hydrothermal pretreatment	Hydrothermal heating in the reactor, 80°C–160°C	20-60 min	The chemical bonds between the cell walls are broken at high temperatures, thermal deformation occurs, and cellulose is decomposed	High decomposition efficiency Short decomposition time Simple operation Easy to control	Monomer sugars degrade into inhibitory by-products High energy consumption	Chozhavendhan et al., Nagarajan et al.[43,52]
Grinding	Biomass is physically collided with ceramic, glass, or quartz beads under high agitation, comminution to fine size	A few minutes	Shear forces break down biomass, making it biodegradable	Does not contaminate products that require pretreatment Produces fewer inhibitory by-products	Unable to decompose macromolecular substances	Chozhavendhan et al., Shanmugam et al.[52,55]
Ultrasonic	Immersion of microalgal suspension in ultrasonic bath, destruction of the cell wall with ultrasound from 10 kHz to 20 MHz	Minutes to hours	Destroys algal cells and speeds up the hydrolysis process	High efficiency No other chemical reagents required	Heat generated by ultrasound needs to be cooled	Shanmugam et al. Snehya et al.[55,56]
Microwave irradiation	Electromagnetic waves in the frequency ranging 0.3–300 GHz.	Minutes to hours	Improves the solubility of cellular organic matter through thermal and non-thermal effects	Fast heating Short radiation time Non-contact heating Minimum space requirements	High energy consumption Unsuitable for scale expansion	Dinesh Kumar et al., Nagarajan et al., Yin et al.[43,57,58]
Acidolysis pretreatment	Pretreatment with inorganic acids, such as HCl, H_2SO_4 and HNO_3 with concentrations ranging from 0.1 to 6.0 M	20–90 min	Changes in pH changes cell membranes, causing cell rupture and dissolution of organic matter	Easy to enlarge Low energy consumption Simple to operate	Discharge of waste pollutes the environment Requires a lot of reagents Corrosion of the reactor	Chozhavendhan et al., Yin et al.[31,52]
Enzyme pretreatment	Utilization of purified or crude enzymes hydrolyze microalgal cells	Hours to a few days	Destroys the structure of cell walls and dissolves carbohydrates	Mild conditions Low energy consumption Less inhibitor production	High cost Enzyme is difficult to recover Enzyme is easily inactivated	Sriyod et al., Zabed et al.[44,59]
Fungal pretreatment	Exposure of biomass to fungi using liquid or solid culture techniques	weeks to months	Produces hydrolases to hydrolyze cellulose and lignin	Mild conditions Simple equipment Low cost Low energy consumption	Long pretreatment time Loss of carbohydrates	Barati et al., Zabed et al.[44,47]
Bacterial pretreatment	Inoculate bacteria into biomass	Several days	Bacteria continuously produce hydrolytic enzymes to hydrolyze cellulose in biomass	Short incubation time Easier to carry out genetic engineering transformation than fungi Higher adaptability More suitable for algae	Acid produced in the hydrolysis process changes the pH value	Barati et al.[47]

Table 2. Comparison of different hydrogen production methods

	Organism	Advantage	Disadvantage	Reference
Photolysis	Green algae	Using light as energy The amount of solar energy fixed is ten times that of trees and crops Pure H_2 produced	Hydrogen production is inhibited by oxygen Short hydrogen production time High-intensity light is required	Bechara et al.[70]
Photofermentation	*Chlorella, Scenedesmus obliquus*	Spectrum range that can be used is wide Substrate is completely converted to H_2 and CO_2 Low energy required compared with photolysis Industrial waste can be used as the substrate	Need for light energy Pretreatment of substrate is required	Anwar et al., Liu et al.[39,71]
Dark fermentation	*Lyngbya limnetica, Scenedesmus*	Low price Does not depend on light Utilizes multiple carbon sources Utilizes waste Produces by-product acids	Produces mixed gas Relatively low H_2 production	Srivastava et al.[72]
Combination of light fermentation and dark fermentation	Microalgae and bacteria	Highest hydrogen production	Difficult to control the reactor conditions	Pandey et al.[73]

of enzymes is necessary to achieve efficient cell disruption and starch hydrolysis.[52,53] The enzymes used mainly include cellulase, hemicellulase, pectinase, protease, and amylase.[36] *Chlorella* biomass co-pretreated with cellulase and macerozyme yielded 2350 mg L^{-1} of hydrogen; when sonicated yeast was used as a source of hydrolases, the hydrogen production efficiency was 4200 mL L^{-1}.[54]

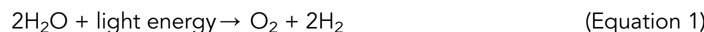
CONVENTIONAL STRATEGIES AND GENERAL REQUIREMENTS FOR BIOHYDROGEN PRODUCTION

Hydrogen production using microalgae is mainly done under ambient temperature and pressure with lower energy consumption than other methods.[60] For decades, microalgal hydrogen production strategies have made great progress toward increasing hydrogen production.[61,62] Typical methods of biohydrogen production include the direct or indirect decomposition of water into hydrogen using light energy, photofermentation, and anaerobic dark fermentation.[44,63] Methods of producing biohydrogen using microalgae are shown in Table 2. Nowadays, biohydrogen production methods are more diversified and efficient.[64] Hydrogen production from algal biomass remains at the laboratory scale, however, and is currently rarely used in large-scale commercial production.[25] Commercial production of microalgal biohydrogen requires multi-stage optimization to achieve low-cost sustainable operations and needs to be carried out in bioreactors.[65] Temperature primarily determines the accumulation of hydrogen, controlling the efficiency of the enzymatic reactions, and pH also plays a role.[60,66] The optimal pH for different strains of microalgae varies but, generally, at pHs under 5, the activity of hydrogenase is inhibited.[34] The most important component in photolysis and photofermentation is light.[34] Bioreactors were used to investigate the effect of light intensity on the continuous production of hydrogen, and different light intensities led to different hydrogen yields.[67,68] The response surface method optimizes several key factors of algal photobioreactor hydrogen production, and designs and builds pilot-scale reactors to produce biofuels.[69]

Direct photolysis of water

Biological photolysis can be categorized as either direct or indirect. Biohydrogen production, as a complex biochemical process, is affected by light, heat, reaction liquid composition, and mass transfer characteristics.[39]

Green algae (e.g., *Chlamydomonas reinhardtii*) exist in the ocean and humid soil and are photosynthetic autotrophs that have the same PS I and PS II systems as plants.[74] Using the hydrogenase PS II photosystem on the thylakoid membrane, microalgal cells decompose water into oxygen and hydrogen using solar energy[75]; the hydrogen ion is then converted into hydrogen by hydrogenases.[66] Overall, the total reaction is as follows:



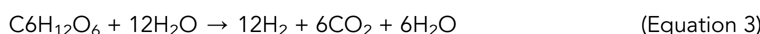
$$2H_2O + \text{light energy} \rightarrow O_2 + 2H_2 \quad \text{(Equation 1)}$$

Hydrogenase activity can be inhibited by 2% oxygen[34]; therefore, in the natural state, the reaction time for hydrogen production is reduced by oxygen inhibition, and efficiency becomes very low.[39] To combat this, genetic engineering or the utilization of antioxidants has been employed to improve hydrogen yield. Particularly popular strategies are the use of stable gene expression and genetic evolution.[76] Hydrogen production pathways from direct photolysis, indirect photolysis, and photo-fermentation are described in Figure 1.

Indirect photolysis

In indirect photolysis, electrons produced by water decomposition are not directly used for hydrogen generation, as hydrogen production is temporarily separated from photosynthesis. Electrons are first used to synthesize carbohydrates and oxygen, after which the carbohydrates are catabolized to produce hydrogen.[79] Indirect photolysis consists of two steps: carbon dioxide fixation by photosynthesis and carbohydrate decomposition.[39] The reaction steps are as follows:

$$12H_2O + 6CO_2 + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O \quad \text{(Equation 2)}$$



$$C6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2 + 6H_2O \quad \text{(Equation 3)}$$

Compared with green microalgae, indirect hydrogen production by cyanobacteria is more attractive.[80] Indirect photolysis is observed in some nitrogen-fixing and non-nitrogen-fixing cyanobacteria, utilizing nitrogenase and hydrogenase enzymes.[76] The problems surrounding the oxygen sensitivities of hydrogenase and nitrogenase are solved by temporal and spatial separation of the production of hydrogen.[81] Non-nitrogen-fixing cyanobacteria such as *Synechocystis*, *Synechococcus*, and *Myxococcus* produce a bidirectional Ni-Fe hydrogenase.[81] Generally, this enzyme exists in the cytoplasm loosely bound to thylakoids and acts effectively under low hydrogen partial pressure.[82,79] These cyanobacteria carry out photosynthesis and carbon dioxide fixation under light, following which carbohydrates are fermented to produce hydrogen without light exposure.[79] There are special structures known as 'heterocysts' in nitrogen-fixing cyanobacteria such as *Chlorella* and *Anabaena*.[80] Compared with hydrogenase, nitrogenase catalyzes an irreversible process and is mainly responsible for reducing nitrogen in the atmosphere to ammonia accompanied by the production of hydrogen.[83] Heterocysts spatially separate photosynthesis from the hydrogen production catalyzed by nitrogenase, keeping the enzyme from contacting oxygen to maintain its activity.[34,84] Nitrogen-fixing cyanobacteria also have Ni-Fe hydrogenases, which catalyze the oxidation of hydrogen to effectively recover the electrons released during nitrogen fixation.[79] Bidirectional hydrogenases are more efficient than nitrogenases, however, because it does not need ATP to generate hydrogen.[79]

Hydrogen production by photofermentation

Photofermentation degrades organic substrates into small molecules in the presence of light[39]:

$$C_6H_{12}O_6 + 6H_2O + \text{light energy} = 6CO_2 + 12H_2 \quad \text{(Equation 4)}$$

Photofermentation is different from photolysis, which requires the reduction of substrates.[39] Nitrogenase is the main enzyme in microalgae photofermentation, which obtains electrons through photocatabolism of organic substrates and heterogeneous fermentation.[85,86] Photofermentation provides electrons through the citric acid cycle and produces hydrogen under the catalysis of nitrogenase and hydrogenase[9,87]; in addition, it requires a large amount of ATP to promote electron transport and hydrogen production.[39] The hydrogen produced by this method is relatively pure, however, so the need for purification of the generated gas is reduced.[88] The fermentation efficiency is affected by light availability, light intensity, carbon source, and microbial fermentation ability.[89] The main disadvantage of photo-fermentation is the inhibition of nitrogenase by oxygen, which requires cells to separate oxygen production and nitrogen fixation; another solution for this is to run the fermentation in the absence of nitrogen.[75]

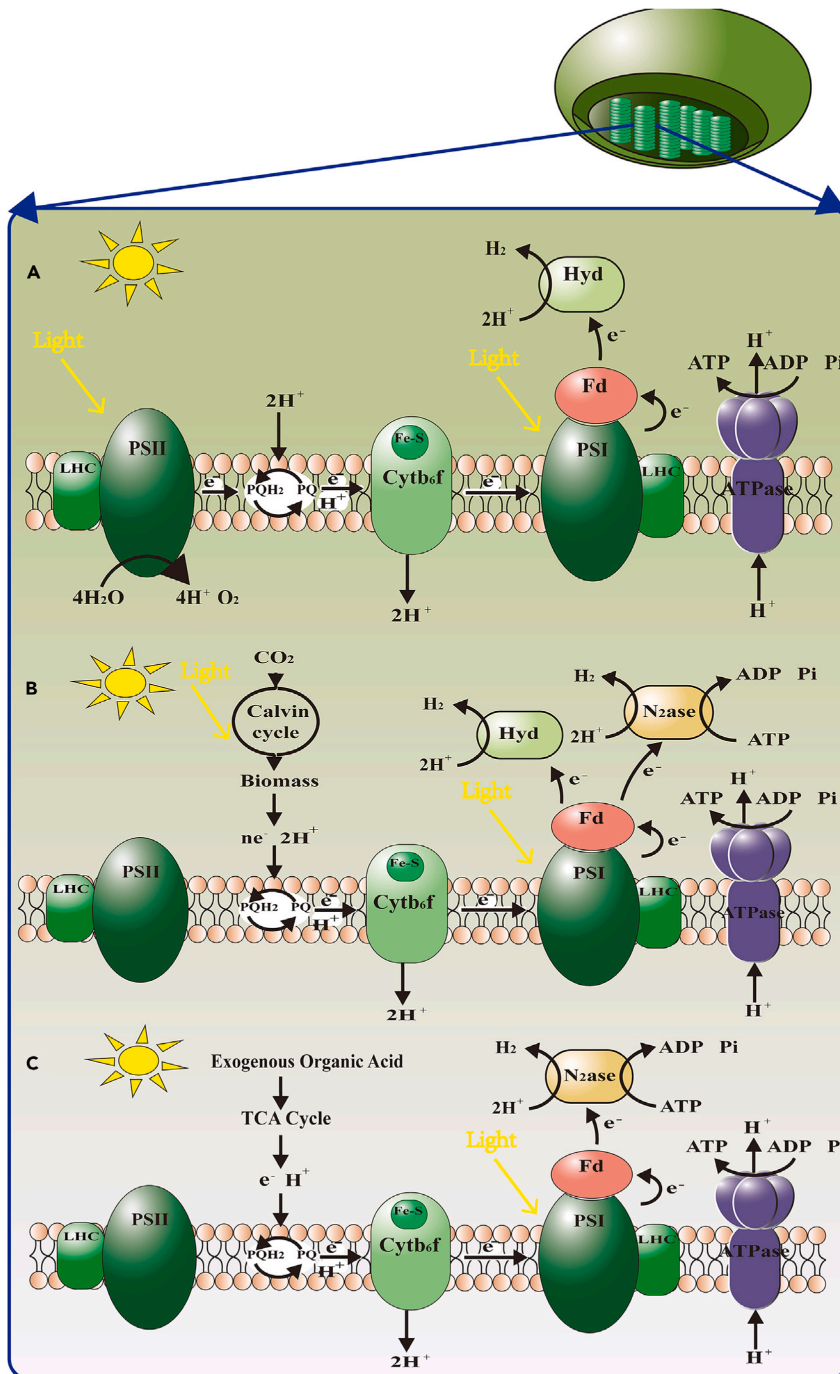


Figure 1. Overview of hydrogen production by microalgal chloroplasts

(A) Direct photolysis process of microalgae.

(B) Indirect photolysis process of microalgae.

(C) Photo fermentation process of microalgae. Figure redrew from reference (A–C).[74,77,78]

Hydrogen production by dark fermentation

Dark fermentation is a light-independent heterotrophic hydrogen production pathway (Figure 2).[90] Biomass with high carbon and water content such as sewage, food waste, and agricultural residues can be used as raw materials.[91]

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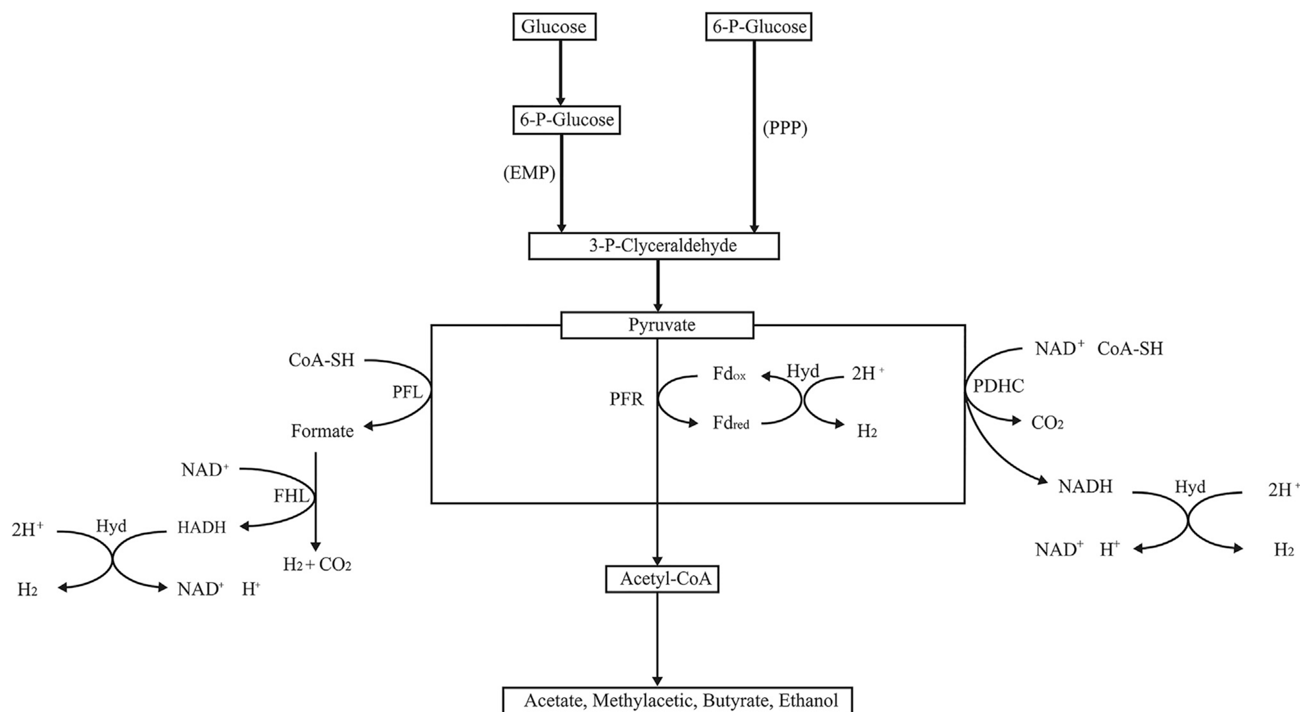

Figure 2. Hydrogen production by dark fermentation of microalgae

The three main dark fermentation processes of microalgae to produce hydrogen are shown. Formic acid catabolism, NADH re-oxidation, and pyruvate ferredoxin oxidoreductase (PFR) oxidation of pyruvate are the three main ways in which microalgae dark fermentation produces hydrogen. Figure redrew from ref.[74,92–95]

In the dark, microalgae obtain energy through the oxidation and reduction of organic compounds.[96] Carbohydrates are first hydrolyzed to glucose, which is then converted with NAD^+ into pyruvic acid and NADH through glycolysis; the pyruvate is then converted into acetyl coenzyme to produce formic acid and NADH.[97] There are three processes for producing biohydrogen through dark fermentation: formic acid catabolism, NADH re-oxidation, and pyruvate ferredoxin oxidoreductase (PFR) oxidation of pyruvate.[90] The redox reaction in microalgal cells also needs to be stabilized through the production of reducing compounds such as ethanol, butanol, and lactic acid.[86] Slow hydrolysis is the rate-limiting step in the production of hydrogen by this method, and thus, pretreatment of biomass is required.[96]

The primary problem facing industrial hydrogen production is insufficient hydrogen production; the most fundamental reasons for this are the limitations of microalgal biology.[86] Hydrogen production from dark fermentation can be optimized through the enhancement of biological activity and improvement of electron transfer during microbial fermentation.[98] The application of genetic engineering, metabolic engineering, molecular biology, and other disciplines is very important to achieve these ends.

GENETIC ENGINEERING FOR MICROALGAE FERMENTATION AS SUBSTRATES TO PRODUCE HYDROGEN

The proportion of carbohydrates in microalgal organic matter determines its applicability for hydrogen production.[97] Microalgae are photosynthetic autotrophs, and carbohydrate accumulation occurs through photosynthesis; carbohydrates manufactured by photosynthesis can be stored as subunits of cell walls or plastids as starch and cellulose.[55] Ideal substrate strains exhibit high biomass productivity and carbohydrate content.[99] Overexpression of carbohydrate synthetase, knockout of carbohydrate suppressor genes, and knock-in of carbohydrate synthesis genes can enhance intracellular carbohydrate productivity.[61]

Enhancement of microalgal carbohydrate content using genetic engineering

Increasing microalgal carbon fixation capacity is very important for increasing biomass.[100] Overexpression of enzymes involved in carbohydrate synthesis can increase the carbohydrate content inside microalgae

cells. A sucrose-phosphate synthase (SPS) encoding-gene was overexpressed in *Anabaena* sp. PCC 7120, and the resulting strains over-accumulated sucrose up to 10% (w/w) on a dry biomass basis under NaCl stress.[101] Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most important enzyme for carbon fixation in photosynthetic organisms, and the rate of carbon fixation is determined by its catalytic ability[102]; in the air, however, it has only 25% of its catalytic capacity.[100] Enhancing Rubisco activity has been shown to improve carbon fixation. An enzyme with ATPase activity, Rubisco Activase (RCA) uses ATP hydrolysis to remove various inhibitors at the Rubisco catalytic site (tightly bound sugar phosphates). Vectors were constructed that overexpressed putative RCA genes revealed through genome-wide analysis in *Nannochloropsis oceanica*, and the resulting transgenic strains had increased Rubisco activity, photosynthesis rates, and biomass productivity.[103] Fructose 1,6-bisphosphate aldolase helps determine carbon allocation in the Calvin cycle, where it catalyzes the reversible conversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate to fructose 1,6-bisphosphate (FBP). Aldolase plays an important role in the branching point of DHAP metabolism and is a key intermediate in starch and sucrose biosynthesis. When the cyanobacterial fructose 1,6-bisphosphate aldolase gene tied to a plastid transit peptide was introduced into *Chlorella vulgaris*, aldolase overexpression induced higher Rubisco activation, resulting in a 1.2-fold increase in photosynthetic capacity.[102]

The atmospheric expansion efficiency of carbon dioxide is very low, and the entry of carbon dioxide into the carbon cycle is very important for carbon fixation. The carbon dioxide fixation capacity of microalgae is 10–50 times higher than that of terrestrial plants.[104] Microalgae prefer to fix carbon dioxide as carbonate.[105] Carbonic anhydrase (CA) is present in most living organisms and catalyzes the conversion of carbon dioxide and carbonat.[78] A CA with high activity (MlCA) from *Mesorhizobium loti* was identified in the BRaunschweig ENzyme DAtabase (BRENDA); when overexpressed in *Chlorella sorokiniana* and *C. vulgaris*, accelerated carbon capture and fixation was observed, resulting in a lipid content 2.2 times higher than that of the wild type strains.[106]

Reduce the breakdown of starch or competition for lipid synthesis

Carbon fixation competes with carbohydrate and lipid synthesis, as glycerol-3-phosphate from glycolysis is the precursor for both.[107] The most important step in starch biosynthesis is the activation of glucose, which is converted into nucleoside-diphosphate-glucose (ADPGlc) by ADP-glucose pyrophosphorylase (AGPase).[108] Overexpression of AGPase in microalgae enhances starch biosynthesis.[109]

Plastidial lysophosphatidic acid acyltransferase (LPAAT) catalyzes the acylation of lysophosphatidic acid (LPA) to produce phosphatidic acid, the second step in the Kennedy pathway responsible for glycerolipid biosynthesis. Nitrogen (N) deficiency is the most widely used strategy to trigger the accumulation of stored metabolites in microalgae.[110] Overexpression of LPAAT in *Chlamydomonas reinhardtii* strains increased lipid content under adequate N, whereas lipid synthesis decreased and starch content increased by 50% when N was depleted. The putative regulatory gene encoding N-acetyltransferase (GNAT19) was identified by transcriptomic analysis and cloned, and was up-regulated 11- to 12-fold under N depletion. Overexpression of GNAT19 significantly increased the starch content of microalgae, showing great potential for enhancing biohydrogen production.[111] Many microalgae alter their metabolism under phosphorus (P) starvation, inducing biosynthesis of stored metabolites. Pi Starvation Response1 (PSR1) is a transcription factor that regulates carbon storage metabolism under P starvation. In *C. reinhardtii* mutants lacking PSR1, P starvation-induced lipid and starch accumulation was inhibited; overexpression led to increased starch content and decreased neutral lipid content, which correlated with higher expression of specific starch metabolism genes.[112]

In microalgae, starch degradation provides nutrients for cell metabolism and growth in the dark; starch also provides a carbon backbone for lipid biosynthesis under adverse conditions.[111] A decline in starch levels is largely attributed to starch catabolism. Manipulation of metabolism through genetic or metabolic engineering to reduce starch degradation is essential to improve biohydrogen production.[113] Knockout of starch-inhibiting genes (glucan-water dikinases) and amylases can inhibit its degradation, increasing the accumulation of intracellular starch.[61] So far, little is known about the regulation of starch metabolic changes in microalgae, and future efforts need to identify metabolism-specific transcriptional regulators.[112]

Increasing photosynthetic efficiency

Microalgae convert sunlight into organic molecules and biomass through photosynthesis. In regards to carbon dioxide fixation efficiency, improving light utilization efficiency helps to reduce the culturing and

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biomass pretreatment costs.[114] Generally, it is difficult to improve the photosynthetic efficiency in traditional crops, but it is much simpler to alter microalgae through genetic engineering.[66,115] Genetic engineering including knockouts, replacements, or insertions to the photosynthetic system has been shown to improve biomass accumulation.[114]

Microalgal cells have a powerful light-harvesting complex (LHC).[115] The LHC complex in *C. reinhardtii* captures light energy before transferring it to the photosystem[116]; the complex also adapts to fluctuations in light quality. The LHC can both capture photons and disperse additional light energy to provide light protection.[117] The light utilization efficiency of microalgae is largely limited by light transmittance. In one study, the efficiency of light energy absorption by large-scale cultured microalgal cells in the upper layer of the culture was higher than the rate of photosynthesis, meaning that 95% of light energy was wasted; the shielded microalgae cells in the lower layer could not carry out photosynthesis.[62] Genetic manipulations overcoming the limitations of LHC antenna size and light saturation have been shown to improve the carbon fixation and light conversion efficiency of photosynthesis.[86] Microalgae adapt to light conditions by regulating LHC expression; the cytoplasmic RNA binding protein NAB1 binds to the mRNA of the LHC gene to prevent its translation; the absence of NAB1 should therefore increase LHC antenna size. To test this, a plasmid expressing a mutated NAB1 coding sequence (C-terminal cysteine residues at positions 181 and 226 were replaced by serine) was constructed; the mutations in these two amino acids resulted in a permanently active variant of NAB1 that reduced antenna size. A suitable NAB1 promoter could then be used to control the antenna size, adjusting according to growth stage and light fluctuation status, improving light transmission.[118] Alternately, an LHC-deficient mutant was generated in *C. reinhardtii* by DNA insertional mutagenesis. The smaller LHC antenna reduced excessive light absorption, allowing light energy to reach the lower microalgae cells in the culture; this mutant showed higher photosynthetic productivity in large-scale culture.[119,120] Theoretically, truncated LHC antenna sizes could increase photosynthetic solar conversion efficiency and productivity by up to three times in large-scale cultivation.

GENETIC ENGINEERING FOR MICROALGAL HYDROGEN PRODUCTION USING THEIR METABOLISM

Compared with fossil fuels, algae biofuels are not competitive in cost.[61] To reduce the cost of hydrogen production, microalgal genetic engineering strategies have attracted more attention.[121] Genetic engineering techniques such as foreign gene expression, RNA interference, gene silencing, and gene disruption have been carried out in many species to improve biohydrogen production.[61,62]; These strategies aim to either change the key enzymes directly or indirectly involved in algal hydrogen production or increase the electron flow to enzymes.[61] Different genetic engineering approaches for improving H_2 yield in microalgae are described in Figure 3.

Improving light capture efficiency

As has been previously discussed, reducing the size of LHC antennas to improve light energy utilization efficiency to increase hydrogen production has shown promise.[114] In addition, a mutant of *C. reinhardtii* with a smaller light capture antenna system was constructed by transforming LHC translation inhibitors; here, the hydrogen yield of the mutant increased by 50% with heterotrophic growth with exogenous glucose.[118]

Reducing the size of chloroplast can also improve photosynthetic efficiency. The chlorophyll content per unit cell volume of a chloroplast in a *C. reinhardtii* mutant was decreased, leading to better light transmittance and higher solar energy conversion efficiency. The maximum hydrogen accumulation of the mutant in pure culture as well as in co-culture with *Pseudomonas* was 1.8–5.2 times and 2.7–3.1 times that of the wild-type, respectively.[123]

Altering hydrogenase and nitrogenase activity

Hydrogenase and nitrogenase are two key enzymes responsible for hydrogen production in microalgae; as these enzymes are easily inhibited by oxygen, genetic engineering has been attempted to improve their activities, including changing the structure of the enzyme, foreign gene expression, altering the absorption of oxygen, and reducing the consumption of hydrogen.[40,98]

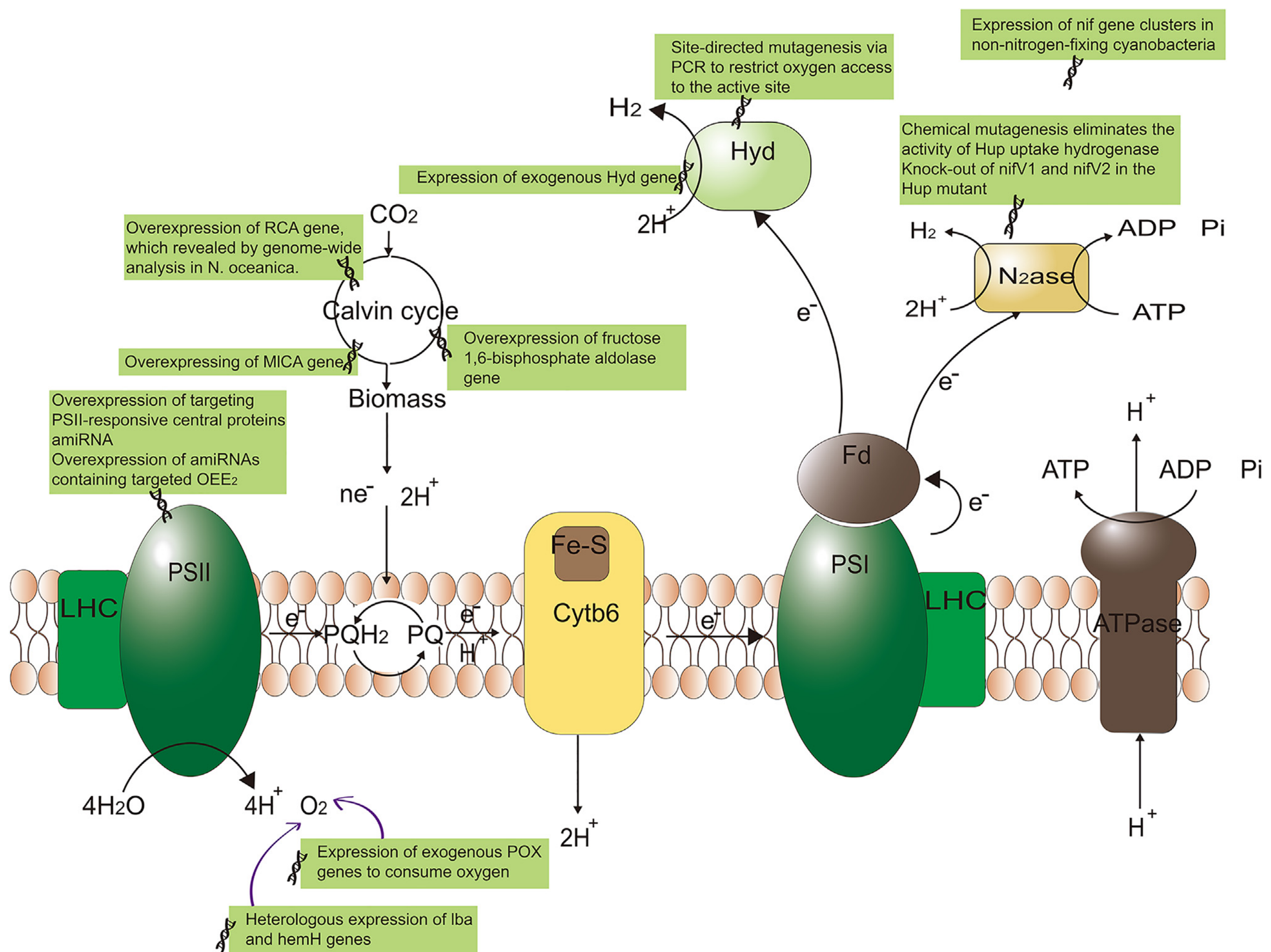

Figure 3. Different Genetic engineering approaches for improvement the H_2 yield

Genetic engineering approaches for microalgae to improve biohydrogen are shown. The genetic engineering approaches shown here include enhancing carbohydrate content, increasing photosynthetic efficiency, altering hydrogenase or nitrogenase activity and optimizing electronic sources. Figure redrew from ref.[122]

Hydrogenase enzymes can be divided into Fe-Fe, Ni-Fe, and Fe types according to the metals present in the enzyme's active site.[73] The activity of Fe-Fe hydrogenase has been demonstrated to be 100 times higher than that of other hydrogenases.[117,124] Of interest, transcription, translation, and enzyme activity of microalgal hydrogenases are controlled by oxygen.[121] Screening for oxygen-tolerant hydrogenases created by random mutation is the most common method currently used to find improved enzymes in green algae[40]; however, this method is time-consuming and high-throughput.[125,126] Overexpression of hydrogenase can also increase hydrogen production, and hydrogenases of different microbes exhibit different levels of activity.[84] As an example, *Clostridium acetobutylicum* is capable of overexpressing various Fe-Fe hydrogenases, however, protein production is quite low; expression vectors from *C. reinhardtii* were successfully heterologously expressed in *C. acetobutylicum* leading to high hydrogenase content (1 mg protein per liter of cells).[124]

The inhibition of hydrogenases by oxygen is caused by the degradation of 2Fe and 4Fe-4S clusters when oxygen binds the enzyme active sites.[126] To help circumvent this, the oxygen gas channel structure of microalgae can be reduced to prevent the entry of oxygen. Overexpression of hydrogenases with high O_2 tolerance and H_2 yield as well as site-directed mutagenesis to replace amino acid residues around the hydrogenase gas tunnel to restrict oxygen access to the active site have been tested; the resulting mutant had improved tolerance to oxygen and 30 times the hydrogen production of the wild-type strain.[127]

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Hydrogenase activity can also be increased by increasing oxygen consumption; although a sulfur-deficient culture can solve this problem, the cost of this method is high.[128] Under normal conditions intracellular oxidases cannot consume all the oxygen produced by photosynthesis. Decreasing cellular oxygen levels is a major area of research. Full-field fragments of pyruvate oxidase genes from *E. coli* (POX) and catalase from *Synechococcus elongatus* (CAT) to prevent toxicity of H_2O_2, a byproduct of POX, were cloned into vectors. Expression in *C. reinhardtii*, resulted in reduced oxygen content and increased hydrogen production in sealed culture; after 48 h, hydrogen production in low light was 100 times higher than that of the wild type.[129] The advantage of this method is that it allows a reduction in oxygen concentration without impacting the photosynthetic rate, enabling the production of more hydrogen.[45] Similarly, genetic manipulation of microalgae using genes from methanotrophs to increase oxygen tolerance and enhance hydrogen production may also be effective.[129]

When sulfur is deficient, hydrogen can be continuously produced. The lack of sulfur limits the synthesis of methionine and cysteine, inactivating photosystem II (PSII) and resulting in photosynthetic oxygen release at a lower rate than respiration.[130] miRNAs are a group of small noncoding RNAs that are ubiquitous in various gene regulatory pathways in eukaryotes that are involved in post-transcriptional gene expression regulation in plants and animals.[131] Changing miRNA expression may affect many metabolic processes.[126] Under sulfur-deficient culture conditions, *C. reinhardtii* microRNA (miRNA) expression was found to be up-regulated, with expression changes being closely related to the level of sulfur deficiency.[131] Artificial miRNA gene sequences were also constructed into a highly expressed blue light-inducible system to target a PSII reaction-center protein in *C. reinhardtii*; after blue light treatment, the PSII response center protein was down-regulated.[132]

Artificial miRNA could also potentially be used to control the expression of the oxygen-evolving enhancer (OEE_2) gene in PSII to increase hydrogen production in *C. reinhardtii*. OEE_2 is a potent PS II-related protein involved in photosynthetic oxygen precipitation, and previous studies have shown that OEE_2 expression decreased significantly after sulfur deprivation[39,27]; A heat-shock inducible expression vector containing miRNA targeting OEE_2 was constructed and successfully transformed into *C. reinhardtii*. After repeated heat induction of the same microalgae culture, hydrogen accumulation was doubled when compared to the control.[133] Compared with sulfur-deficient culture conditions, genetic engineering is advantageous as culture media does not need to be changed, making this method more suitable for large-scale production.[133]

In addition to hydrogenases, cyanobacteria contain nitrogenase to fix nitrogen.[134] Different from the reversible formation of hydrogen using protons, nitrogenase catalyzes the unidirectional production of hydrogen.[135] Owing to the unidirectional nature of the catalytic reaction, it was previously thought that hydrogen could be produced more easily with nitrogenases compared to hydrogenases[83]; however, it has since been shown that the catalytic efficiency of nitrogenase is much lower. Nitrogenase requires a large number of electrons, a reducing agent, and ATP to produce large volumes of hydrogen[117]; therefore, genetic engineering is key to hydrogen production utilizing this enzyme.[136]

In nitrogen-fixing cyanobacteria, a Hup uptake hydrogenase reduces the net hydrogen production of nitrogen-fixing cyanobacteria.[137] A promising method for improving biohydrogen production in nitrogen-fixing cyanobacteria in the presence of oxygen is to select strains with high nitrogenase activity and eliminate the activity of the Hup uptake hydrogenase.[135] A mutant lacking Hup was created by chemical mutagenesis (*Nostoc* sp. *PCC 7120*)[138]; elimination of Hup activity was very effective in increasing hydrogen accumulation by the strain.[139] *Nostoc* sp. *PCC 7422* had the highest nitrogenase activity out of the strains examined in the study, and a mutant of this strain was constructed that contained a *hupL* gene insertional disruption (Δ*hupL*). The knock-out improved the hydrogen production rate in the optimal hydrogen production stage to 4–7 times higher than that of the wild-type strain.[140]

Hydrogen production can also be increased by reducing the fixed electron distribution to nitrogen by replacing nitrogen gas with argon, but the cost of this method is very high, making it unsuitable for large-scale production.[135] Although the inactivation of uptake hydrogenase can increase nitrogenase activity, the high nitrogenase activity of the mutant in the previous study was only stable in the air for around 10 h. Citrate combined with the Fe-Mo cofactor of nitrogenase is important for efficient nitrogen fixation, but not necessary for hydrogen production. There are two homocitrate synthase genes (*nifV1* and *nifV2*)

in cyanobacteria[139]; using these, homocitrate synthase gene knock-out mutations were performed in the Hup mutant of *Nostoc* sp. *PCC 7120* (Δ*hupL*). Compared with the Δ*hupL* mutant, the double mutant had higher hydrogen production capacity and nitrogenase activity. Changing homocitrate synthase activity may therefore be an effective strategy to improve the sustained hydrogen production capacity of nitrogen-fixing cyanobacteria and other microalgal strains.[141]

To achieve high expression of this gene, nitrogenase must be expressed in cyanobacteria lacking the *nif* gene cluster.[142] The minimal *nif* gene cluster from *Bacteroides* was integrated into the genome of the non-nitrogen-fixing *S. elongatus PCC 7942* for nitrogenase expression. This minimal *nif* gene cluster is divided into six segments, each of which has an independent promoter and terminator; this solves the problem of decreased gene expression levels caused by the long *nif* gene cluster. A bioelectrochemical nitrogen fixation (e-BNF) system was formed by coupling the biological and electrochemical systems of the recombinant strain; methyl viologen (MV) provided the reducing power for nitrogenase, and PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was used to inhibit photosynthetic oxygen production. The recombined *S. elongatus PCC 7942* was able to continuously carry out nitrogen fixation and hydrogen production; after 48 h, the nitrogen fixation efficiency was 21 times that of purely photosynthetic nitrogen fixation systems.[143]

Hemoglobin is an oxygen-binding protein that exists widely in nature. Leguminous hemoglobin (encoded by the *lba* gene) combines with oxygen in root nodules during nitrogen fixation in leguminous plants and plays an important role in nitrogen metabolism.[144] The heterologous expression of hemoglobin could provide a solution for the inactivation of microalgal nitrogenase when oxygen is present[145]; however, few studies have examined recombinant *lba* expressed in transgenic algae. After optimizing codon bias, *lba* and *hemH* (ferrochelatase gene from *Bradyrhizobium japonicum*) were transformed into the chloroplasts of *C. reinhardtii*; the hydrogen yield of the codon-optimized transgenic *C. reinhardtii* was 22% higher than that of the non-optimized strain.[146]

Electronic supply optimization

There are two main ways microalgae produce hydrogen from light; PSII water decomposition and electron transfer to hydrogenase, or oxidation of organic substrates.[147] The first pathway uses photosynthesis, which consists of a light-utilizing reaction and a reaction that does not use light. The light reaction obtains electrons from water decomposition that are transferred from PSII to ferredoxin (FDX) through the electron transport chain, resulting in the production of ATP and NAD(P)H during the dark reaction; carbon dioxide is therefore reduced and fixed to an organic form as starch or glycogen.[148] Overall, during direct photolysis, FDX provides electrons for hydrogenase and H_2 come from protons and electrons produced by PSII water decomposition. Indirect photolysis hydrogen production is the result of intracellular carbohydrate degradation.[148]

Insufficient electron supply is a barrier to H_2 synthesis.[149] The hydrogenase iron-sulfur cluster (FeS) with redox activity in the center of FDX plays a role in electron transfer in microalgal cells.[150] Through photosynthesis, H^+ is released into the thylakoid lumen to establish a proton gradient.[151] Electrons are transferred from water to FDX through the electron transfer chain.[152] Proton carrier enhancement combined with thylakoid proton power dissipation promotes the availability of protons and electrons to Fe-Fe hydrogenase, forming hydrogen.[147] FDX acts as a switch between carbon fixation and hydrogen production, as well as other environment-related functions.[149] Hydrogenase needs to compete with other metabolic processes for electrons[153]; to circumvent this, genetic manipulation can increase electron flow to hydrogenase.[134]

Naturally expressed hydrogenases exist as a defense mechanism against excess electrons in microalgae.[154] FDX directly supplies electrons to hydrogenase, but FDX availability can be limited because of other competitive processes[155,150]; Reducing electron competition at the FDX level can improve the affinity of hydrogenase for FDX.[156] In one study, FDX was fused with Fe-Fe hydrogenase and expressed in *C. reinhardtii*; the resulting mutation improved the electron competitiveness of the hydrogenase, and the photosynthetic hydrogen production rate was 4.5 times higher than that of the strain containing the natural enzyme.[154] Other genes can also be modified to improve electron flow to hydrogenase. Ferredoxin $NADP^+$ reductase (FNR) is an enzyme in microalgae that catalyzes the conversion of FDX to $NADP^+$; however, under anaerobic conditions, the reaction catalyzed by this enzyme competes with that of hydrogenase. Using RNA interference (RNAi), double-stranded RNA targeting the FNR gene was introduced

into *C. reinhardtii* to inhibit the expression of FNR; hydrogen production in the mutant was 2.5 times higher than that in the wild-type.[157]

The fixation of carbon dioxide is the main competitor to hydrogenase electron flow.[158] Rubisco, as discussed earlier, is a key enzyme responsible for carbon dioxide fixation. Although overexpression of Rubisco greatly improved carbon fixation and biomass production, it also led to an increase in oxygen release, which negatively impacts hydrogenase activity.[159] When Rubisco was inhibited, the corresponding hydrogenase activity increased.[160] In Rubisco mutant Y67A of *C. reinhardtii*, the tyrosine residues at positions 67, 68, and 72 were replaced by alanine, weakening the enzyme's stability; the resulting mutant had a high potential for hydrogen production under normal light without nutrition, with 10–15 times higher hydrogen production than that of the wild-type. At the beginning of the culture, Rubisco protein content increased before decreasing rapidly as the protein degraded. The degradation of Rubisco inhibited carbon fixation, resulting in an excessive reduction in photosynthetic electron transport; hydrogenase was then widely expressed to relieve the metabolic pressure in cells. With the same photosynthetic and respiratory rates, Y67A could reach anaerobic conditions under normal light without the deactivation of hydrogenase.[158]

ATP synthase is a multi-subunit complex that combines proton transfer and ATP synthesis. Although the synthesis of ATP is very important for carbon dioxide fixation, the demand for ATP decreases in the hydrogen generation stage.[150] When the electron transport chain is disconnected from ATP generation, carbon fixation is reduced because of insufficient energy being available.[149] Robertson et al. characterized a chloroplast mutant of *C. reinhardtii* that produced an epsilon truncated polypeptide because of a frameshift mutation; the epsilon subunit protein is necessary for ATP synthase, preventing proton leakage and aiding assembly. Owing to this mutation, the assembly and function of ATP synthase were impaired, leading to the inactivation of the main metabolic pathway competing with hydrogenase for electrons, causing more electron flow to be used for hydrogen production.[161,162]

The photosynthetic electron transport chain driven by PSII, cytochrome *b*6f (Cytb6f) complex, and photosystem I (PSI) is on the thylakoid membrane[163]; PSII and PSI are located in the stacked thylakoid membrane and stromal layer, respectively. The reason for the spatial separation of PSII and PSI is to prevent energy from being transmitted from PSII to PSI because of the close contact of their antenna systems. In anaerobic microalgal culture, the protein on PSII attaches to PSI, sacrificing PSII to increase light absorption capacity. When an increase in PSI cyclic electron flow (CEF) compared with PSII linear electron flow (LEF), the proton gradient produced by CEF was found to limit the electron supply of hydrogenase leading to an increase in ATP synthesis and a decrease in hydrogen production.[151,155] Therefore, inhibition of CEF can improve photosynthetic hydrogen production efficiency.[151] Random insertion of resistance genes to produce PRGL1 knockout mutants (pgrl1-ko) of *C. reinhardtii* resulted in a lack of photosynthetic CEF in the mutant; electron flow then went to hydrogenase, acting as a safety mechanism for cellular health. Under higher light intensity, the hydrogen production rate of the mutant remained high, whereas that of their wild-type counterparts decreased rapidly.[163] The same findings were also found using a mutant strain of *C. reinhardtii* where proton gradient regulatory protein (PGR5) was affected; the mutant lacked CEF and had high respiratory activity, breaking through the two bottlenecks of hydrogen production. The mutant was able to prolong hydrogen production for 12 days under mixed nutrition conditions and was an ideal strain for hydrogen production.[164]

Metabolic engineering regulation

Genome editing enables metabolic engineering to reconstruct metabolic pathways to produce biological products and has been used to build highly efficient microbial factories.[165] In recent years, great progress has been made in the production of bio-based chemicals with the help of advanced tools and strategies for systems metabolic engineering.[166]

Gene manipulation of hydrogenase can improve enzyme activity, and is suitable for use with both light and dark fermentation. Biohydrogen production by microalgae is transient under natural conditions and is mainly controlled by the cell's metabolic network.[167] Metabolic engineering has great potential for increasing the production of microbial biological hydrogen.[94] Microalgal metabolic networks are highly complex, nonlinear, and closely coordinated; microalgal cells carry out various metabolic processes organized to meet the needs caused by their environment. Cellular metabolism is first applied to growth rather than hydrogen production, but with the help of metabolic engineering, the hydrogen production pathway

can be systematically and quantitatively improved.[94,168] Microalgae have been transformed to eliminate bottlenecks, increase substrate utilization, and alternate substrate metabolic pathways can be introduced into the metabolic network. The modified microalgae have all been shown to continuously produce hydrogen in a reactor environment.[10] Some metabolic engineering approaches have also achieved good results by emphasizing hydrogen production by blocking other metabolic processes. A wide range of combined strategies have been shown to significantly increase hydrogen, though combinatorial approaches have been primarily confirmed with hydrogen production in *Escherichia coli*.[94] The application of advanced metabolic engineering in microalgae is expected to remove the limitation of low metabolic efficiency. These engineering approaches include providing energy to break thermodynamic barriers, expressing heterologous proteins, achieving more complete substrate conversions, and increasing proton reduction electron flux.[169]

The natural metabolic pathways of microalgae are diverse. Dark fermentation in particular has a low energy demand and is simple to run.[20,170] In industrial production, dark fermentation is easier to control and has shown higher industrial stability and feasibility.[171] The primary problem with this approach is that only one-third of the substrate can be used for hydrogen production, as the remaining two-thirds are used to produce additional fermentation products.[172] The nitrate assimilation pathway in unicellular cyanobacteria (*Synechocystis* sp.) has the potential to compete with hydrogenase for electrons in dark fermentation. To improve hydrogen production, Baebprasert et al. deleted the nitrate assimilation pathway; the hydrogen production of the mutant strain was 140 times higher than that of the wild-type.[173]

The hexose transporter is a membrane protein that transports glucose into the cytoplasm. The plasma membrane of *C. reinhardtii* lacks hexose transporter proteins, but some microalgae can obtain nutrition through the overexpression of some hexose transporters.[174] Expression of *Chlorella* HUP1 hexose transporter in *C. reinhardtii* allowed for carbohydrates from external sources to provide protons and electrons to hydrogenase, improving hydrogen production.[175] Glucose transporters 1 (GLUT1) are a group of human uniporter protein, which facilitates bidirectional glucose transport. The GLUT1 on the human erythrocyte membrane is superior to that found in plants or algae; when introduced into *C. reinhardtii*, it led to very high glucose transport efficiency.[174]

With the development of a microalgal metabolism model (AlgaGEM) based on *C. reinhardtii*, it has become simpler to predict the effect of known mutations on hydrogen production. Using this metabolic model, the physiological pathway for hydrogen production under dark conditions was predicted, showing that when the circulating electron flow is prevented, hydrogen production will increase. The impact of this result on increasing hydrogen production is consistent with the results of studies in the literature.[176]

ECONOMIC AND TECHNICAL FEASIBILITY

The commercialization of biohydrogen faces severe economic and technical challenges. The production cost is mainly determined by the bioreactor and the selected process.[177] In microalgal large-scale cultivation, open pond systems are used more frequently as they are lower cost, however, the high water quality requirements increase operating costs. Cultivating microalgae for hydrogen production in 140 ha of open ponds or 14 ha of photobioreactors has been reported to cost US$6 m^{-2} and US$100 m^{-2}, respectively, with annual operating costs of US$43 million and US$10 million; the cost of producing biohydrogen was calculated to be US$10 GJ^{-1}, much higher than that of gasoline (US$2.5 GJ^{-1}).[86,178] Unfortunately, this analysis was too simplistic, as it did not include the cost of harvesting, purifying, and transporting hydrogen. With the current hydrogen production process, biohydrogen from microalgae is rather costly. An economic analysis report shows that the cost of hydrogen production by photolysis is between US$1.42 kg^{-1} and US$7.24 kg^{-1}, and the cost of hydrogen production by fermentation is between US$7.54 kg^{-1} and US$7.61 kg^{-1}[186] In addition, cultivation and reactor designs play a decisive role in the overall cost; overall, the average cost of biohydrogen production from algae is no less than US$10 GJ^{-1} (1.3kg^{-1}) under current conditions.[86,178]

For biohydrogen to be used as fuel, purification is required. Purification using a two-stage carbon film system can produce >99.5 vol % of high-purity biohydrogen at US$0.67/kg, which is more competitive with the state-of-the-art hydrogen purification technologies of pressure swing adsorption and cryogenic distillation.[179] With the expected future increase in hydrogen demand, hydrogen purification will develop scalable production at industrial capacities.

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Cost-effective biohydrogen requires low operational cost and high yield; the main limitation to commercialization is production cost. Although progress has been made, improving production efficiency using genetic or metabolic engineering remains challenging.[117] Genetic engineering can also be applied to other aspects of microalgal commercialization to reduce harvest costs, improve bioflocculant production, and allow biofuel production from wastewater[180]; expression of limonene synthase in microalgae has been shown to increase cell hydrophobicity and drive cell aggregation for sedimentation and harvest.[181] Future research should focus on the use of high-throughput genome-wide analysis in combination with the design of efficient bioreactors.[122] This requires the joint efforts of scientists to make fully establish biohydrogen as a future energy source.[19]

CONCLUSION

To combat resource depletion and environmental concerns, microalgal biohydrogen must be commercialized. The application of genetic and metabolic engineering along with synthetic biology has been shown to make biohydrogen production more cost-effective. With the understanding of algal genome sequences and metabolic functions, as well as the development of advanced tools and software, the combination of genetic engineering and bioinformatics will no doubt help to develop microalgae strains that are more suitable for biohydrogen production than those available currently. Although measures have been taken to improve the competitiveness of biohydrogen production, the infant stage of this technology has not been applied to large-scale hydrogen production. A tremendous amount of effort is still required before this technology could occupy a large market share.

Future directions and challenges

As a sustainable energy source, microalgal biohydrogen has attracted increasing international attention. It can be produced in two ways, with the most common being hydrogen fermentation using microalgal biomass as raw material as a sustainable and robust feedstock for large-scale energy production. Genetic and metabolic engineering can be used to improve the efficiency of photosynthesis, improve carbon sequestration by driving carbon fluxes into energy-rich compounds that can be used as hydrogen energy sources, and develop robust microalgae strains to allow for low-cost large-scale cultivation to reduce operating costs.[115,182]

Hydrogen can also be produced from microalgae through metabolism. There are two main processes involved in the production of biohydrogen; increasing biomass, and photolysis, photofermentation, or dark fermentation under anaerobic conditions.[183] Improving the efficiency of photosynthesis, rapidly accumulating carbohydrates, and maintaining cellular stability are important for the efficient use of water and organic matter. In addition, it is necessary to increase the activity of enzymes associated with hydrogen production through genetic manipulation, and reconstruct metabolic pathways through metabolic engineering to establish more efficient production strains.[121,165] Currently, there are bottlenecks in the large-scale production of biohydrogen, as production costs depend not only on the microorganisms used and metabolic pathways present[84]; it is also necessary to improve the technology used for microalgae biomass cultivation and harvesting. Adjustments to culture conditions may have a large impact on the biomass and carbohydrate contents, and optimal production can be achieved by a proper combination of genetic manipulation and culture condition engineering.[115]

Gene editing technology can overcome many obstacles, but the stability and safety of genetically-modified microalgae are also primary factors to consider, requiring further evaluation to ensure that they do not pose future risks to human and environmental health.[115] Another primary challenge in facing metabolic engineering is the limited number of available genes and the complex genotype-phenotypic relationships resulting from their combination with host organism metabolic pathways.[165] For downstream biohydrogen production, it is necessary to weaken the demand for light in downstream processes and consider more energy-efficient dark fermentation strategies. Biological hydrogen production also does not produce only one gas product, impacting hydrogen purity. Low-cost gas separation technologies need to be developed to remove by-products such as carbon dioxide.[184] For continuous production, periodicity is required, and the integration of steps in the production process is crucial. It is necessary to specifically understand the impact of each variable in each step on hydrogen production to achieve the highest possible hydrogen production.[108]

Recommendations

Application of genetic engineering in the field of biofuels is expanding rapidly. Genetic engineering tools solve many problems, and provide industrially capable microalgae strains. A systematic review of biofuel production in genetically modified (GM) algae between 2008 and 2019 found that one of the areas that received the least attention was environmental risk.[185] There are differences in biotechnology laws and regulations between countries; because of the lack of internationally harmonized regulations, the commercialization of genetically modified biofuels cannot be effectively applied. The use of genetic engineering can provide high yields, high growth rates, and insect resistance, but the impact of released modified plasmid or chromosomal DNA into surrounding water bodies has been overlooked.[186] Farming without a comprehensive risk assessment of GM strains can pose a serious threat to human and environmental health. More work should be done in regards to safety testing of GM organisms to assess the potential risks posed to the environment from the growth and processing of GM microalgae.

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J.Z.: Conceptualization, Methodology, Software, Data curation and processing, Validation, Writing–original draft. D.X.: Data curation, Investigation. C.W.: Validation, Writing–review and editing. D.F.: Software, Validation. L.C.: Methodology, Formal analysis. C.G.: Conceptualization, Methodology, Validation, Writing–review and editing, Supervision, Funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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