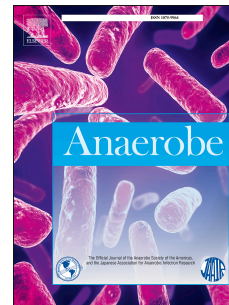


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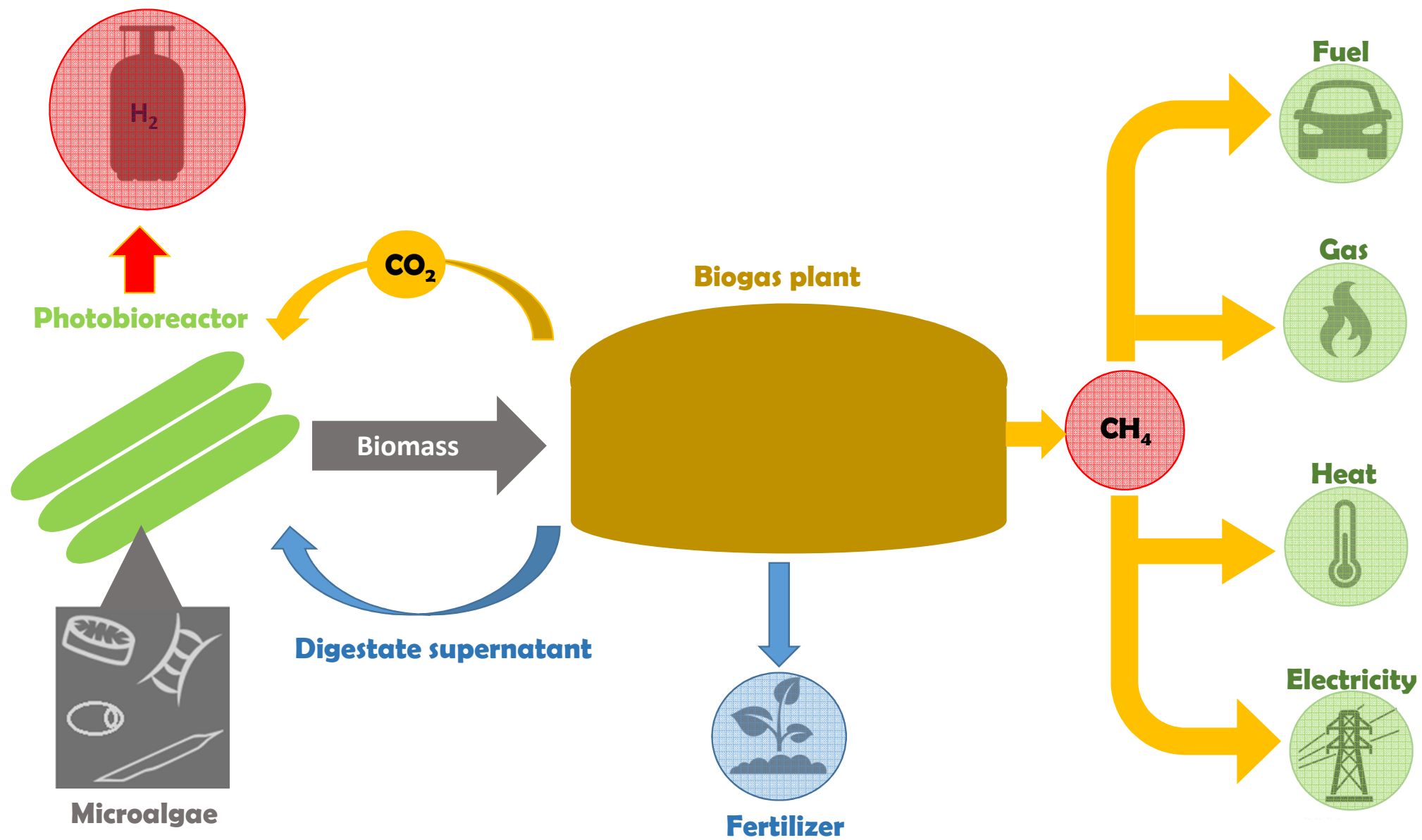
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1 **Anaerobic gaseous biofuel production using microalgal biomass – a review**

2

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25

26 **Abstract**

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

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

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43 **Highlights:**

- 44 • Microalgal biomass is a promising source for carbon-neutral biofuels.
- 45 • H₂ production: autotrophic, heterotrophic and photoheterotrophic approaches are
46 available.
- 47 • The CH₄ potential of algal biomass depends on the species and conditions.
- 48 • Combination of anaerobic H₂ and biogas production is recommended.

49

50 **1. Introduction**

51 Nowadays, global climate change and world energy crisis are among the most
52 concerned problems. These issues are mainly due to the fast industrialization, population
53 growth and increased use of fossil fuels [1]. Replacement or supplementation of fossil fuels
54 with alternative energy sources could help address this problem. For electricity production,
55 wind turbines and photovoltaic technologies have grown rapidly in recent years. The
56 requirements for liquid biofuels have been partially satisfied by mass production of first-
57 generation corn or sugarcane ethanol and biodiesel from soy, sunflower or rapeseed. To avoid
58 the food versus fuel debate in the production of agricultural commodities, next generation
59 biofuels from algal biomass, organic wastes and lignocellulose-rich materials have to replace
60 energy plants [2–5]. Algal biomass cultivation has advantages against agricultural crops. This
61 alternative biomass has fast growth rate, high contents of lipids, carbohydrates, and proteins,
62 and do not contain recalcitrant lignin. Moreover, it can be cultivated on lands that are not
63 suitable for traditional agriculture [6–8]. Interest in gaseous fuels, such as hydrogen (H₂) and
64 methane (CH₄), has increased in recent years due to their zero, or even carbon dioxide
65 negative production-and-use cycle [9–12]. Biohydrogen and biogas production from algal
66 biomass is therefore intensively studied with a goal of reducing the nutrients, energy
67 requirements and increasing the production efficiency [13–16]. In this review we summarized
68 the recent developments in the utilization of algal biomass for the production of gaseous
69 biofuels such as biohydrogen and biogas and the exploitation of anaerobic microbiology.

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

72 **2. Algal biohydrogen: Strategies for handling the oxygen sensitivity of** 73 **algal hydrogenases**

74 The advantage of the application of eukaryotic green microalgae for hydrogen
75 production is the remarkable efficiency of their [FeFe]-hydrogenases at ambient temperature
76 and pressure [20]. However, the wild-type algal [FeFe]-hydrogenases function only in
77 anaerobic environment [21] (Figure 1). The oxygen produced by photosynthesis rapidly and
78 irreversibly inactivates the active center of algal [FeFe]-hydrogenases [22]. Various
79 approaches have been proposed and tested to overcome this issue [23]. The task is to sustain
80 the alga alive while aerobic photosynthesis is suppressed and H₂ production takes place via
81 anaerobic fermentation of storage materials.

82 **2.1. Depletion strategies**

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

91 **2.1.1. Sulfur deprivation**

92 Sulfur (S) deprivation is the most studied strategy to achieve sustainable H₂ production
93 in green algae [26,27,35–37]. The D1 protein in the reaction center of photosystem-II (PSII)
94 undergoes a rapid degradation caused by the reactive oxygen radicals in response to S-
95 deprivation [30]. This results in an efficient but not complete inhibition of PSII activity (30-
96 75%) [28,38,39]. The PSII inhibition leads to a gradual decline of O₂ evolution. In the
97 presence of acetate the unaffected mitochondrial respiration consumes the residual O₂ until

98 the cultures become fully anaerobic between days 1 and 3 following S-deprivation [21,39–
99 42]. The disadvantage of the PSII inactivation is the gradual inhibition of the electron flow
100 towards the hydrogenases. Approximately 60-90% of the total electrons used for H₂ evolution
101 derive directly from PSII activity, only the remaining 20-30% of the electrons originate from
102 the previously accumulated starch [29,40,43–45].

103 2.1.2. Nitrogen deprivation

104 Nitrogen (N) deprivation has also been tested for micro-algal H₂ production
105 [25,33,46]. There are clear similarities between the S- and N-deprivation approaches.
106 Photosynthetic activity significantly decreases, while there is a general increase in the starch
107 and lipid content of the algae cells, especially in the presence of acetate [47,48]. However, the
108 aerobic phase in N-deprived cultures was conspicuously longer compared to that in S-
109 deprivation, which resulted in a delayed H₂ production [33]. The accumulation of starch and
110 lipids, and the degradation of proteins (e.g. cytochrome b6f complex) were more efficient in
111 N-deprivation than in S-deprivation [49]. Moreover, ammonium production is observed
112 during the H₂ evolution period indicating significant protein degradation [50].

113 2.1.3. Phosphorus deprivation

114 Sulfur deprivation is impossible in seawater due to the high concentration of sulfates
115 [31,32]. However, phosphorus (P) deprivation in seawater is possible. Similarly to S-
116 deprivation, the P deficiency results in decreased PSII activity, although the inactivation
117 process is considerably slower due to the slower consumption of the stored P reserves
118 compared to S-deprivation [38,51,52]. P-deprivation also created anaerobic environment in
119 the presence of acetate, which was consumed in the aerobic phase and starch accumulated. In
120 the anaerobic phase most of the starch was degraded resulting in fermentative H₂ production,
121 while acetate consumption slowed down but remained incessant. H₂ production could be
122 achieved by the inoculation of *Chlamydomonas* sp. or *Chlorella* sp. cultures into P-free
123 medium, allowing the algae to efficiently deplete the intracellular P reserves [31].

124 **2.1.4. Magnesium deprivation**

125 The magnesium (Mg)-controlled algal H₂ production is the most recent nutrient
126 deprivation method [34,53]. Mg occupies an essential position in the photosynthetic apparatus
127 as a constituent of the chlorophyll molecule. Mg-deprivation resulted in decreased
128 photosynthetic activity by ~20% [34,54], which was accompanied by the slow-down of the
129 electron transport and a concomitant reduction of the plastoquinon-pool [53-56]. H₂
130 production under Mg²⁺ deficiency is mainly linked to the PSII-dependent pathway [34]. The
131 photosynthetic antenna size and the total amount of chlorophyll molecules also decreased by
132 approximately 60%. The mitochondrial respiration was active and starch accumulation
133 increased. These activities enhanced the establishment of anaerobiosis and the continuous
134 flow of the electrons necessary for H₂ evolution. H₂ production lasted for approximately 7
135 days. The disadvantage is the requirement of a preceding 7-day long Mg-depletion period
136 under aerobic environment [34].

137 **2.2. Acetate regulation**

138 The majority of the studies on light dependent H₂ production of *Chlamydomonas* spp.
139 employed nutrient depleted algae cultures as summarized above [57,58]. These methods
140 always require two temporary separated phases. The algal biomass must be first cultivated,
141 followed by the replacement of the growth media to achieve the required nutrient shortage
142 and to promote H₂ production. Therefore these approaches are time- and energy-consuming
143 and make the process economically unfeasible [26].

144 H₂ photoproduction could also be enhanced by acetate addition in nutrient-repleted
145 media in some algal species adapted to light and anaerobiosis [21,59–61]. This way, the
146 parallel production of H₂ and substantial biomass was possible in a single step. The major
147 shortcoming of this strategy was the significantly lower H₂ production rate compared to the
148 nutrient depletion methods. Nonetheless, the establishment of the anaerobic environment took
149 place within a day as opposed to the 2-8 days under nutrient-depleted conditions [62].

150 Moreover, in aerated fed-batch bioreactors, periodic supplementation of acetate and addition
151 of O₂ greatly enhanced H₂ production and allowed semi-continuous H₂ and biomass
152 production [62].

153 **2.3. Algal-bacterial co-cultures**

154 The low H₂ production efficiency of the axenic *Chlamydomonas* spp. cultures could be
155 improved by the addition of bacterial partner(s) to the H₂ producing algae [15,63]. This way,
156 the net mitochondrial respiration of the algal cells becomes significantly elevated, allowing
157 the efficient application of stronger light regimes during H₂ production. The higher light flux
158 prompted more active water splitting reaction in PSII, which generated more electrons for H₂
159 generation. The bacterial partner consumed the excess O₂, which enabled the establishment of
160 anaerobiosis in 2-12 hours allowing quick start of H₂ evolution depending on the gas-to-liquid
161 phase ratio [15,16,63]. H₂ accumulation rates can be further elevated by lowering the
162 competing bacterial H₂-uptake activity, e.g. using uptake-hydrogenase deficient bacterial
163 strains. Using both the bacterial partners and S-depleted algae cultures doubled the H₂ yield
164 by shortening the aerobic phase [63]. Increased volumetric hydrogen production rate was
165 achieved by the application of a *Chlorella* sp. strain, which has remarkably smaller cell size
166 than that of the commonly investigated *Chlamydomonas* spp. strains [16]. In addition to the
167 rapid O₂ consumption and early start of H₂ production, the algal biomass grew more
168 efficiently in symbiosis with its bacterial partner than in axenic cultures in complete media
169 [64,65].

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

175 **3. Anaerobic digestion of microalgal biomass**

176 The decomposition of organic materials is carried out under anaerobic conditions and
177 a great variety of diverse microbes participate in the microbial food chain gradually, which
178 degrades the complex molecules essentially to a mixture of CH₄ and CO₂ [68–70]. The idea of
179 using microalgal biomass substrate in anaerobic digestion (AD) dates back to the 1950s [71]
180 (Figure 2), when a mixed culture of *Chlorella* sp. and *Scenedesmus* sp., grown in wastewater,
181 was utilized. In the sporadic follow-up work, biogas composition and AD process stability of
182 different microalgae species were investigated [72–81].

183 **3.1. Strain selection**

184 Biogas productivity from representatives of various microalgal groups were compared,
185 including fresh- and seawater strains [82–85]. As a general feature in mesophilic conditions,
186 the CH₄ content of the biogas from the microalgae was ~7-13% higher than that from maize
187 silage, the most widespread substrate in biogas industry [82]. Albeit the higher CH₄ content,
188 the overall biogas yields varied depending on the cell wall structure of the algae strains.
189 Easily biodegradable species either lack cell wall, as in the case of *Dunaliella salina*
190 halophilic microalgae [86], or their cell wall is rich in easily-biodegradable protein
191 substances, as in the case of *Chlamydomonas reinhardtii* [87]. Other species such as *Chlorella*
192 *kessleri* and *Scenedesmus obliquus* have hemicellulose-rich, more recalcitrant cell walls,
193 making them difficult to hydrolyse [88-93].

194 **3.2. Physico-chemical pre-treatments**

195 In addition to strain selection, biogas yield from algae can be improved by suitable
196 pre-treatments, i.e. disruption or solubilisation of the cell wall. The possibilities have been
197 recently reviewed [94]. The main pre-treatment strategies include mechanical, thermal,
198 chemical and biological methods. The key limiting parameter determining large scale
199 application of these technologies is their energy consumption. Mechanical pre-treatments,
200 including sonication, are efficient to disrupt the cell wall, but the energy requirement render

201 them economically unfeasible [95]. Thermal treatment provided promising results in biogas
202 production enhancement although concentrated biomass is needed to reach positive energy
203 balance [80,96–99]. The heat induced polymerization of available reducing sugars and amino
204 acids to complex molecules may explain this phenomenon [80,82,100]. Chemical
205 solubilisation of microalgal biomass presented higher effectiveness compared to thermal
206 treatment but biogas production did not increase accordingly [82,84,100,101].

207 **3.3. Biological pre-treatments**

208 Biological methods involve the application of various enzymes to decompose the cell
209 wall polymers effectively. Protease pre-treatment of *S. obliquus* and *C. vulgaris* enhanced the
210 CH₄ yields 1.72-fold and 1.53-fold, respectively [103]. In a similar approach an enzyme
211 cocktail, including β -glucanase, xylanase, cellulase and hemicellulase, was efficient in
212 facilitating AD of algal biomass [104,105]. The main restricting factor of the biological pre-
213 treatment methods is the cost of enzyme production. Therefore, *in situ* enzyme production has
214 been suggested. This could be done by separating the hydrolytic-acidogenic stage from the
215 methanogenesis stage in a two-stage AD design [67]. Bioaugmentation of biogas formation
216 from algal biomass employing *Clostridium thermocellum* improved the degradation of
217 *Chlorella vulgaris* biomass. In this two-step process *C. thermocellum* was added first and
218 methanogenic sludge subsequently beneficially increased the bioenergy yield [106].
219 Significant improvements in the methane yield were observed through biological pre-
220 treatment of mixed microalgal cultures (mainly *Oocystis* sp.) using *Trametes versicolor* fungi
221 and commercial laccase. The CH₄ yield increased by 20% for commercial laccase and 74%
222 for fungal broth in batch tests, as compared to non-pretreated biomass [82,106]. An
223 interesting novel approach has been explored when genes of foreign lytic enzymes, involved
224 in cell division and programmed cell death, were expressed in algae to enhance cell disruption
225 [108]. A recent review summarized numerous studies on pretreatments [80].

226 **3.4. Salt effects**

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

236 **3.5. C/N ratio**

237 The C/N ratio has a very significant impact on the methane yield and on productivity
238 in all microalgae-based AD. The optimal C/N ratio of AD is between 20 and 30 [113]. AD of
239 substrates having lower C/N results in increased free ammonia, which may become inhibitory
240 [114]. Microalgal species usually contain higher proportion of proteins compared to terrestrial
241 plants. The C/N ratio of green microalgae is generally low (C/N ~10), while terrestrial plants
242 have higher ratios (depending on the plant species and season, C/N ~20-40) [115]. This has
243 been corroborated in studies in microalgae from natural reservoir (mainly *Chlorella* sp. and
244 *Scenedesmus* sp.), which had a C/N ratio of 6.7, *C. vulgaris* having a C/N ratio of 5, and *S.*
245 *obliquus* possessing C/N of 8.9 [15,116,117]. Ammonia accumulation at low C/N ratio has
246 been observed in various studies [71,118,119]. The use of ammonia-tolerant inoculum could
247 be a promising solution to effectively digest the protein-rich microalgal biomass in a
248 continuous biogas-producing process [120]. AD of algal biomass generated under N-
249 limitation showed efficient CH_4 production due to the favourable C/N ratio of the substrate
250 [84,85].

251 **3.6. Effects of OLR and HRT**

252 A proper organic loading rate (OLR) and hydraulic retention time (HRT) can diminish
253 the negative effects of inhibitory conditions. HRT is the time allowed for any given substrate
254 to be digested. OLR is the amount of volatile solids to be fed into the digester daily in a
255 continuous AD process. The biogas yield rises upon increasing the OLR, but above the
256 optimal OLR the volatile solids degradation and biogas yield decrease due to overloading
257 [121]. In order to reduce operation costs and achieve optimum performance, biogas reactors
258 should be designed to operate at maximum methane production at lowest HRT and highest
259 OLR [122]. An effective OLR of *Chlorella* biomass at mesophilic conditions was found at 5g
260 VS L⁻¹ d⁻¹ [123]. Higher OLR increased the level of valeric and butyric acids resulting process
261 inhibition. Other studies also confirmed that highest biogas yields were attained at the low
262 OLR, i.e., 0.6g VS L⁻¹ d⁻¹ (mixed culture containing *Chlamydomonas reinhardtii* and
263 *Pseudokirchneriella subcapitata* in mesophilic conditions) [124]. Typical OLRs are between
264 1–6 g VS L⁻¹ d⁻¹ and HRT varies between 10 and 30 days [83,122,125].

265 **3.7. Co-digestion**

266 Co-digestion is a promising strategy to increase the performance of a digester by
267 ensuring optimal substrate composition, which can enhance biogas productivity from
268 microalgal biomass. Significant enhancement of methane production upon addition of waste
269 paper to the algal sludge has been reported [116]. Long-term experiments using mixtures of
270 maize silage and marine microalga *Nannochloropsis salina* were investigated under batch and
271 semi-continuous conditions. The biogas yields were significantly increased and the semi-
272 continuous AD was stable for more than 200 days [126]. Increased CH₄ production was
273 observed in a mixture of *Chlorella* sp. microalgal biomass and food waste [127]. The elevated
274 CH₄ production was probably due to the multi-stage digestion of different substrates having
275 different degrees of degradability. Co-digestion of algal biomass with sewage sludge or liquid
276 manure has been shown to be advantageous in several cases [125,128]. In a laboratory scale

277 fed-batch co-fermentation experiment of algal-bacterial mix, the cumulative methane yield
278 was $\sim 350 \text{ mL CH}_4 \text{ g VS}^{-1}$ (OLR: $1 \text{ g VS L}^{-1} \text{ d}^{-1}$; HRT: 1 d, mesophilic conditions) [15]. In
279 another study from the same research group, microbiologically pure *Scenedesmus obliquus*
280 and maize silage were subjected to co-fermentation (OLR: $1 \text{ g VS L}^{-1} \text{ d}^{-1}$; HRT: 1 d). The
281 observed methane yield was $\sim 280 \text{ mL CH}_4 \text{ g VS}^{-1}$. It is noteworthy that co-digestion resulted
282 in significantly higher methane productivity in both cases relative to the microalgal biomass
283 mono-substrate [15,66]. The addition of used cooking oil, maize silage, and mill residue to
284 AD of the microalga *Chlorella vulgaris* was studied in semi-continuous, laboratory-scale
285 digestions by Rétfalvi et al. [117]. The volumetric methane yields were in the range of 300 to
286 $500 \text{ mL CH}_4 \text{ g VS}^{-1}$ (OLR: $0.78\text{-}2.15 \text{ g VS L}^{-1} \text{ d}^{-1}$; HRT: 88-383 d). Triple co-digestion of oil-
287 extracted *Chlorella vulgaris* microalgal biomass, glycerol and chicken litter in various
288 proportions was studied under mesophilic conditions [129]. Oil-extracted microalgae in co-
289 digestion with chicken litter enhanced the biochemical methane potential. The highest CH_4
290 yield was $131 \text{ mL CH}_4 \text{ g VS}^{-1}$ (HRT: 90 d). Based on these results, co-digestion may be the
291 recommended approach to degrade microalgal biomass effectively and sustainably without
292 pre-treatment.

293 **4. Conclusions and outlooks**

294 Utilization of solar energy stored in microalgal biomass is a promising source for
295 anaerobic gaseous biofuel production. Despite the technological challenges the interest in
296 microalgae-based biofuels increases [13,14,130,131]. Innovative developments in microalgal
297 cultivation will reduce biomass production costs. Aqueous waste streams are inexpensive and
298 efficient growth media for mixed algal-bacterial biomass production, which is a suitable
299 substrate for biohydrogen and biological CH_4 production via anaerobic fermentation [132–
300 137]. Natural habitat of microalgae may expand the limits of deprivation methods. The
301 efficiency of AD using microalgal biomass depends on various factors, such as strain

302 selection, pre-treatment, OLR, HRT, reactor design, temperature and pH [79,80]. In
303 microalgae-based biogas production the goal is to maintain effective and balanced operation.
304 An emerging and effective strategy to improve technical and economic feasibility is co-
305 digestion with organic wastes or by-products to optimize process parameters. The coupling of
306 biohydrogen and biogas production processes, using algal-bacterial co-cultures, is
307 recommended.

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763 Figure legends:

764 Figure 1. Schematic link between oxygenic photosynthesis and hydrogen production.

765 Abbreviations: PS II: Photosystem II; PS I: Photosystem I; Pheo: pheophytin; PQ:
766 plastoquinon; Cytb/Cytf: Cytochrome bf complex; PC: Plastocyanin; FD: ferredoxin;
767 H₂ase: hydrogenase; NPQR: NADP quinone reductase; PFOR: pyruvate ferredoxin
768 oxidoreductase; FDox: oxidized ferredoxin; FDred: reduced ferredoxin.

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

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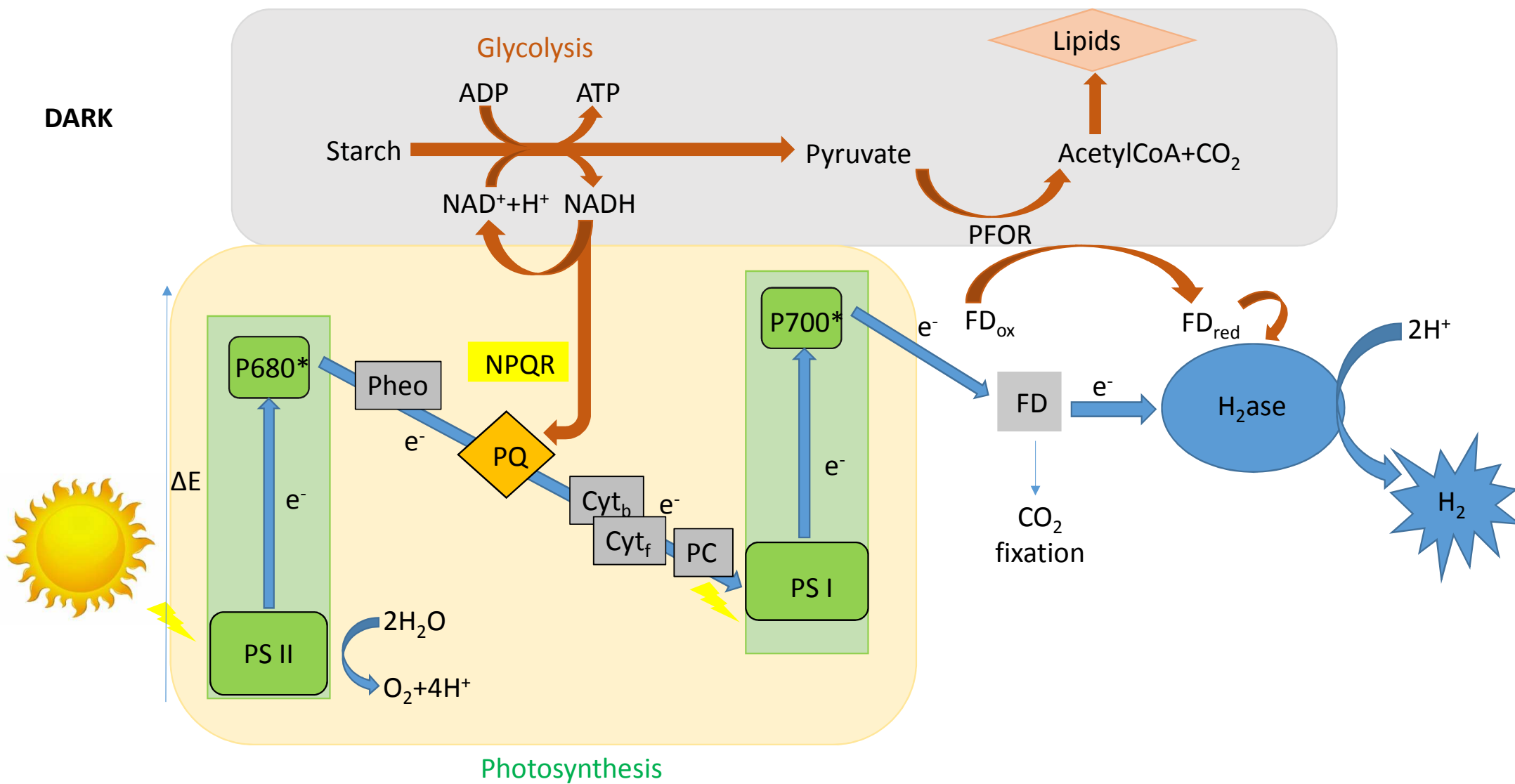
773 Table 1. Summary of depletion-induced photosynthetic biohydrogen strategies.

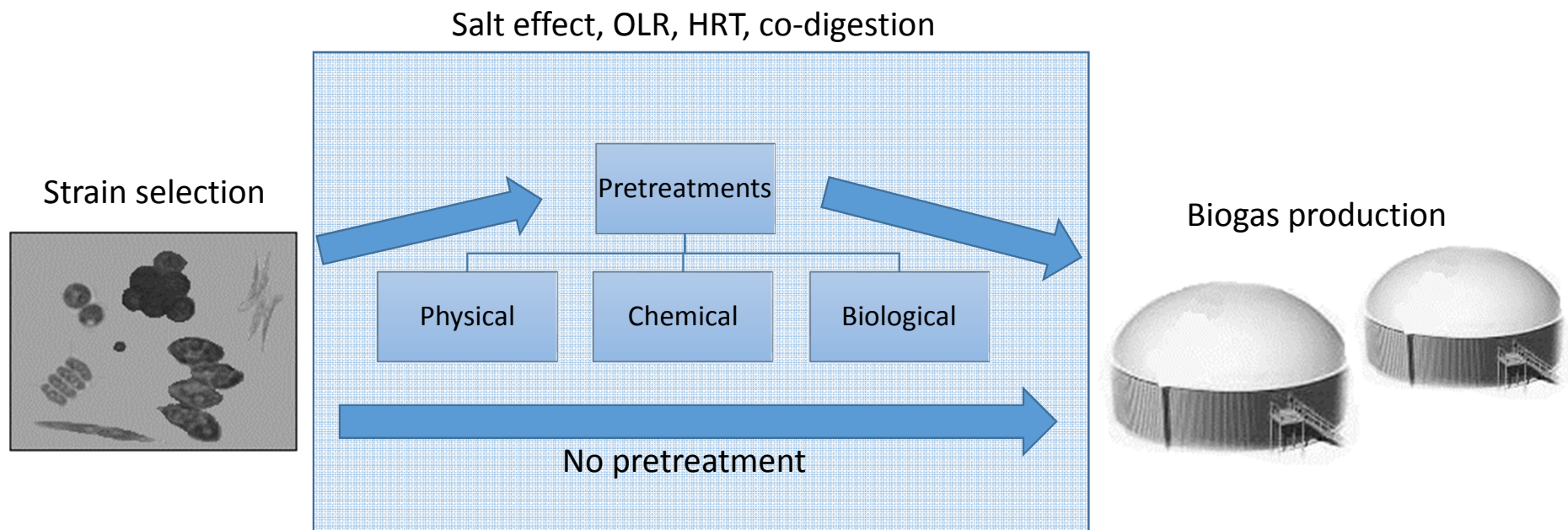
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| Strategy | Lag of H ₂ production (hours) | Accumulated H ₂ yield (ml H ₂ l ⁻¹ culture h ⁻¹) | Effects of treatment | References |
|---------------------------------|--|---|--|---|
| Sulfur deprivation | 24-72 | 1.6-3 | Down-regulated photosynthesis 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 <i>b6f</i> 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 |

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Highlights:

- Microalgae are promising source of alternative carbon neutral biofuels.
- H₂ production: autotrophic, heterotrophic and photoheterotrophic approaches.
- The CH₄ potential of algal biomass depends on the species and AD conditions.
- Combination of anaerobic H₂ and biogas production is recommended.