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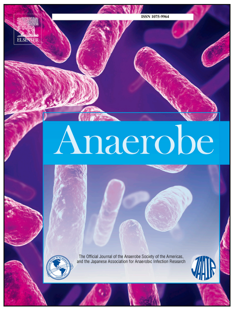

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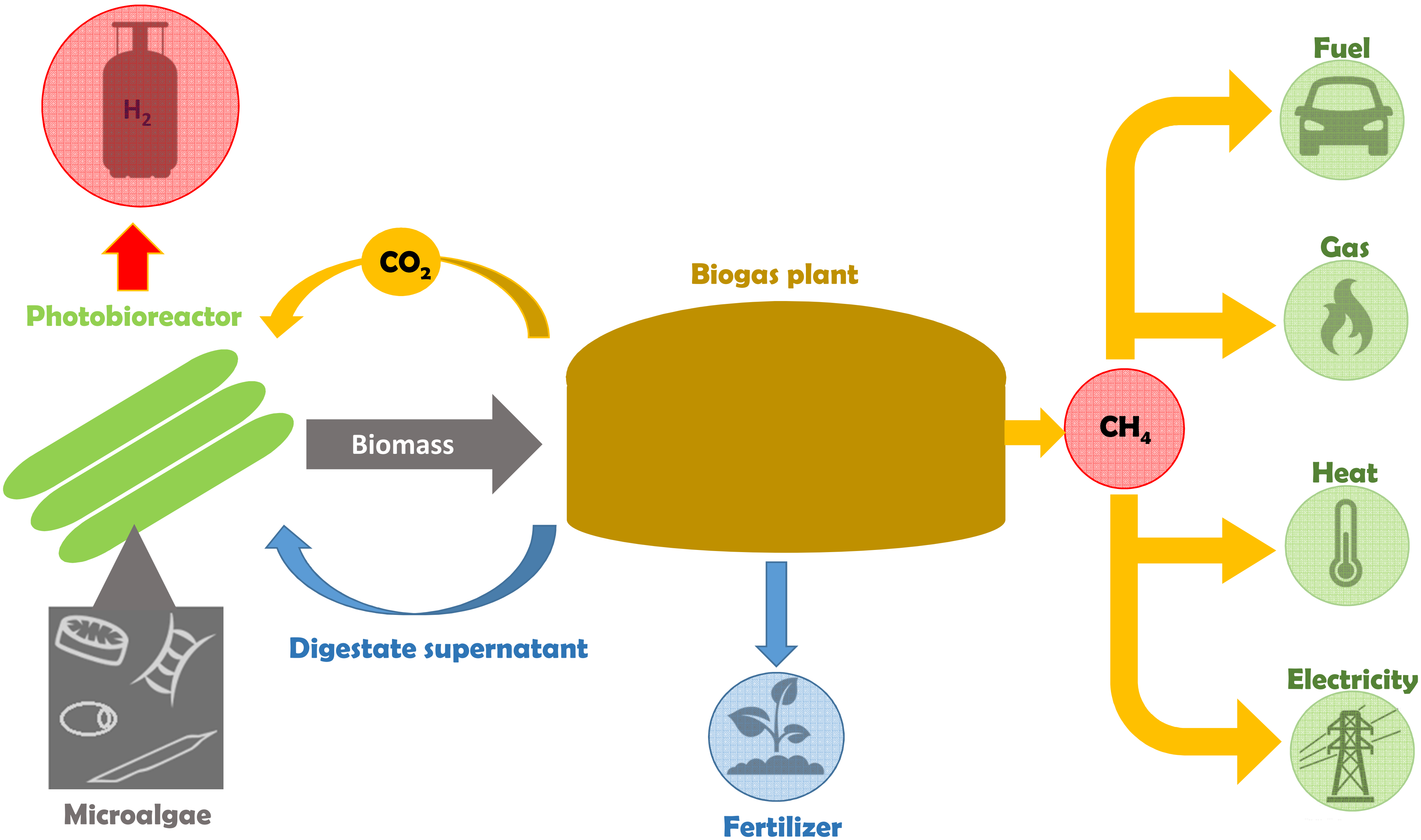

H2
Photobioreactor
CO2
Biogas plant
Biomass
Digestate supernatant
Microalgae
Fertilizer
CH4
Fuel
Gas
Heat
Electricity

Anaerobic gaseous biofuel production using microalgal biomass – a review

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Abstract

Most photosynthetic organisms store and convert solar energy in an aerobic process and produce biomass for various uses. Utilization of biomass for the production of renewable energy carriers employs anaerobic conditions. This review focuses on microalgal biomass and its use for biological hydrogen and methane production. Microalgae offer several advantages compared to terrestrial plants. Strategies to maintain anaerobic environment for biohydrogen production are summarized. Efficient biogas production via anaerobic digestion is significantly affected by the biomass composition, pretreatment strategies and the parameters of the digestion process. Coupled biohydrogen and biogas production increases the efficiency and sustainability of renewable energy production.

Key words: microalgae, biohydrogen, biogas, anaerobic fermentation, biomass conversion, renewable energy

Highlights:

- Microalgal biomass is a promising source for carbon-neutral biofuels.
- H_2 production: autotrophic, heterotrophic and photoheterotrophic approaches are available.
- The CH_4 potential of algal biomass depends on the species and conditions.
- Combination of anaerobic H_2 and biogas production is recommended.

1. Introduction

Nowadays, global climate change and world energy crisis are among the most concerned problems. These issues are mainly due to the fast industrialization, population growth and increased use of fossil fuels [1]. Replacement or supplementation of fossil fuels with alternative energy sources could help address this problem. For electricity production, wind turbines and photovoltaic technologies have grown rapidly in recent years. The requirements for liquid biofuels have been partially satisfied by mass production of first-generation corn or sugarcane ethanol and biodiesel from soy, sunflower or rapeseed. To avoid the food versus fuel debate in the production of agricultural commodities, next generation biofuels from algal biomass, organic wastes and lignocellulose-rich materials have to replace energy plants [2–5]. Algal biomass cultivation has advantages against agricultural crops. This alternative biomass has fast growth rate, high contents of lipids, carbohydrates, and proteins, and do not contain recalcitrant lignin. Moreover, it can be cultivated on lands that are not suitable for traditional agriculture [6–8]. Interest in gaseous fuels, such as hydrogen (H_2) and methane (CH_4), has increased in recent years due to their zero, or even carbon dioxide negative production-and-use cycle [9–12]. Biohydrogen and biogas production from algal biomass is therefore intensively studied with a goal of reducing the nutrients, energy requirements and increasing the production efficiency [13–16]. In this review we summarized the recent developments in the utilization of algal biomass for the production of gaseous biofuels such as biohydrogen and biogas and the exploitation of anaerobic microbiology.

Although macroalgae and cyanobacteria are also considered as promising biomass source for energy production [17-19], we restrict our discussion to microalgae.

2. Algal biohydrogen: Strategies for handling the oxygen sensitivity of algal hydrogenases

The advantage of the application of eukaryotic green microalgae for hydrogen production is the remarkable efficiency of their [FeFe]-hydrogenases at ambient temperature and pressure [20]. However, the wild-type algal [FeFe]-hydrogenases function only in anaerobic environment [21] (Figure 1). The oxygen produced by photosynthesis rapidly and irreversibly inactivates the active center of algal [FeFe]-hydrogenases [22]. Various approaches have been proposed and tested to overcome this issue [23]. The task is to sustain the alga alive while aerobic photosynthesis is suppressed and H_2 production takes place via anaerobic fermentation of storage materials.

2.1. Depletion strategies

A good portion of the approaches to achieve this goal are based on various nutrient depletion strategies [19,21,24,25] (Table 1). These strategies rely on the depletion of either sulfur [26–30], phosphate [31,32], nitrogen [33,34] or magnesium [34] from the growth medium. These nutrient stresses are accompanied with the decline of cell proliferation, photosynthetic activity and carbon fixation. A considerable drawback of the nutrient depletion methods is that the aerobic biomass generation phase must be temporally separated from the anaerobic hydrogen production phase, which represents costly technological difficulties and often leads to an irreversible decaying process of the algae cultures.

2.1.1. Sulfur deprivation

Sulfur (S) deprivation is the most studied strategy to achieve sustainable H_2 production in green algae [26,27,35–37]. The D1 protein in the reaction center of photosystem-II (PSII) undergoes a rapid degradation caused by the reactive oxygen radicals in response to S-deprivation [30]. This results in an efficient but not complete inhibition of PSII activity (30-75%) [28,38,39]. The PSII inhibition leads to a gradual decline of O_2 evolution. In the presence of acetate the unaffected mitochondrial respiration consumes the residual O_2 until the cultures become fully anaerobic between days 1 and 3 following S-deprivation [21,39–42]. The disadvantage of the PSII inactivation is the gradual inhibition of the electron flow towards the hydrogenases. Approximately 60-90% of the total electrons used for H_2 evolution derive directly from PSII activity, only the remaining 20-30% of the electrons originate from the previously accumulated starch [29,40,43–45].

2.1.2. Nitrogen deprivation

Nitrogen (N) deprivation has also been tested for micro-algal H_2 production [25,33,46]. There are clear similarities between the S- and N-deprivation approaches. Photosynthetic activity significantly decreases, while there is a general increase in the starch and lipid content of the algae cells, especially in the presence of acetate [47,48]. However, the aerobic phase in N-deprived cultures was conspicuously longer compared to that in S-deprivation, which resulted in a delayed H_2 production [33]. The accumulation of starch and lipids, and the degradation of proteins (e.g. cytochrome b6f complex) were more efficient in N-deprivation than in S-deprivation [49]. Moreover, ammonium production is observed during the H_2 evolution period indicating significant protein degradation [50].

2.1.3. Phosphorus deprivation

Sulfur deprivation is impossible in seawater due to the high concentration of sulfates [31,32]. However, phosphorus (P) deprivation in seawater is possible. Similarly to S-deprivation, the P deficiency results in decreased PSII activity, although the inactivation process is considerably slower due to the slower consumption of the stored P reserves compared to S-deprivation [38,51,52]. P-deprivation also created anaerobic environment in the presence of acetate, which was consumed in the aerobic phase and starch accumulated. In the anaerobic phase most of the starch was degraded resulting in fermentative H_2 production, while acetate consumption slowed down but remained incessant. H_2 production could be achieved by the inoculation of *Chlamydomonas* sp. or *Chlorella* sp. cultures into P-free medium, allowing the algae to efficiently deplete the intracellular P reserves [31].

2.1.4. Magnesium deprivation

The magnesium (Mg)-controlled algal H_2 production is the most recent nutrient deprivation method [34,53]. Mg occupies an essential position in the photosynthetic apparatus as a constituent of the chlorophyll molecule. Mg-deprivation resulted in decreased photosynthetic activity by ~20% [34,54], which was accompanied by the slow-down of the electron transport and a concomitant reduction of the plastoquinon-pool [53-56]. H_2 production under Mg^{2+} deficiency is mainly linked to the PSII-dependent pathway [34]. The photosynthetic antenna size and the total amount of chlorophyll molecules also decreased by approximately 60%. The mitochondrial respiration was active and starch accumulation increased. These activities enhanced the establishment of anaerobiosis and the continuous flow of the electrons necessary for H_2 evolution. H_2 production lasted for approximately 7 days. The disadvantage is the requirement of a preceding 7-day long Mg-depletion period under aerobic environment [34].

2.2. Acetate regulation

The majority of the studies on light dependent H_2 production of *Chlamydomonas* spp. employed nutrient depleted algae cultures as summarized above [57,58]. These methods always require two temporary separated phases. The algal biomass must be first cultivated, followed by the replacement of the growth media to achieve the required nutrient shortage and to promote H_2 production. Therefore these approaches are time- and energy-consuming and make the process economically unfeasible [26].

H_2 photoproduction could also be enhanced by acetate addition in nutrient-repleted media in some algal species adapted to light and anaerobiosis [21,59–61]. This way, the parallel production of H_2 and substantial biomass was possible in a single step. The major shortcoming of this strategy was the significantly lower H_2 production rate compared to the nutrient depletion methods. Nonetheless, the establishment of the anaerobic environment took place within a day as opposed to the 2-8 days under nutrient-depleted conditions [62].

Moreover, in aerated fed-batch bioreactors, periodic supplementation of acetate and addition of O_2 greatly enhanced H_2 production and allowed semi-continuous H_2 and biomass production [62].

2.3. Algal-bacterial co-cultures

The low H_2 production efficiency of the axenic *Chlamydomonas* spp. cultures could be improved by the addition of bacterial partner(s) to the H_2 producing algae [15,63]. This way, the net mitochondrial respiration of the algal cells becomes significantly elevated, allowing the efficient application of stronger light regimes during H_2 production. The higher light flux prompted more active water splitting reaction in PSII, which generated more electrons for H_2 generation. The bacterial partner consumed the excess O_2, which enabled the establishment of anaerobiosis in 2-12 hours allowing quick start of H_2 evolution depending on the gas-to-liquid phase ratio [15,16,63]. H_2 accumulation rates can be further elevated by lowering the competing bacterial H_2-uptake activity, e.g. using uptake-hydrogenase deficient bacterial strains. Using both the bacterial partners and S-depleted algae cultures doubled the H_2 yield by shortening the aerobic phase [63]. Increased volumetric hydrogen production rate was achieved by the application of a *Chlorella* sp. strain, which has remarkably smaller cell size than that of the commonly investigated *Chlamydomonas* spp. strains [16]. In addition to the rapid O_2 consumption and early start of H_2 production, the algal biomass grew more efficiently in symbiosis with its bacterial partner than in axenic cultures in complete media [64,65].

The generated algal-bacterial biomass could be further utilized as feedstock for biogas production [15,66]. Another novel approach is offered by Ding et al. In this process the algal biomass is fermented in both hydrogen and methane production stages. Co-fermentation of carbon-rich macro-algae and nitrogen-rich micro-algae in two stages markedly increased the energy conversation efficiencies [67].

3. Anaerobic digestion of microalgal biomass

The decomposition of organic materials is carried out under anaerobic conditions and a great variety of diverse microbes participate in the microbial food chain gradually, which degrades the complex molecules essentially to a mixture of CH_4 and CO_2 [68–70]. The idea of using microalgal biomass substrate in anaerobic digestion (AD) dates back to the 1950s [71] (Figure 2), when a mixed culture of *Chlorella* sp. and *Scenedesmus* sp., grown in wastewater, was utilized. In the sporadic follow-up work, biogas composition and AD process stability of different microalgae species were investigated [72–81].

3.1. Strain selection

Biogas productivity from representatives of various microalgal groups were compared, including fresh- and seawater strains [82–85]. As a general feature in mesophilic conditions, the CH_4 content of the biogas from the microalgae was ~7-13% higher than that from maize silage, the most widespread substrate in biogas industry [82]. Albeit the higher CH_4 content, the overall biogas yields varied depending on the cell wall structure of the algae strains. Easily biodegradable species either lack cell wall, as in the case of *Dunaliella salina* halophilic microalgae [86], or their cell wall is rich in easily-biodegradable protein substances, as in the case of *Chlamydomonas reinhardtii* [87]. Other species such as *Chlorella kessleri* and *Scenedesmus obliquus* have hemicellulose-rich, more recalcitrant cell walls, making them difficult to hydrolyse [88-93].

3.2. Physico-chemical pre-treatments

In addition to strain selection, biogas yield from algae can be improved by suitable pre-treatments, i.e. disruption or solubilisation of the cell wall. The possibilities have been recently reviewed [94]. The main pre-treatment strategies include mechanical, thermal, chemical and biological methods. The key limiting parameter determining large scale application of these technologies is their energy consumption. Mechanical pre-treatments, including sonication, are efficient to disrupt the cell wall, but the energy requirement render them economically unfeasible [95]. Thermal treatment provided promising results in biogas production enhancement although concentrated biomass is needed to reach positive energy balance [80,96–99]. The heat induced polymerization of available reducing sugars and amino acids to complex molecules may explain this phenomenon [80,82,100]. Chemical solubilisation of microalgal biomass presented higher effectiveness compared to thermal treatment but biogas production did not increase accordingly [82,84,100,101].

3.3. Biological pre-treatments

Biological methods involve the application of various enzymes to decompose the cell wall polymers effectively. Protease pre-treatment of *S. obliquus* and *C. vulgaris* enhanced the CH_4 yields 1.72-fold and 1.53-fold, respectively [103]. In a similar approach an enzyme cocktail, including ß-glucanase, xylanase, cellulase and hemicellulase, was efficient in facilitating AD of algal biomass [104,105]. The main restricting factor of the biological pre-treatment methods is the cost of enzyme production. Therefore, *in situ* enzyme production has been suggested. This could be done by separating the hydrolytic-acidogenic stage from the methanogenesis stage in a two-stage AD design [67]. Bioaugmentation of biogas formation from algal biomass employing *Clostridium thermocellum* improved the degradation of *Chlorella vulgaris* biomass. In this two-step process *C. thermocellum* was added first and methanogenic sludge subsequently beneficially increased the bioenergy yield [106]. Significant improvements in the methane yield were observed through biological pre-treatment of mixed microalgal cultures (mainly *Oocystis* sp.) using *Trametes versicolor* fungi and commercial laccase. The CH_4 yield increased by 20% for commercial laccase and 74% for fungal broth in batch tests, as compared to non-pretreated biomass [82,106]. An interesting novel approach has been explored when genes of foreign lytic enzymes, involved in cell division and programmed cell death, were expressed in algae to enhance cell disruption [108]. A recent review summarized numerous studies on pretreatments [80].

3.4. Salt effects

Alternatives to fresh water, algal strains habitating the saline seawater have been studied in order to preserve freshwater supplies. Alkaline earth metal salts are needed in very low concentration for bacteria and methanogenic archaea, while higher concentrations can be toxic for both of them [109]. In seawater, the sodium ions (Na^+) are particularly inhibitory to AD [110]. Sodium concentrations of 5, 10 and 14 g L^{-1} caused 10, 50, and 100% inhibition of acetoclastic methanogens [111]. Moderate inhibition of AD was observed at sodium concentrations ranging from 3.5 to 5.5 g L^{-1}. However, total AD inhibition was detected above 8 g L^{-1} of Na^+ [109]. An adapted microbial community containing halophilic methanogens digested *Dunaliella salina* successfully at 35 g L^{-1} of salinity [112].

3.5. C/N ratio

The C/N ratio has a very significant impact on the methane yield and on productivity in all microalgae-based AD. The optimal C/N ratio of AD is between 20 and 30 [113]. AD of substrates having lower C/N results in increased free ammonia, which may become inhibitory [114]. Microalgal species usually contain higher proportion of proteins compared to terrestrial plants. The C/N ratio of green microalgae is generally low (C/N ~10), while terrestrial plants have higher ratios (depending on the plant species and season, C/N ~20-40) [115]. This has been corroborated in studies in microalgae from natural reservoir (mainly *Chlorella* sp. and *Scenedesmus* sp.), which had a C/N ratio of 6.7, *C. vulgaris* having a C/N ratio of 5, and *S. obliquus* possessing C/N of 8.9 [15,116,117]. Ammonia accumulation at low C/N ratio has been observed in various studies [71,118,119]. The use of ammonia-tolerant inoculum could be a promising solution to effectively digest the protein-rich microalgal biomass in a continuous biogas-producing process [120]. AD of algal biomass generated under N-limitation showed efficient CH_4 production due to the favourable C/N ratio of the substrate [84,85].

3.6. Effects of OLR and HRT

A proper organic loading rate (OLR) and hydraulic retention time (HRT) can diminish the negative effects of inhibitory conditions. HRT is the time allowed for any given substrate to be digested. OLR is the amount of volatile solids to be fed into the digester daily in a continuous AD process. The biogas yield rises upon increasing the OLR, but above the optimal OLR the volatile solids degradation and biogas yield decrease due to overloading [121]. In order to reduce operation costs and achieve optimum performance, biogas reactors should be designed to operate at maximum methane production at lowest HRT and highest OLR [122]. An effective OLR of *Chlorella* biomass at mesophilic conditions was found at 5g VS L^{-1} d^{-1} [123]. Higher OLR increased the level of valeric and butyric acids resulting process inhibition. Other studies also confirmed that highest biogas yields were attained at the low OLR, i.e., 0.6g VS L^{-1} d^{-1} (mixed culture containing *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* in mesophilic conditions) [124]. Typical OLRs are between 1–6 g VS L^{-1} d^{-1} and HRT varies between 10 and 30 days [83,122,125].

3.7. Co-digestion

Co-digestion is a promising strategy to increase the performance of a digester by ensuring optimal substrate composition, which can enhance biogas productivity from microalgal biomass. Significant enhancement of methane production upon addition of waste paper to the algal sludge has been reported [116]. Long-term experiments using mixtures of maize silage and marine microalga *Nannochloropsis salina* were investigated under batch and semi-continuous conditions. The biogas yields were significantly increased and the semi-continuous AD was stable for more than 200 days [126]. Increased CH_4 production was observed in a mixture of *Chlorella* sp. microalgal biomass and food waste [127]. The elevated CH_4 production was probably due to the multi-stage digestion of different substrates having different degrees of degradability. Co-digestion of algal biomass with sewage sludge or liquid manure has been shown to be advantageous in several cases [125,128]. In a laboratory scale fed-batch co-fermentation experiment of algal-bacterial mix, the cumulative methane yield was ~350 mL CH_4 g VS^{-1} (OLR: 1 g VS L^{-1} d^{-1}; HRT: 1 d, mesophilic conditions) [15]. In another study from the same research group, microbiologically pure *Scenedesmus obliquus* and maize silage were subjected to co-fermentation (OLR: 1 g VS L^{-1} d^{-1}; HRT: 1 d). The observed methane yield was ~280 mL CH_4 g VS^{-1}. It is noteworthy that co-digestion resulted in significantly higher methane productivity in both cases relative to the microalgal biomass mono-substrate [15,66]. The addition of used cooking oil, maize silage, and mill residue to AD of the microalga *Chlorella vulgaris* was studied in semi-continuous, laboratory-scale digestions by Rétfalvi et al. [117]. The volumetric methane yields were in the range of 300 to 500 mL CH_4 g VS^{-1} (OLR: 0.78-2.15 g VS L^{-1} d^{-1}; HRT: 88-383 d). Triple co-digestion of oil-extracted *Chlorella vulgaris* microalgal biomass, glycerol and chicken litter in various proportions was studied under mesophilic conditions [129]. Oil-extracted microalgae in co-digestion with chicken litter enhanced the biochemical methane potential. The highest CH_4 yield was 131 mL CH_4 g VS^{-1} (HRT: 90 d). Based on these results, co-digestion may be the recommended approach to degrade microalgal biomass effectively and sustainably without pre-treatment.

4. Conclusions and outlooks

Utilization of solar energy stored in microalgal biomass is a promising source for anaerobic gaseous biofuel production. Despite the technological challenges the interest in microalgae-based biofuels increases [13,14,130,131]. Innovative developments in microalgal cultivation will reduce biomass production costs. Aqueous waste streams are inexpensive and efficient growth media for mixed algal-bacterial biomass production, which is a suitable substrate for biohydrogen and biological CH_4 production via anaerobic fermentation [132–137]. Natural habitat of microalgae may expand the limits of deprivation methods. The efficiency of AD using microalgal biomass depends on various factors, such as strain selection, pre-treatment, OLR, HRT, reactor design, temperature and pH [79,80]. In microalgae-based biogas production the goal is to maintain effective and balanced operation. An emerging and effective strategy to improve technical and economic feasibility is co-digestion with organic wastes or by-products to optimize process parameters. The coupling of biohydrogen and biogas production processes, using algal-bacterial co-cultures, is recommended.

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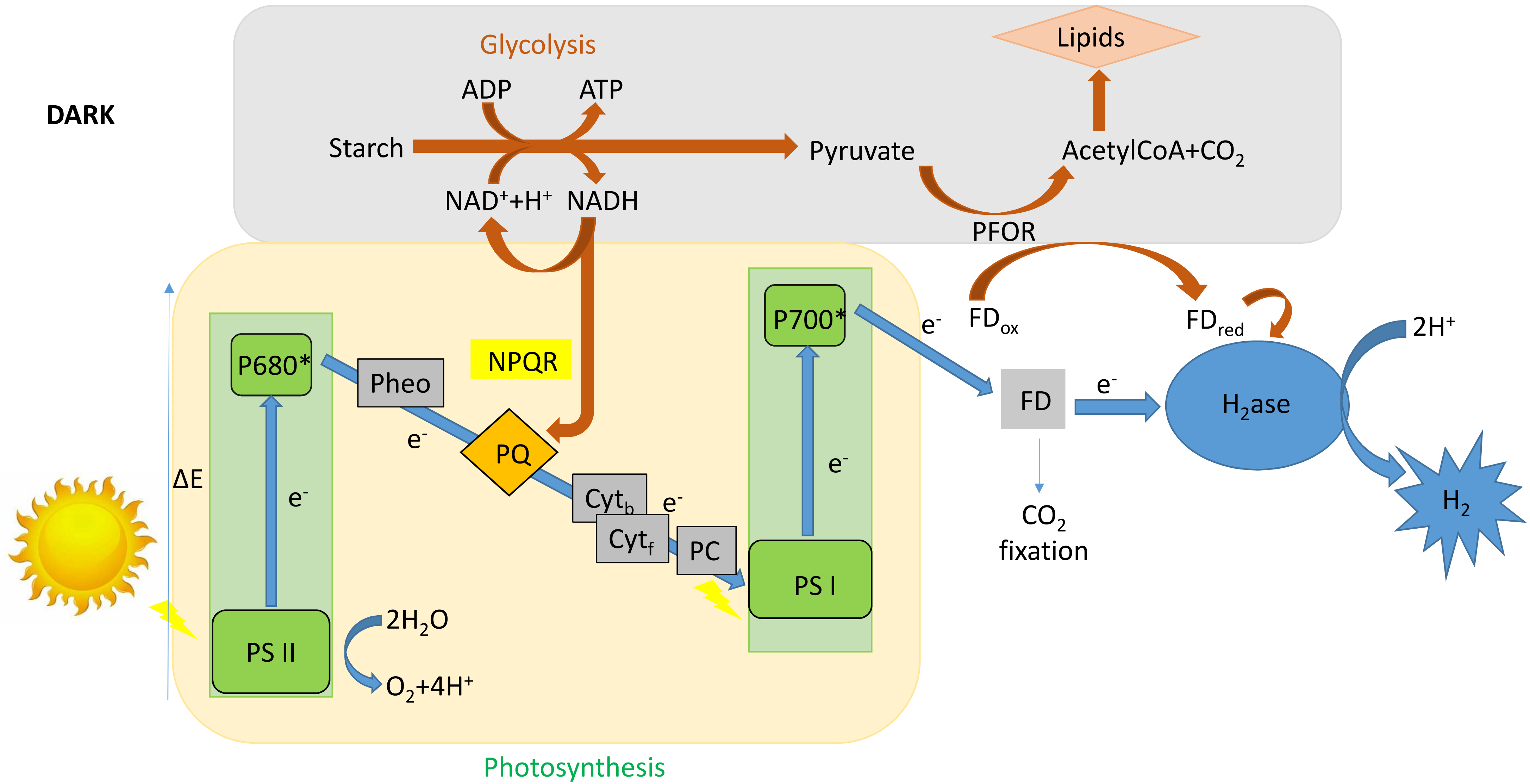
Figure legends:

Figure 1. Schematic link between oxygenic photosynthesis and hydrogen production. Abbreviations: PS II: Photosystem II; PS I: Photosystem I; Pheo: pheophytin; PQ: plastoquinon; Cytb/Cytf: Cytochrome bf complex; PC: Plastocyanin; FD: ferredoxin; H2ase: hydrogenase; NPQR: NADP quinone reductase; PFOR: pyruvate ferredoxin oxidoreductase; FDox: oxidized ferredoxin; FDred: reduced ferredoxin.

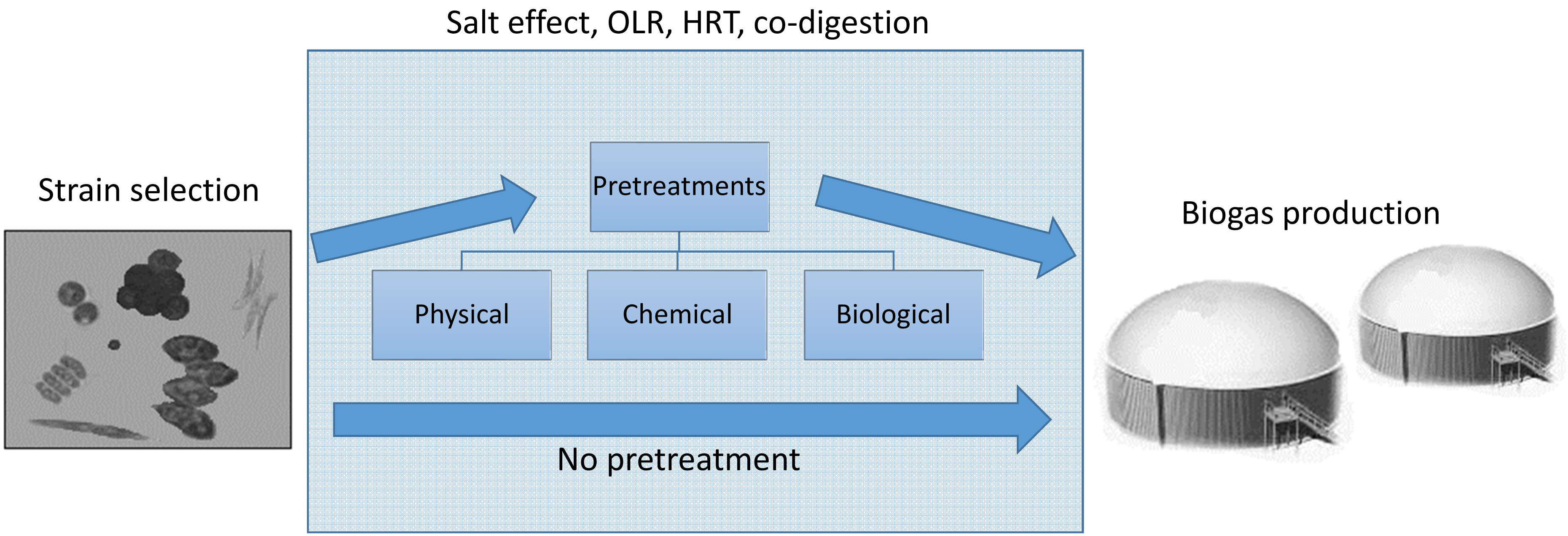
Figure 2. The principle of alga-based biogas production. Abbreviations: OLR: organic loading rate, HRT: hydraulic retention time.

Table 1. Summary of depletion-induced photosynthetic biohydrogen strategies.

Strategy	Lag of H_2 production (hours)	Accumulated H_2 yield (ml H_2 l^{-1} culture h^{-1})	Effects of treatment	References
Sulfur deprivation	24-72	1.6-3	Down-regulated photosynthesis Elevated starch content Reduced amount of Rubisco and PSII	Melis et al., 2000; Melis and Happe, 2001; Kruse et al., 2005; Toepel et al., 2013; Wijffels et al., 2013
Nitrogen deprivation	30-54	0.5-4.25	Chlorosis Loss of Cyt *b6f* complex; Inhibition of carbon fixation Reduced amount of Rubisco; Elevated starch content	Philipps et al., 2012; Li et al., 2015; Saroussi et al., 2017
Phosphorus deprivation	120-288	0.18-0.43	Elevated starch content; Inactivation of PSII	Batyrova et al., 2012, 2015
Magnesium deprivation	216	0.72	Decrease of Chl content	Volgusheva et al., 2015, 2017
Acetate regulation	<24	0.29-0.39	none	Fan et al., 2016; Jurado-Oller et al., 2015
Alga-bacteria co-culture	2-12	0.125-0.25	Elevated biomass production rate	Lakatos et al., 2014; Wirth et al., 2015b



DARK
Glycolysis
ADP
ATP
Starch
Pyruvate
AcetylCoA+CO2
Lipids
NAD+H+
NADH
PFOR
FDox
FDred
e-
2H+
NPQR
P680*
Pheo
e-
PQ
Cytb
e-
Cytf
PC
P700*
e-
FD
e-
H2ase
H2
CO2
fixation
ΔE
e-
PS II
2H2O
O2+4H+
PS I
Photosynthesis


Salt effect, OLR, HRT, co-digestion
Strain selection
Pretreatments
Physical
Chemical
Biological
No pretreatment
Biogas production

Highlights:

- Microalgae are promising source of alternative carbon neutral biofuels.
- H_2 production: autotrophic, heterotrophic and photoheterotrophic approaches.
- The CH_4 potential of algal biomass depends on the species and AD conditions.
- Combination of anaerobic H_2 and biogas production is recommended.