Fermentative hydrogen production using pretreated microalgal biomass as feedstock

Jianlong Wang1,2* and Yanan Yin1

Abstract
Microalgae are simple chlorophyll containing organisms, they have high photosynthetic efficiency and can synthesize and accumulate large quantities of carbohydrate biomass. They can be cultivated in fresh water, seawater and wastewater. They have been used as feedstock for producing biodiesel, bioethanol and biogas. The production of these biofuels can be integrated with CO2 mitigation, wastewater treatment, and the production of high-value chemicals. Biohydrogen from microalgae is renewable. Microalgae have several advantages compared to terrestrial plants, such as higher growth rate with superior CO2 fixation capacity; they do not need arable land to grow; they do not contain lignin. In this review, the biology of microalgae and the chemical composition of microalgae were briefly introduced, the advantages and disadvantages of hydrogen production from microalgae were discussed, and the pretreatment of microalgal biomass and the fermentative hydrogen production from microalgal biomass pretreated by different methods (including physical, chemical, biological and combined methods) were summarized and evaluated. For the production of biohydrogen from microalgae, the economic feasibility remains the most important aspect to consider. Several technological and economic issues must be addressed to achieve success on a commercial scale.

Keywords: Microalgae, Biohydrogen, Pretreatment, Fermentation

Background
The fossil fuels are depleting and resulting in serious environment issues. Hydrogen gas is regarded as a potential candidate for a future energy economy. Hydrogen is the only carbon-free fuel, with water as its final combustion product. Therefore the application of hydrogen will greatly contribute to the reduction of the energy-related environmental issues, such as greenhouse emission or acid rain [1, 2].

Biohydrogen is defined as hydrogen produced biologically, most commonly by algae, bacteria and archaea from both cultivation and from waste organic materials [3]. Most biologically produced hydrogen in the biosphere is evolved in microbial fermentation processes. These organisms decompose organic matter to carbon dioxide and hydrogen.

Microalgal biomass, being rich in carbohydrates, has great potential as feedstock for the production of various biofuels such as biodiesel, bioethanol, biohydrogen and biogas (Fig. 1), in an economically effective and environmentally friendly way [4]. Microalgae are a high-potential source of biomass for the production of food, industrial materials, pharmaceuticals and energy [5].

Microalgae like cyanobacteria and green algae can produce biohydrogen after derivation of their photosynthetic metabolism. Besides, microalgae can also be used as feedstock for biohydrogen production by microbial dark fermentation.

Biohydrogen from microalgae is renewable. The production of biohydrogen by microalgae through photofermentation is of interest, because it generates hydrogen gas from the most plentiful resources, light and water. However, the adaptation of the algae to an anaerobic atmosphere is prerequisite. Unfortunately, hydrogen production by this process is quite ineffective since the simultaneously produced oxygen would inhibit the hydrogenase enzyme. Therefore, accumulation of oxygen will stop the hydrogen production process.
The production of biohydrogen from microalgae through dark fermentation has received increasing attention in recent years [6]. However, biohydrogen potentials are usually low and a pretreatment step is often required to convert polymeric carbohydrates into monomeric sugars, to increase the microbial accessibility and further the biohydrogen production. Thus, physical, chemical and biological pretreatments are usually employed in order to facilitate carbohydrates de-polymerization and enhance biohydrogen production from microalgae.

The present mini-review will briefly introduce the biohydrogen production from microalgal biomass through dark fermentation, focusing on the pretreatments of microalgae to enhance hydrogen production.

Biology of microalgae

Microalgae in this review refer to all microscopic oxygenic phototrophs. Microalgae are primitive plant, which are one of the oldest life forms on earth. They are lack of roots, stems and leaves, have chlorophyll a as their primary photosynthetic pigment. Microalgae are commonly photosynthetic organisms that primarily use water, carbon dioxide, and sunlight to produce biomass and oxygen (Fig. 2).

Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms, which are normally found in marine and freshwater habitats. They can be grouped into prokaryotic microalgae (Cyanobacteria), eukaryotic microalgae (green algae Chlorophyta), red algae (Rhodophyta), and diatoms (Bacillariophyta), which are capable of growing rapidly due to their low nutrient requirement and simple structure. Besides to natural environments, microalgae can be cultivated in freshwater, seawater, and wastewater within open ponds (raceway) and closed photo-bioreactors.

Microalgae structures are primarily for energy conversion, and their simple development makes them to adapt to prevailing environmental conditions.

Microalgae are autotrophic, heterotrophic and mixotrophic. The autotrophic algae require only inorganic carbon source such as CO₂, salts and a light energy source for growth; while the heterotrophic ones are non-photosynthetic, they require an external source of organic compounds as an energy source; the mixotrophic algae are capable of performing photosynthesis and acquiring exogenous organic nutrients. For autotrophic algae, photosynthesis is a key component of their survival, whereby they convert solar light and CO₂ into adenosine triphosphate (ATP) and O₂, which is then used in respiration to produce energy to support growth.

Microalgal chemical composition

Components of microalgae vary according to their species and cultivation environment. Microalgae contain approximately 50% carbon by dry weight, which is typically derived from carbon dioxide. Production of 100 g of microalgal biomass can fix about 183 g of carbon dioxide.

In terms of chemical composition, microalgal biomass is mainly composed of proteins, carbohydrates and lipids. In general, proteins account for 40–60% of dry biomass, followed by carbohydrate (20–30%) and lipids (10–20%). Table 1 presents the general compositions of different microalgae [7–9].

Table 1 shows that the distribution of biochemical fractions of a microalgal cell is as follows: proteins 28–71%, carbohydrates 10–57%, lipids 4–22%. It is worth noting that the figures presented in Table 1 are estimates, since the proportion of individual cell constituents largely depends on environmental parameters. The chemical
composition of microalgae is high variable, largely depending on species, environmental conditions and cultivation methods. For instance, nutritional limitation and deprivation can induce and maximize lipid and carbohydrates synthesis by changing the metabolic strategies of microalgae. Microalgal cells tend to synthesize lipid instead of the starch at nitrogen-limited and high light conditions. In addition to these three major components, microalgal cells also contain small amount of nucleic acids (1–5%), and other valuable components (for example, pigments, anti-oxidants, fatty acids and vitamins) [10–14].

Advantages and disadvantages of hydrogen production from microalgae

The components of microalgae are valuable for a wide range of applications. Carbohydrates in microalgae can exist in the form of glucose and some polysaccharides like starch, agar, carrageenan, etc., which are considered to be an appropriate feedstock for generation of various fermentation products. Algal lipids are composed of glycerol, sugars or bases esterified to saturated or unsaturated fatty acids, which can be used for biodiesel production. The related long-chain fatty acids, pigments, and proteins have their own nutraceutical and pharmaceutical applications.

Comparing with the cellulose-based biomass and waste activated sludge produced from wastewater treatment plant, microalgae are a relatively new energy source. They have many advantages, for example, they have high growth rate with the fixation of CO₂, cultivation of microalgae can be beneficial to the environment by combining with wastewater treatment, they can be easily used as substrate with high carbohydrate content and simple structure and so on (Table 2) [7, 9, 10, 14].

Table 1 General composition of different microalgae (% of dry matter)

| Microalga         | Protein | Carbohydrate | Lipid |
|-------------------|---------|--------------|-------|
| Anabaena cylindrica | 43–56   | 25–30        | 4–7   |
| Chlamydomonas reinhardtii | 48     | 17           | 21    |
| Chlorella vulgaris  | 51–58   | 12–17        | 14–22 |
| Dunaliella salina   | 57      | 32           | 6     |
| Porphyridium cruentum | 28–39  | 40–57        | 9–14  |
| Scenedesmus obliquus | 50–56  | 10–17        | 12–14 |
| Spirulina maxima    | 60–71   | 13–16        | 6–7   |
| Synechococcus sp.   | 63      | 15           | 11    |

Nevertheless, these problems are expected to be overcome or minimized by technology development. Given the vast potential of microalgae as the most efficient primary producers of biomass, there is little doubt that they will eventually become one of the most important alternative energy sources.

Pretreatment of microalgal biomass

Since the hydrolytic enzymatic activity of hydrogen-producing bacteria is usually low, in order to enhance the biohydrogen production efficiency of fermentation process, the pretreatment step is often required for the hydrolysis of algal biomass to release the organic substances from the algal cells and make them readily biodegraded. A variety of pretreatment technologies that are researched and developed for treating other waste materials (e.g., animal waste and municipal sewage sludge) can be used to pretreat microalgal biomass for biohydrogen production.

Pretreatment methods can be divided into four categories: physical (mechanical, heat and ultrasonic treatment), chemical (acid, base and ozone), biological (enzymatic and microbiological treatment) and a combination of different treatments.

The most commonly used for pretreatment of microalgae to enhance carbohydrates hydrolysis include milling, ultrasonic, microwave, steam explosion, chemical oxidation and enzymatic hydrolysis.

In fact, the objective of all these pretreatment methods is the disruption of the cell wall to release the organic substances from the cells. Therefore they are applicable to biohydrogen production.

Hydrogen production from microalgae

Microalgae have been used as feedstock for producing biodiesel, bioethanol and biogas. Various microbial species have been used as feedstock for biohydrogen production, among which Chlorella sp., Scenedesmus sp. and Saccharina sp. have been extensively studied. To enhance the hydrogen production efficiency, different pretreatment methods were explored.
Table 2 Advantages and disadvantages of microalgae as feedstock for biohydrogen production

| Advantages                                                                 | Disadvantages                                                                 |
|---------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| 1. High growth rate                                                       | 1. Low biomass concentration                                                 |
| Microalgae can proliferate rapidly and are capable of all year round      | The low biomass concentration in the microalgal culture, in combination with   |
| production, and be obtained in large amount easily, which makes it         | the small size of algal cells, makes the harvest of algal biomasses           |
| possible to satisfy the massive demand on biofuels using limited land      | relatively costly                                                               |
| resources without causing potential biomass deficit. Their exponential     |                                                                               |
| growth rates can double their biomass in periods as short as 3.5 h         |                                                                               |
| 2. Superior CO₂ fixation capacity                                         | 2. Large water content                                                        |
| Microalgae are quite efficient in utilizing inorganic carbon sources to    | The large water content of harvested algal biomass suggests that its drying    |
| synthesize cell biomass, and their tolerance to high CO₂ content in gas    | process would be energy-consuming                                               |
| streams allows high-efficiency CO₂ mitigation (1 kg of dry algal biomass   |                                                                               |
| utilize about 1.83 kg of CO₂)                                              |                                                                               |
| 3. Benefit to the environment                                             | 3. Higher capital cost                                                         |
| The cultivation of microalgae does not require herbicides or pesticides    | The higher capital costs and the rather intensive care required by           |
| application. Nitrous oxide release could be minimized when they are         | microalgal cultivation facility compared to a conventional agricultural       |
| used for biofuel production                                                | farm would impede the commercial application of the biofuels from             |
|                                                                           | microalgae                                                                     |
| 4. Strong adaptation to environment                                       |                                                                               |
| Microalgae have strong adaptation to various environments without         |                                                                               |
| competing with fertile soils for agriculture                              |                                                                               |
| 5. Growth in aqueous media, less water required                            |                                                                               |
| Microalgae do not need arable land to grow, they grow in aqueous media,    |                                                                               |
| therefore may not incur land-use change, minimizing the associated         |                                                                               |
| environmental impacts, and their cultivation consumes less water than      |                                                                               |
| terrestrial crops, thus reducing the load on freshwater sources            |                                                                               |
| 6. High carbohydrate content                                              |                                                                               |
| Microalgae have high carbohydrate content, which is helpful in enhancing   |                                                                               |
| the hydrogen production efficiency                                         |                                                                               |
| 7. Simple structure                                                       |                                                                               |
| Microalgae are lack of hemicellulose and lignin, thus, the required        |                                                                               |
| pretreatments can be milder                                               |                                                                               |
| 8. Easy cultivation                                                       |                                                                               |
| Microalgae are unicellular or simple-multicellular microorganisms, which   |                                                                               |
| are adaptive to various environment conditions, and can be cultivated      |                                                                               |
| in fresh water, seawater and wastewater. The biochemical composition of   |                                                                               |
| the algal biomass can be modulated by varying growth conditions. The      |                                                                               |
| nutrients for microalga cultivation (especially nitrogen and phosphorus)   |                                                                               |
| can be obtained from wastewater. Therefore, apart from providing growth   |                                                                               |
| medium, there is dual potential for treatment of wastewater                 |                                                                               |

Hydrogen production from un-pretreated microalgae

Table 3 summarizes the hydrogen production from microalgae without pretreatment. It can be seen that *Chlorella vulgaris* is the most widely used as substrate for hydrogen production without treatment. Hydrogen yield obtained ranges from 0.37 to 19 mL H₂/g VS, and highest hydrogen yield was achieved from *C. vulgaris* [15], followed by the lipid extracted *Scenedesmus* sp. [16].

Hydrogen production from physically and chemically pretreated microalgae

The physical and chemical pretreatments, including mechanical, heat, ultrasonic, acid, base and ozonation, have been widely applied to disrupt and disintegrate the cell wall of microalgal biomass for enhancing the subsequent biological conversion process. For example, Ortigueira et al. [24] investigated fermentative hydrogen production using dry ground *Scenedesmus obliquus* biomass as feedstock. Usually, the use of microalgae biomass as a fermentable feedstock is determined by the recovery of the intracellular sugars and those that constitute the cell walls. Thermal pretreatment normally involves some additional pretreatment. For instance, when increasing temperature by autoclaving or microwaving, side pretreatments such as pressure build-up or electromagnetic radiation, respectively, will also have an effect on pretreated biomass. Chemical pretreatment of different types of wastes was shown to improve hydrogen production.

Table 4 summarizes the hydrogen production from microalgae pretreated by physical and chemical
| Substrate               | Substrate concentration (g/L TS) | Inoculum     | Operational conditions | Hydrogen yield (mL H₂/g VS) | Comments                                                                                                                                                                                                 | References |
|-------------------------|----------------------------------|--------------|------------------------|----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| *Chlorella vulgaris*    | 5                                | Anaerobic sludge | pH = 7.0, 37 °C; batch | 10.8                      | Due to the activities of satellite bacteria associated with algal cultures, hydrogen can be produced with and without inocula. Addition of BESA inhibited both hydrogen production and methane production.                                      | [17]       |
| *Chlorella vulgaris*    | 5–30                             | Anaerobic sludge | pH = 7.5, 60 °C; batch | 1.75–19                    | Combination of hydrogen production from microalgae and methane production from hydrogen fermentation residues was investigated. Effects of different enzymatic pretreatment on hydrogen and methane yield were examined.                                                                 | [15]       |
| *Chlorella vulgaris*    | 3–117                            | Anaerobic sludge | pH = 4.2–98, 35 °C; batch | 14.6–31.2[^b]              | Hydrogen production from microalgae biomass via dark fermentation was optimized by response surface methodology (CCD). The optimal condition was found at 76 g TS/L and initial pH of 7.4.                                                                 | [18]       |
| *Chlorella sp.*         | 4–40                             | Anaerobic sludge | pH = 6.5, 35 °C; batch | 0.37–7.13                  | Influences of inoculum–substrate ratio, VFAs and NADH on anaerobic hydrogen production from *Chlorella* sp. were examined. Results showed that inoculum–substrate ratio and NADH had a negative correlation with hydrogen production and increase of VFA formation was accompanied with increased hydrogen production. 3D EEM fluorescence spectrometry was used to determine NADH. | [19]       |
| *Nannochloropsis* sp. NANNO-2 | 2.5–10                          | Enterobacter aerogenes ATCC 13048 | 30 °C; batch | 26.4–60.6[^b] | Hydrogen was produced from *Nannochloropsis* sp. biomass before or after lipid extraction. Higher hydrogen yield was obtained from lipid extracted microalgae biomass.                                                                 | [20]       |
| *Nannochloropsis oceanica* | 50                               | Anaerobic sludge | pH = 6.0, 35 °C; batch | 2                         | The flue gas–cultivated microalgal biomass (*N. oceanica*) is efficiently used as feedstock to cogenerate hydrogen and methane through a novel three-stage method comprising dark fermentation, photo-fermentation and methanogenesis. | [21]       |
Table 3 (continued)

| Substrate                              | Substrate concentration (g/L TS) | Inoculum                  | Operational conditions | Hydrogen yield (mL H₂/g VS) | Comments                                                                                                                                                                                                 | References |
|----------------------------------------|----------------------------------|---------------------------|------------------------|----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Scenedesmus sp. (lipid extracted)      | 18<sup>a</sup>                   | Anaerobic sludge          | pH = 6.3, 37 °C, batch | 16.99                      | Different treatment methods on hydrogen production from microalgae biomass were examined. Include base, heat and combination of base and heat treatment. Treatment methods except base treatment all led to a significant increase in hydrogen production from microalgae biomass                                                                 | [16]       |
| Scenedesmus sp. (lipid extracted)      | 4.5–45<sup>a</sup>               | Anaerobic sludge          | pH = 5.0–70, 37 °C, batch | 0.42–40.27                | Effects of inoculum treatment, inoculum concentration, initial pH and substrate concentration on hydrogen production were investigated. Optimum condition was determined to be initial pH 6.0–6.5, heat treated inoculum concentration of 2.35 g VSS/L and the microalgae biomass concentration of 36 g VS/L                                                                 | [22]       |
| Chlamydomonas reinhardtii              | 50                               | Clostridium butyricum NCBI 9576 | pH = 6.0, 37 °C, batch | 16.6<sup>b</sup>          | Anaerobic hydrogen production from Chlamydomonas reinhardtii biomass was followed by photo fermentation, increased hydrogen yield from 2.58 mol H₂/mol starch–glucose to 8.30 mol H₂/mol starch–glucose equivalent algal biomass                                                                 | [23]       |
| Dunaliella tertiolecta                 | 5                                | Anaerobic sludge          | pH = 7.0, 37 °C, batch | 12.6                      | The high salinity of the Dunaliella tertiolecta slurry was prohibitive to methanogens, result in low methane production and high hydrogen yield                                                                 | [17]       |

<sup>a</sup> g/L VS  
<sup>b</sup> ml H₂/g TS
Table 4 Hydrogen production from microalgae pretreated by physical and chemical methods

| Treatment methods | Substrate          | Substrate concentration (g/L TS) | Inoculum                          | Operational conditions | Hydrogen yield (mL H₂/g VS) | Comments                                                                 | References |
|-------------------|--------------------|----------------------------------|------------------------------------|------------------------|-----------------------------|--------------------------------------------------------------------------|------------|
| Milling           | Scenedesmus obliquus | 10–50                            | Clostridium butyricum DSM 10702    | pH = 70, 37 °C; batch  | 28.1–35.0                   | Pure culture showed better hydrogen production than mixed culture       | [24]       |
| Milling           | Scenedesmus obliquus | 10–50                            | Anaerobic sludge                   | pH = 70, 37 °C; batch  | 5.4–34.8                    | Hydrogen production by mixed culture showed lower H₂/CO₂ ratio than pure culture | [24]       |
| Milling           | Scenedesmus obliquus | 10–50                            | Anaerobic sludge                   | pH = 70, 58 °C; batch  | 0.7–15.3                    | Higher hydrogen production was achieved at higher temperature           | [24]       |
| Milling           | Scenedesmus obliquus | 10–50                            | Anaerobic sludge + Clostridium butyricum DSM 10702 | pH = 70, 58 °C; batch  | 32.7–48.9                   | Co-culture of microorganisms achieved the highest hydrogen yield         | [24]       |
| Heat: 100 °C, 8 h | Scenedesmus sp. (lipid extracted) | 18°                               | Anaerobic sludge                   | pH = 63, 37 °C; batch  | 35.38                       | Hydrogen production from microalgae biomass was increased by over 2 times after heat treatment at 100 °C for 8 h | [16]       |
| Heat: 121 °C, 15 min | Scenedesmus obliquus | 2.5–50                           | Enterobacter aerogenes ATCC 13048   | pH = 68, 30 °C; batch  | 10.8–56.5                   | With the increase of substrate concentration, hydrogen yield decreased while cumulative hydrogen production and hydrogen production rate increased. Better hydrogen production was obtained from wet biomass than dried microalgae | [26]       |
| Heat: 121 °C, 15 min | Scenedesmus obliquus | 2.5–50                           | Clostridium butyricum DSM 10702    | pH = 68, 37 °C; batch  | 94.3–113.1                  | Hydrogen yield, cumulative hydrogen production and hydrogen production rate increased with the increase of substrate concentration. Better hydrogen production was obtained from wet biomass than dried microalgae | [26]       |
| Treatment methods             | Substrate                  | Substrate concentration (g/L TS) | Inoculum          | Operational conditions               | Hydrogen yield (mL H₂/g VS) | Comments                                                                                                                                                                                                 | References |
|------------------------------|----------------------------|----------------------------------|-------------------|--------------------------------------|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Heat: 121 °C, 20 min         | *Chlorella sorokiniana*    | 14                               | Anaerobic sludge  | pH = 65, 60 °C; batch                | 338                         | Different treatment methods on hydrogen production from microalgae biomass were examined. XRD and SEM were used to examine the rupture effect on cells by different treatment methods.                                | [26]       |
| Heat: 121 °C, 4 h            | *Scenedesmus* sp. (lipid extracted) | 18 a                             | Anaerobic sludge  | pH = 63, 37 °C; bath                | 35.58                       | Increasing treating temperature from 100 to 121 °C can achieve similar hydrogen production but shorter treating time was needed.                                                                         | [16]       |
| Base: NaOH 8 g/L, 24 h       | *Scenedesmus* sp. (lipid extracted) | 18 a                             | Anaerobic sludge  | pH = 63, 37 °C; batch                | 16.89                       | Base treatment alone showed little effect on hydrogen production from microalgae biomass.                                                                                                                    | [16]       |
| Chemical: H₂O₂ 2%, 12 h     | *Chlorella sorokiniana*    | 14                               | Anaerobic sludge  | pH = 65, 60 °C; batch                | 63                          | H₂O₂ showed better effect in treating microalgae biomass than sonication, but not as effective as other methods like heat and heat-acid treatment.                                                      | [25]       |
| Sonication: 130 W, 10 min   | *Chlorella sorokiniana*    | 14                               | Anaerobic sludge  | pH = 65, 60 °C; batch                | 52                          | Sonication showed little effect on cell disruption, and hydrogen production from sonication treated microalgae was not obviously increased.                                                               | [25]       |

*a g/L VS*
methods. It can be seen that hydrogen yield obtained
from the physically and chemically treated microalgae
were obviously higher than microalgae without treat-
ment, indicating that both physical and chemical treat-
ment can help to disrupt the microalgal cell. Relatively
higher hydrogen yield was obtained from the heat
treated microalgae (94.3–338 mL H₂/g VS) and high-
est hydrogen yield was obtained from the heat treated
*Chlorella sorokiniana* [25].

**Hydrogen production from biologically pretreated
microalgae**

The biological approaches use microbes and enzymes to
disrupt biomass and release intracellular materials, which
enhances the biohydrogen production rate. Depending
on cell wall composition, enzymes election is crucial.
Additionally, pH, temperatures, and the microalgae/
enzyme ratio are important parameters to control dur-
ing enzymatic treatment. The electrostatic bind enzyme-
microalgae are affected by acid or alkali conditions.
Under inappropriate pH conditions, enzymes can even
be inactivated by denaturing. Similarly, higher tempera-
ture results in increasing interactions enzyme-microalgae
until a certain level at which denaturalization may hap-
pen. Finally, the enzyme/microalgae ratio influences the
enzyme activity efficiency. High loading of microalgae
may result in high viscosity due to the release of insoluble
matter which in turn can hinder enzymatic activity.

Composition of microalgae cell walls include cellulose,
mucopolysaccharide and peptidoglycan, etc., therefore
research on microalgal biomass focus on the application
of macerozyme. Cellulases were proven to be suitable for
disruption of *C. sorokiniana* cell wall, and lysozyme was
found to be able to dissolve Cyanobacteria cell wall. Cell
wall lysis was supported by microscopic observation.

Table 5 summarizes the hydrogen production from
microalgae pretreated by biological method. It can be
seen that hydrogen yield varies greatly in the range of
11–135 mL H₂/g VS. Higher hydrogen yield was obtained
by enzyme treated microalgae than microbial consort-
tium treated microalgae, and a combination of different
enzymes can significant enhance the hydrogen yield [15].

**Hydrogen production from microalgae pretreated
by combined methods**

To disintegrate biomass more efficiently and to take
advantage of various pretreatment methods, the combi-
nation of different pretreatment methods has been used.
Most combined pretreatment methods comprise a physi-
tical treatment method and a chemical treatment method.

Combined heat and acid pretreatment is the most com-
monly used method. Besides acid pretreatment, heat pre-
treatment has also been combined with other methods
such as base pretreatment, enzymatic treatment and
oxidizing agent addition. Other combination of pretreat-
ment methods has been also applied, such as combi-
ing ozone with ultrasonication and enzyme hydrolysis,
respectively; combining microwave with base and acid
pretreatment, respectively; combining ionizing radiation
and base pretreatment. All of them achieved enhanced
hydrogen production from pretreated biomass wastes. In
some cases, combinations of three or more pretreatment
methods were also used, such as acid-heat-enzyme pre-
treatment [28, 29], acid-microwave-enzyme pretreatment
[30], base-heat-enzyme pretreatment [31] and so on.

Table 6 summarized the hydrogen production from
microalgae pretreated by the combined methods. It can
be seen that the hydrogen yield varies in the range of
33.56–958 mL H₂/g VS. The combination of acid and heat
showed the highest potential in enhancing the hydrogen
production from microalgae, and *Chlorella* sp. are more
preferable in achieving higher hydrogen yield.

**Concluding remarks and perspectives**

Microalgae are capable of producing high levels of car-
bohydrates such as starch or cellulose as reserve materi-
als, which are ideal feedstocks for hydrogen production.
Microalgae can potentially be employed for the produc-
tion of biohydrogen in an economically affective and
environmentally sustainable manner. The production
of biohydrogen from microalgae can be integrated with
flue gas (CO₂) mitigation, wastewater treatment, and the
production of high-value chemicals. There is increasing
interest in using microalgae as the renewable feedstock
for the production of biohydrogen. In comparison with
terrestrial biofuel feedstocks, microalgae can convert
solar energy into fuels with higher photosynthetic effi-
ciency, can synthesize and accumulate large quantities of
carbohydrate biomass, and can thrive in seawater system.

Studies have shown that fermentative hydrogen pro-
duction from microalgae shows great potential in sus-
tainable energy generation. Hydrogen production can be
modified through disrupting the microalgal cells by some
pretreatment methods, and a proper combination of dif-
ferent treatment methods can achieve a synergistic effect
and thus significantly enhance the hydrogen yield.

However, there still remain some obstacles hindering
the wide application of hydrogen production from micro-
algae, and several technological and economic issues
must be addressed to achieve success on a commercial
scale. Studies have shown great variance in the hydro-
gen yield, some of the hydrogen yields are high, like 958
and 760 mL H₂/g VS obtained from acid-heat treated *C.
sorokiniana* while some are far from industrial applica-
tion. Thus, further studies are needed to enhance the cost
effectiveness of the biohydrogen from microalgae, like
| Treatment methods                                      | Substrate                   | Substrate concentration (g/L TS) | Inoculum             | Operational conditions    | Hydrogen yield (mL H₂/g VS) | Comments                                                                 | References |
|--------------------------------------------------------|-----------------------------|----------------------------------|-----------------------|--------------------------|-----------------------------|---------------------------------------------------------------------------|------------|
| Biological: Onozuka R-10 enzyme                         | *Chlorella vulgaris*        | 10                               | Anaerobic sludge      | pH = 7.5, 60 °C; batch   | 39                          | Onozuka R-10 enzyme treatment increased hydrogen production from *Chlorella vulgaris* biomass from 19 to 39 mL/g VS | [15]       |
| Biological: macerozyme R-10 enzyme                      | *Chlorella vulgaris*        | 10                               | Anaerobic sludge      | pH = 7.5, 60 °C; batch   | 62                          | Macerozyme R-10 enzyme showed better effect on hydrogen production from *Chlorella vulgaris* biomass than Onozuka R-10 enzyme | [15]       |
| Biological: Onozuka R-10 enzyme + macerozyme R-10 enzyme | *Chlorella vulgaris*        | 10                               | Anaerobic sludge      | pH = 7.5, 60 °C; batch   | 135                         | Combination of Onozuka R-10 enzyme and macerozyme R-10 enzyme treatment resulted in significant increase in hydrogen yield from *Chlorella vulgaris* biomass than single enzyme treatment | [15]       |
| Biological: microbial consortium TC60, 60 °C, 10 days   | *Chlorella vulgaris*        | 0.14⁴                           | TC60 from compost     | pH = 7.0, 60 °C; batch   | 11                          | *Chlorella* biomass showed recalcitrance to anaerobic digestion by TC60, and hydrogen was produced by satellite heterotrophs from *C. vulgaris* | [27]       |
| Microbial consortium TC60, 60 °C, 10 days              | *Dunaliella tertiolecta*    | 0.094⁴                          | TC60 from compost     | pH = 7.0, 60 °C; batch   | 13                          | Hydrogen yields increased at least 10% after biological treatment process. Digestion of *Dunaliella tertiolecta* provided additional nutrients for cellulolytic activity | [27]       |
Table 6 Hydrogen production from microalgae pretreated by combined methods

| Treatment methods          | Substrate          | Substrate concentration (g/L TS) | Inoculum                      | Operational conditions | Hydrogen yield (mL H₂/g VS) | Comments                                                                 | References |
|----------------------------|--------------------|----------------------------------|--------------------------------|------------------------|----------------------------|----------------------------------------------------------------------------|------------|
| Acid: HCl 2.0%, 12 h; Heat: 121 °C, 20 min | Chlorella sorokiniana | 10                               | Enterobacter cloacae IIT-BT 08 | pH = 7.0, 37 °C batch | 201.6²                     | Algal biomass of C. sorokiniana was produced by CO₂ sequestration in continuous mode, and then used as substrate for anaerobic hydrogen production. Substrate concentration was optimized to enhance the hydrogen yield from C. sorokiniana       | [32]       |
| Acid-heat: HCl 5%, 121 °C, 20 min | Chlorella sorokiniana | 14                               | Anaerobic sludge              | pH = 6.5, 60 °C batch | 760                        | Better hydrogen production was achieved from microalgae biomass treated by combined treatment than single treatment method including autoclave, sonication and H₂O₂ treatment                                                                | [25]       |
| Acid-heat: HCl 20%, 121 °C, 20 min | Chlorella vulgaris  | 20                               | Clostridium acetobutylicum B-1787 | pH = 6.8, 37 °C batch | 958                        | Hydrogen yield was increased from 760 to 958 mL/g VS when HCl concentration was increased from 5 to 20%                                                                 | [25]       |
| Acid-heat: H₂SO₄ 0.1 mM, 108 °C, 30 min | Nannochloropsis sp. rse-msu-N-1 | 20                               | Clostridium acetobutylicum B-1787 | pH = 6.8, 37 °C batch | 0.90–9.52²                | Immobilized Clostridium acetobutylicum cells were used for hydrogen production from various microalgae species                                                                 | [33]       |
| Acid-heat: H₂SO₄ 0.1 mM, 108 °C, 30 min | Arthrospira platensis | 20                               | Clostridium acetobutylicum B-1787 | pH = 6.8, 37 °C batch | 2.24–8.06² | Different microalgae species were used as substrate, and highest hydrogen yield was obtained from wet Nannochloropsis sp. biomass                                                                 | [33]       |

² Heating temperature range of 100–121 °C, with and without acid addition were applied in treating microalgae biomass, most efficient treatment condition was determined to be 108 °C, 30 min with 0.1 mmol/L H₂SO₄.
| Treatment methods | Substrate | Substrate concentration (g/L TS) | Inoculum | Operational conditions | Hydrogen yield (mL H₂/g VS) | Comments | References |
|-------------------|-----------|---------------------------------|----------|------------------------|-----------------------------|----------|------------|
| Acid-heat: H₂SO₄ 0.1 mM, 108 °C, 30 min | Dunaliella tertiolecta | 20 | Clostridium acetobutylicum B-1787 | pH = 6.8, 37 °C, batch | 0.22–1.46 | Immobilized Clostridium acetobutylicum cells were used for hydrogen production from various microalgal species | [33] |
| Acid-heat: H₂SO₄ 0.5 mol/L, 100 °C, 30 min | Scenedesmus obliquus | – | Clostridium butyricum | pH = 7.0, 37 °C, batch | 2.9 | Potential of H₂ production from microalgal biomass and the respective energy consumption and CO₂ emissions in the bioconversion process were evaluated. Energy consumption of 7270 MJ/MJH₂ and 670 kg CO₂/MJH₂ were achieved, 98% of which owed to microalgal culture process due to the use of artificial lighting | [34] |
| Acid-heat: H₂SO₄ 0.5%, 121 °C, 60 min | Spirulina platensis | 10 | Bacillus firmus NMBL-03 | pH = 6.5, 38 °C, batch | 0.38 | A wide variety of substrates (glucose, xylose, arabinose, lactose, sucrose, and starch) and carbohydrate rich waste products (bagasse hydrolysate, molasses, potato peel and cyanobacterial mass) were used for dark fermentative hydrogen production. Abundant VFA were present in spent medium of hydrogen production from cyanobacterial mass, which can be further used as substrate for photo fermentative hydrogen production | [35] |
| Treatment methods                          | Substrate                          | Substrate concentration (g/L TS) | Inoculum                  | Operational conditions | Hydrogen yield (mL H\textsubscript{2}/g VS) | Comments                                                                                                                                                      | References |
|-------------------------------------------|------------------------------------|----------------------------------|---------------------------|------------------------|---------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Acid-heat: H\textsubscript{2}SO\textsubscript{4} 1%, 135 °C, 15 min | Chlorella pyrenoidosa               | 20                               | Clostridium butyricum     | pH = 6.0, 35 °C; batch | 81.2                                        | Heat and acid treated Chlorella pyrenoidosa biomass was used as substrate for hydrogen production. Energy was further removed through following photo hydrogen production and methane fermentation                                      | [36]       |
| Acid-heat: H\textsubscript{2}SO\textsubscript{4} 1%, 135 °C, 15 min | Chlorella pyrenoidosa               | 10 (additional cassava starch 10 g/L) | Clostridium butyricum     | pH = 6.0, 35 °C; batch | 276.2                                       | Hydrogen production from microalgae biomass was significantly increased from 81.2 to 276.2 mL/g VS by the addition of cassava starch to get an optimum C/N ratio                                                                 | [36]       |
| Acid-heat: H\textsubscript{2}SO\textsubscript{4} 3%, 121 °C, 60 min | Lipid extracted algae cake (collected from a lake) | 5\textsuperscript{b}          | Anaerobic sludge          | pH = 6.0, 29 °C; batch | 122\textsuperscript{d}                     | Comparison of hydrogen production from algae untreated, liquid fraction of treated algae, solid fraction of treated algae and treated algae mixture was examined. Best hydrogen and VFA generation was achieved from liquid fraction of treated algae | [37]       |
| Acid-microwave: H\textsubscript{2}SO\textsubscript{4} 0–2.0%, 80–180 °C, 5–25 min | Nannochloropsis oceanica            | 50                               | Anaerobic sludge          | pH = 6.0, 35 °C; batch | 39                                          | Hydrogen production from microalgae biomass was significantly increased by combined acid and microwave treatment                                                                                                  | [21]       |
| Base-heat: NaOH, 8 g/L, 100 °C, 8 h       | Scenedesmus (lipid extracted)       | 18\textsuperscript{a}          | Anaerobic sludge          | pH = 6.3, 37 °C; batch | 45.54                                      | For the combined treatment, lower temperature and longer treating time was preferred than higher temperature and shorter time                                                                                          | [15]       |
| Base-heat: NaOH, 8 g/L, 121 °C, 4 h       | Scenedesmus (lipid extracted)       | 18\textsuperscript{a}          | Anaerobic sludge          | pH = 6.3, 37 °C; batch | 37.42                                      | Better hydrogen production was achieved from microalgae biomass treated by combined treatment than single treatment method                                                                                         | [16]       |
| Treatment methods | Substrate                             | Substrate concentration (g/L TS) | Inoculum          | Operational conditions | Hydrogen yield (mL H₂/g VS) | Comments                                                                                                                                                                                                 | References |
|-------------------|--------------------------------------|----------------------------------|-------------------|------------------------|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Acid-heat: pH 1.4, 140 °C, 15 min; biological: cellulase 0.05 g/g TVS, 48 h; glucoamylase 0.05 g/g VS, 24 h | Mixed algae (collected from algae bloom in Taihu Lake) | 25                 | Anaerobic sludge      | pH = 6.0, 35 °C, batch   | 33.56–43.84                 | Steam with acid treatment showed better reducing sugar release than steam with alkaline treatment. The energy conversion efficiency was significantly increased through 3-stage process: dark-fermentation, photo-fermentation, and methanogenesis. | [30]       |
| Acid-microwave: pH 1.4, 140 °C, 15 min; biological: cellulase 0.05 g/g TVS, 48 h; glucoamylase 0.05 g/g TVS, 24 h | Mixed algae (collected from algae bloom in Taihu Lake) | 25                 | Anaerobic sludge      | pH = 6.0, 35 °C, batch   | 42.4–47.07                 | Microwave with diluted acid treatment degraded algal cells into smaller fragments (< 5 mm), and resulted in higher saccharification efficiency of microalgae | [30]       |
| Acid-microwave: H₂SO₄ 0.2 mL, 140 °C, 15 min; biological: glucoamylase 0.2% | *Arthrospira platensis* 10–40 | Anaerobic sludge      | pH = 6.5, 35 °C, batch | 86.5–96.6d            | Hydrogen yield was significantly enhanced from 96.6 to 337.0 mL H₂/g DW using a combination of dark- and photo-fermentation. Removal of harmful byproducts from hydrolysis pretreatment and dark fermentation can further enhance the overall hydrogen yield. | [38]       |

*a* g/L VS  
*b* g/L COD  
*c* mL H₂/g COD  
*d* mL H₂/g TS  
*e* mol H₂/mol sugar
the improvement in microalgal cultivation and downstream processing (e.g., harvesting, concentrating and drying), optimization of nutritional structure of microalgae for hydrogen production through adding protein-rich or mineral nutrient-rich wastes, operational conditions optimization including inoculum, initial pH, temperature as well as reactor structure, etc.

Authors’ contributions
YY has collected the literatures and drawn figures. JW finished the writing of this paper. Both authors read and approved the final manuscript.

Author details
1 Collaborative Innovation Center for Advanced Nuclear Energy Technology, INET, Energy Science Building, Tsinghua University, Beijing 100084, People’s Republic of China. 2 Beijing Key Laboratory of Radioactive Wastewater Treatment, Tsinghua University, Beijing 100084, People’s Republic of China.

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