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Algae as promising feedstocks for fermentative biohydrogen production according to a biorefinery approach: A comprehensive review

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ABSTRACT

Interest is growing in the production of biohydrogen from algae through dark fermentation, as alternative to fossil fuels. However, one of the limiting steps of biohydrogen production is the conversion of polymeric carbohydrates into monomeric sugars. Thus, physical, chemical and biological pretreatments are usually employed in order to facilitate carbohydrates de-polymerization and enhancing biohydrogen production from algae. Considering the overall process, biohydrogen production through dark fermentation leads generally to negative net energy balances of the difference between the energy produced as biohydrogen and the direct ones (heat and electricity) consumed to produce it. Thus, to make the overall process economically feasible, dark fermentation of algae must be integrated in a biorefinery approach, where the outlets are valorized into bioenergy or value added biomolecules.The present study reviews recent findings on pretreatments and biohydrogen production through dark fermentation of algae looking at the perspectives of integrating side streams of dark fermentation from algal biomass, according to a biorefinery approach.

Contents

Abbreviations: AD, anaerobic digestion; BHP, biological hydrogen potential; BBD, Box–Behnken design; BESA, 2-bromoethanesulfonic acid; COD, chemical oxygen demand; DF, dark fermentation; ECE, energy conversion efficiency; FHP, fermentative hydrogen potential; HMF, hydroxylmethylfurfural; HPB, hydrogen producing bacteria; HRT, hydraulic retention time; LHW, liquid hot water; MEC, microbial electrolysis cells; MFC, microbial fuel cell; MOW, mariculture organic waste; OLR, organic loading rate; PHA, polyhydroxyalkanoates; PF, photofermentation; RSM, response surface methodology; S/L, solid to liquid ratio; TS, total solids; VFA, volatile fatty acids; VS, volatile solids

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1. Introduction

Depletion of fuels, environmental pollution and climate change compels the search for sustainable and environmental sources of energy to sustain the industrial economy and society [1]. In this context, macro and micro-algae offers a huge potential for the production of biofuels. If used in a sustainable way, algal biomass could be beneficial for the reduction of the world's dependency on oils, as well as the global emission of greenhouse gases [2]. Solid (pellets, wood chips), liquid (bioethanol, vegetable oil and biodiesel) or gaseous (biogas, biohydrogen) biofuels can be categorized into 1st, 2nd and 3rd generations according to the origin of the biomasses used [3].

Recently, the use of first generation biofuels from agricultural substrates, traditionally destined for food and animal purposes, raised controversial debates due to the "food versus fuel" dilemma [4,5]. This led to the development of 2nd generation biofuels (bioethanol, biohydrogen, methane) produced from non-food biomass, such as crop residues (corn stover, manure, straw, waste wood) and energy crops cultivated on no arable lands (miscanthus, sorghum, poplar, willow, switchgrass) [6–8]. The use of these lignocellulosic substrates presents several advantages, since they are abundant renewable non-food materials and do not create competition for lands with food crops. Nevertheless, contrarily to first generation biofuels, the production of second generation fuels is still not cost effective. Indeed, expensive pretreatment steps are required to defeat the intrinsic compositional and physical barriers of lignocellulosic matrices in order to convert such substrates into biofuels [9,10].

In this light, 3rd generation biofuels derived from algae could be considered as another viable alternative energy source that is devoid of the major drawbacks associated with the first and second-generation biofuels [3,11–13]. Indeed, algae present several advantages compared to terrestrial plants: (i) higher growth rate with superior CO_2 fixation capacity; (ii) they do not need arable land to grow; (iii) they do not contain lignin. Nevertheless, the cultivation of microalgae requires high water use and high initial investment that can make the process still not economically appealing [14].

Biohydrogen production trough dark fermentation (DF) process has gained increased attention in last years, mainly due to process simplicity and possibility to convert a wide range of substrates [7]. Moreover, hydrogen gas presents a high-energy yield (122 kJ g^{-1}); its combustion generated only water vapour and its surplus can be stored and used when needed [15]. Like starch- and lignocellulosic-based substrates, algal biomass as a feedstock for DF biohydrogen production requires firstly the conversion of polymeric carbohydrates into readily accessible monomeric sugars [16]. Anaerobic mixed cultures are characterized by low hydrolytic enzymatic activity; consequently a pre-treatment step is often required to enhance the hydrolysis of algae biomass [7,17]. So far, among the different pretreatment categories (physical, chemical and biological), the most commonly used to enhance carbohydrates hydrolysis of micro and macroalgae are physical (milling, ultrasonic, microwave), thermal (LHW, steam explosion,) and thermo-chemical pretreatments. However, the major drawback of using thermal and thermo-chemical pretreatments is the possible formation of by-products, such as aliphatic acids and furan derivatives (furfurals and 5-HMF), which can inhibit the action of enzymes and/or further reduce the sugar conversion into H_2 [18,19].

Finally, biohydrogen production through dark fermentation leads generally to negative net energy balances as of the difference between the energy produced and the direct ones (heat and electricity) consumed to produce it [15]. Furthermore, during carbohydrates conversion, only 1/3 is directed to H_2 producing pathways and most of the hydrogen equivalents are still incorporated in Volatile Fatty Acids (VFAs) (i.e. butyrate, acetate) or solvents (i.e. ethanol, acetone) [20]. Acid metabolites accumulation in DF process can also cause a sharp drop of pH, resulting in the failure of the process [21]. Consequently, to be sustainable, dark fermentation must be integrated in a biorefinery approach where the outlets are valorised for bioenergy production or valuable added compounds (Fig. 1). For this purpose, the solid phase could be partially converted into methane through anaerobic digestion process or into other biofuels using thermo-chemical conversion processes (i.e. co-gasification, co-pyrolysis) [22,23]. Recently, the valorization of liquid effluents which are rich in VFAs (mainly acetate and butyrate) has attracted a lot of attention [24,25]. Among them, biogas production through anaerobic digestion [26], photofermentation [27,28] and bio-electrochemical systems such as microbial fuel cells (MFC) or microbial electrolysis (ME) [21,29] as well as heterotrophic microalgae cultivation [30] and value added molecules (PHAs) [31] have been reported.

The objective of the present study is to review recent findings on biohydrogen production from algae through dark fermentation. For this purpose, the following points are discussed: (a) chemical characterization of macro and microalgae; (b) screening of biohydrogen potentials of different algal species and limiting factors that influence their efficient conversion into biohydrogen; (c) effect of pretreatments to enhance biohydrogen production from algae; (d) investigation of various valorisation routes of dark fermentation outlets of algae according to a biorefinery approach.

2. Algae biomass

Microalgae and macroalgae are simple chlorophyll containing organisms which are able to photosynthetically convert atmospheric carbon dioxide into a wide range of metabolites and chemicals including proteins, hydrogen, polysaccharides and/or lipids [32].

For these reasons, they have received great attention as novel biomasses to produce biofuels (i.e. biodiesel, bioethanol, biogas,

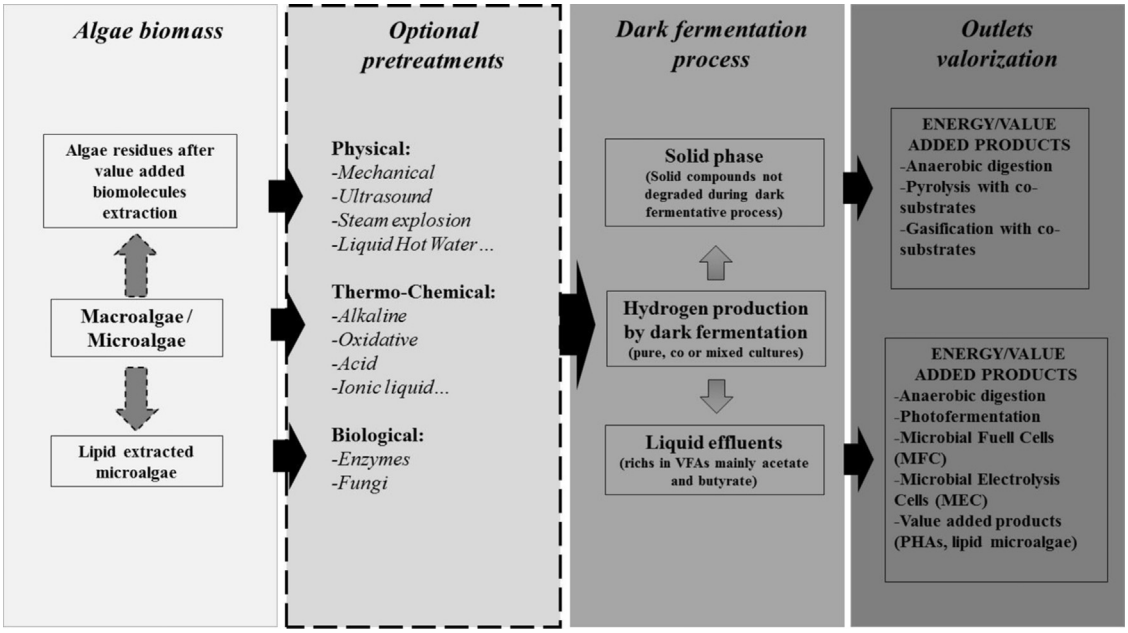

Fig. 1. Algae biomass or co-products conversion into biohydrogen through dark fermentation process integrated in a biorefinery approach concept.

biohydrogen) and/or biomolecules (i.e. agars, alginates, carrageenans and phytosterols) [33].

2.1. Macroalgae

Macroalgae, also called seaweeds, constitute the most important organisms in marine ecosystems for the prevention of eutrophication and pollution [34]. They are photoauxotrophic organisms and thus they produce and store organic carbons by utilizing CO_2 and HCO_3, with very high productivity rates [35,36]. In their natural environment, they grow on rocky substrates and they are fast growing, reaching sizes up to 60 m in length [37]. Thanks to their high capacity to generate and store carbon resources, they have recently attracted attention as possible substrates for biorefinery.

Indeed, the world amount of mass-cultivated macroalgae has continuously increased during the last ten years, reaching a total amount 16×10^6 wet metric ton in 2010 [38,39]. The pigment, growth, and chemical composition of macroalgae are significantly affected by their habitat conditions (i.e. light, temperature, salinity, nutrient, pollution, and even water motion) [40]. Light represents the most important factor that affects pigmentation allowing to classify macroalgae into Phaeophyta (brown), Rhodophyta (red) and Chlorophyta (green) algae [41]. According to the respective pigments, algae are able to selectively absorb the light with specific wavelengths. For instance, some red algae are usual to grow in the deep sea [42].

In terms of chemical composition, macroalgae, except green algae, do not have high content of starch and oil [43]. They do not contain lignin [44], have high contents of water (70–90% fresh wt.) and minerals such as alikali metals (10–50% dry wt.) [45]. They have also low amount of proteins (7–15% dry wt.) and lipids (1–5% dry wt.) [46], but a high content of carbohydrates.

Carbohydrates of green, red, and brown algae differ in quality and quantity, according their species. Generally the amount of carbohydrates vary between 25–60%, 30–60%, and 30–50% dry wt. for green, red, and brown algae, respectively [45–48].

As recently reviewed by Jung et al. [49], green algae are mainly composed of mannan, ulvan, starch and cellulose. While, starch (i.e., α-1-4-glucan) accounts for 1–4%, ulvan, which is mainly composed of D-glucuronic acid, D-xylose, L-rhamnose and sulphate, is a distinctive feature of green macroalgae [50].

The main polysaccharides of brown algae are alginate, laminarin, fucoidan, cellulose and mannitol [51]. Alginate is the principal constituent of the cell wall and accounts for the 40% dry wt. of brown algae [52]. It is composed of mannuronic and guluronic acid blocks [50]. Laminarin in brown algae accounts for the 35% dry wt. and it is composed of β-1,3-glucans units [53]. Alginates from brown algae are used mostly in textile (50%) and food (30%) industries [37].

Finally, red algae are mainly composed of carrageenan (up to 75% dry wt.) and agar (up to 52% dry wt.), as polysaccharides [54]. Carrageenan is composed of repeating D-galactose and anhydro-galactose units, while agar consists of alternating β-D-galactose and α-L-galactose with scarce sulfactions [50]. Purified carrageenans from red algae are usually used to produce gel; agar from red algae is used to produce hydrocolloids in food, pharmaceutical and biological industries [50,55,56].

2.2. Microalgae

Microalgae are photosynthetic unicellular or simple-multicellular microorganisms that are normally found in marine and fresh water habitats. They can be grouped into prokaryotic microalgae (cyanobacteria *Chloroxybacteria*), eukaryotic microalgae (green algae *Chlorophyta*), red algae (*Rhodophyta*), and diatoms (*Bacillariophta*). Besides to natural environments, microalgae can be cultivated in freshwater, seawater, and wastewater within open ponds (raceway) and closed photo-bioreactors. Certain microalgae can tolerate and adapt to a wide variety of environmental conditions (in terms of pH, temperature, light, etc.) and they can be produced all year round [57]. Under optimal conditions, they double in number within hours, permitting a short harvesting cycle (less than ten days) [58]. In terms of chemical composition, microalgae are mainly composed of proteins (40–60%), carbohydrates (8–30%), lipids (5–60%), and other valuable components (pigments, anti-oxidants, fatty acids, and vitamins) [57].

Chemical composition of microalgae is high variable, depending mainly 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 algae [59,60]. Carbon dioxide

concentration, salts stress, temperature and light affect the carbohydrates accumulation in microalgae [60]. Izumo et al. [61] obtained a 2.5 fold increase in carbohydrates content when the supplied carbon dioxide concentration was reduced from 3% to 0.04%. It is general known that higher light intensities and temperature increase the carbohydrates content of microalage [62,63]. For instance, the level of carbohydrates in *Spirulina sp.* was slightly enhanced by increasing the temperature from 25 to 40 °C [63].

Finally, it is important to note that for the production of bioenergy from microalgae, the economic feasibility remains the most important aspect to consider. Indeed, there are many operational parameters that can make the conversion process more or less economical appealing. First of all, the use of water: growing microalgae in wastewaters for biofuel and bioenergy production seems a viable and economical friendly option. Another aspect is related to the method of microalgae cultivation. Indeed, cultivation of algae in open ponds seems to be the most economical and preferable way, even if the possibility of contamination with other organisms is the main disadvantage.

Last but not least, harvesting biomass costs remains an important issue in the case of low value added product like biofuels. It was estimated that harvesting microalgae accounts for 20–30% of the total biofuel production cost [64]. Nowadays, centrifugation remains the most common harvesting method, though very expensive. Other possible methods are filtration, sedimentation, flocculation and floatation. The choice of the harvesting method has to be chosen carefully according the final end products. Indeed, in the case of low value added end products like biofuels, low cost harvesting methods are recommended. Among them, the most promising low-cost method is gravity settling enhanced by flocculation, without the use of chemical flocculants [65].

An integrated approach can be also devised, in which microalgae substrates after lipid extraction, still rich in carbohydrates (i.e. cellulose, starch) and proteins can be processed by dark fermentation [66–68] and/or anaerobic digestion [69]. Various techniques of lipids extraction have been recently reviewed by [33]. These consist of expeller/oil press, ultrasound methods, solvent extraction and supercritical fluid extraction.

3. Dark fermentation process

3.1. Principles of dark fermentation

Dark H_2 fermentation is a simple process that requires low energy and can use various kinds of organic waste [7,70]. Monosaccharides (i.e., glucose, mannose, galactose etc.) and polymers such as starch or cellulose can be used as hydrogen feedstocks. There are two common pathways in the production of hydrogen by dark H_2 fermentation: one producing acetate and the second butyrate (Fig. 2).

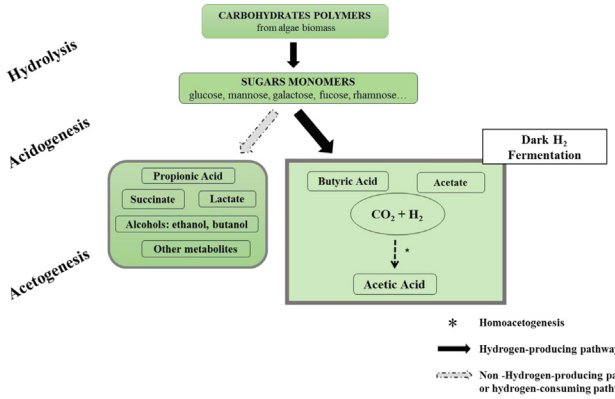

Fig. 2. Scheme of carbohydrate polymers degradation through dark fermentation process operated with mixed cultures (adapted from [71]).

Theoretically, the hydrolytic fermentation of 1 mol of glucose yields to 4 and 2 mol of H_2 through acetate and butyrate pathways, respectively [72]:

1) $C_6H_{12}O_6+2H_2O=4H_2+2CO_2+2CH_3COOH$
(hydrogen fermentation to acetate pathways)
2) $C_6H_{12}O_6=2H_2+CH_3CH_2CH_2COOH+2CO_2$
(hydrogen fermentation to butyrate pathways)

Dark fermentation can be realized either by pure or mixed cultures of hydrogen producing bacteria (HPB). The use of pure cultures for hydrogen production trough dark fermentation has been extensively evaluated and reported in literature (for a comprehensive review see [73]). When pure cultures are used with sugar substrates, a H_2 yield up to 3.5–3.8 mol H_2 mol^{-1} glucose equivalent can be achieved, either by mesophilic (*Enterobacter colacae* DM11) or termophilic (*Caldicellulosiruptor saccharolyticus*) strains [74]. The main advantage is the possibility to optimize the operational parameters ($T°$, pH…) in order to maximize the single strain attitude to produce higher hydrogen yield. Moreover, metabolic engineering approach can be applied to increase the H_2 yield and kinetics by genetic manipulation of selected strains [75,76]. The drawbacks are linked to the different performance of the same strain with different substrates, the need to sterilize the matrices to avoid contamination of the inoculum and the low resilience of a single H_2-producing strain when operational condition changes. About pure or co-cultures, Rittman and Herwig [77] comprehensively reviewed strains and processes adopted in pure and defined co-culture dark fermentative HPB since 1901. By this wide survey, they evidenced that thermophilic strains comprise high substrate conversion efficiency, but mesophilic strains achieve high volumetric productivity.

Mixed cultures generally originating from natural environments such as soils and anaerobic sludge to produce hydrogen [7]. Mixed cultures are easier to use because they are simpler to operate and do not require sterile conditions compared to pure cultures [7]. Mixed cultures, mainly characterized by microorganisms belonging to the genus Clostridia and Bacillus, are more suitable for degrading a wide range of complex and unsterile substrates [78]. However, specific treatments are needed to select HPB and inhibit H_2 competing or consuming bacteria from the starting inoculum [70,79]. Methanogens, which are considered as the main hydrogen-consuming microorganisms, can be easily eradicated by using pre-treatments such as heat shock and pH control (with acid/alkali) whereas in such conditions, hydrogen producing bacteria (i.e. *Clostridia* species) can sporulate [79,80–83]. However, it was shown that the effect of inoculum pretreatment on DF performances is highly dependent of the origin of mixed cultures [84,85]. Apart from the clear advantage to select for HPB from the complex microbial community of a common inocula (e.g. sewage sludge), it has to be noted that a reduced diversity of HPB is produced (lack of mesophilic, non-spore forming HPB) and that the phylogenetic and metabolic heterogeneity of *Clostridium* spp. itself [86] cannot guarantee the maximization of final H_2 (H_2 yield higher than 2.5 mol per mol of hexose are still never observed using mixed cultures [71]).

3.2. Operational parameters of dark fermentation

Besides the kind of inoculum sources, operational process parameters (i.e. temperature, pH and organic loading rate) can affect significantly the performances and efficiency of HPB and

Table 1
Biohydrogen production yields obtained from macro and microalgae.

Feedstocks	Preparation of feedstocks	Fermentative processes characteristics	Inoculum sources	H_2 yield ($mL\ g^{-1}$ TS)	Ref.
Macroalgae					
Green macroalga *Codium fragile*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	24.4	[102]
Green macroalga *Chlamydamonas reinhardtii*	Wash with fresh water	Batch bottles, T=75 °C	Hyperthermophilic eubacterium *Thermotoga neapolitana*	0	[107]
Green macroalga *Ulva Lactuca*	Wash with fresh water, dry at room temperature, milling size not specified	Batch bottles, T=35 °C, no pH control	WWTP anaerobic digester sludge, no heat shock treatment	10[a,c]	[108]
Red macroalga *Gelidium amansii*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	43.1	[102]
Red macroalga *Porphyra tenera*	Wash with fresh water, dry at room temperature, milling size not specified	Batch bottles, T=35 °C, no pH control	WWTP anaerobic digester sludge, no heat shock treatment	9[a,c]	[108]
Red macroalga *Porphyra tennera*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	15.4	[102]
Red macroalga *Gracilaria verrucosa*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	26.7	[102]
Brown macroalga *Hizikia fusiforme*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	10.3	[102]
Brown macroalga *Laminaria Japonica*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	67	[102,109]
Brown macroalga *Ecklonia stolonifera*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	26.6	[102]
Brown macroalga *Undaria pinnatifida*	Wash with fresh water, dry at 105 °C, milled at 0.5 mm	Batch bottles, pH=5.5, T=35 °C	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	20.4	[102]
Brown macroalga *Undaria Pinnatifida*	Wash with fresh water, dry at room temperature, milling size not specified	Batch bottles, T=35 °C, no pH control	WWTP anaerobic digester sludge, no heat shock treatment	13[a,c]	[108]
Brown macroalga *Laminaria Japonica*	Wash with fresh water, dry at room temperature, milling size not specified	Batch bottles, T=35 °C, no pH control	WWTP anaerobic digester sludge, no heat shock treatment	25[a,c]	[108]
Brown macroalga *Laminaria Japonica*	Dried at room temperature, ground into 0.5 mm particle size.	Batch bottles, T=35 °C, pH=5.5	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	71.4	[110]
Brown macroalga *Laminaria Japonica*	Wash with fresh water, dried at 105 °C, ground into 0.5 mm particle size.	Anaerobic Sequencing Batch Reactor (ASBR), HRT=6 days, OLR=3.4 g COD L^{-1} d^{-1}, pH=5.5, T=35 °C.	WWTP anaerobic digester sludge (operated at 36 °C), heat pretreated: 90 °C, 20 min	61.3	[17]
Brown macroalga *Laminaria Japonica*	Wash with fresh water, dried at room temperature, ground into 0.5 mm particle size.	Anaerobic Sequencing Batch Reactor (ASBR), HRT=6 days, OLR=3.4 g COD L^{-1} d^{-1}, pH=5.5, T=50 °C.	WWTP anaerobic digester sludge (operated at 36 °C), heat pretreated: 90 °C, 20 min	34.1	[17]
Brown macroalga *Laminaria Japonica*	Dried at room temperature, ground into 0.5 mm particle size.	Anaerobic Sequencing Batch Reactor (ASBR), HRT=6 days, pH=5.5, T=35 °C.	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	58.5[b]	[110]
Brown macroalga *Laminaria Japonica*	Wash with fresh water, dried at room temperature, ground into 0.5 mm particle size.	Anaerobic Sequencing Batch Reactor (ASBR), HRT=6 days, OLR=3.4 g COD L^{-1} d^{-1}, pH=5.5, T=65 °C.	WWTP anaerobic digester sludge, (operated at 36 °C), heat pretreated: 90 °C, 20 min	43.5	[110]
Microalgae					
Microalga *Chlorella Vulgaris*	Stored at 4 °C	Batch bottles, T=35 °C, pH initial=7.4	WWTP anaerobic digester sludge, heat pretreated: 90 °C, 20 min	31.2	[104]
Microalga *C. Vulgaris sp.*	Harvested by floculation followed by centrifugation, pH adjusted to 7 and stored at −20 °C	Bacth bottles, T=30 °C	Enrichment of compost pile	114[a]	[111]
Microalga *C. vulgaris sp.*	Harvested by floculation followed by centrifugation, pH adjusted to 7 and stored at −20 °C	Batch bottles, T=37 °C	Produced by bacteria naturally present in microalgal biomass slurry, methanogenesis suppressed by 20 mM BESA	11[a]	[106]
Microalga *Chlorella sp.*	n.d	Batch bottles, T=35 °C, pH=6.5; S/I=3.3	Anaerobic digested sludge, heat pretreated: 80 °C, 30 min	6.1	[105]
Microalga *C. Pyrenoidosa sp.*	Harvested by centrigfugation, spray dried	Batch bottles, T=35 °C, pH initial=6	Hydrogen-producing bacteria isolated from a sludge anaerobic digester	8.8	[27]
Microlaga *Chlamydomonas reinhardtii*	Harvested by centrigugation (6,000 g, 15 min).	Batch bottles, T=37 °C	Pure culture *C. butyricum NCIB 9576*	40	[112]
Microalga *D. tertiolecta sp.*	harvested by floculation followed by centrifugation, pH adjusted to 7 and stored at −20 °C	Batch bottles, T=37 °C	Produced by bacteria naturally present in microalgal biomass slurry, methanogenesis suppressed by 20 mM BESA	13[a]	[106]
Microalga *D. tertiolecta sp.*	Harvested by floculation followed by centrifugation, pH adjusted to 7 and stored at −20 °C	Bacth bottles, T=30 °C	Enrichment of compost pile	58[a]	[110]
Microalga *Nannochloropsis* Oceanica *sp.*	Harvested by centrigfugation (5000 g), oven dried and powdered to 0.02 mm mesh size	Batch bottles, T=35 °C, pH initial=6	Hydrogen-producing bacteria isolated from a sludge anaerobic digester	0–2	[113]
Microalga *Nannochloropsis sp.*		Bacth bottles, T=30 °C	*Enterobacter aerogenes ATCC 13048*	48[a]	[99]

	Harvested by centrifugation at 10,000 rpm for 10 min, dried in an oven at 70 °C					
Lipid extracted Microalga *Scenedesmus sp.*	n.d	Batch bottles, T=37 °C, pH=6.5	WWTP anaerobic digester sludge, heat pretreated: 95 °C, 30 min	21	[67]	
Pigments and lipid extracted Microalga *Nannochloropsis sp.*	Harvested by centrifugation at 10,000 rpm for 10 min, dried in an oven at 70 °C, grounding, extraction of lipids by soxhlet extraction	Bacth bottles, T=30 °C	*Enterobacter aerogenes ATCC 13048*	50.5[a]	[99]	
Pigment and lipid extracted Microalga *Nannochloropsis sp.*	Harvested by centrifugation at 10,000 rpm for 10 min, dried in an oven at 70 °C, grounding, extraction of lipids by supercritical fluid extraction	Bacth bottles, T=30 °C	*Enterobacter aerogenes ATCC 13048*	49.5–60.6[a]	[99]	

d: estimated from biohydrogen curves.

[a] mL H_2 g^{-1} algae.

[b] mL H_2 g^{-1} COD.

[c] mL H_2 g^{-1} TS.

thus are important parameters to take into account [17,79]. Recently Shi et al. [17] investigated the impact of operational conditions (temperature, pH and organic loading rate) during DF process of *Laminaria Japonica* macroalgae, using anaerobic mixed cultures. Three temperatures were investigated: 35, 50 and 65 °C to respectively represent mesophilic, thermophilic and hyperthermophilic conditions. Highest hydrogen production was observed at mesophilic conditions, followed by the hyperthermophilic condition, with the thermophilic condition resulting in the lowest. The different conditions seriously affected the distribution of organic acids produced, with the temperature having the most influent impact on the microbial diversity and composition, and consequently the metabolic pathways involved. Concerning medium pH, under mesophilic condition, the highest H_2 yield was obtained for pH 5.5 whereas for the thermophilic and hyperthermophilic conditions, pH 6 was found to be optimal [17]. Among the various operational conditions, the maximum H_2 yield of 61.3 mL H_2 g^{-1} TS was observed under mesophilic condition at an OLR of 3.4 g COD L^{-1} d^{-1} and pH 5.5 [17]. In addition to operational parameters, some intrinsic factors of algae (chemical composition, *C*/*N* ratio, salinity, heavy metals) can affect the dark fermentation process performances and will be discussed in the following part.

3.3. Algae intrinsic factors influencing dark fermentation

Macro and microalgae are rich in nitrogen, phosphorous and microelements that are essential for the growth of HPB [87]. However, some intrinsic parameters of algae could affect the activity of HPB during dark fermentation. Among them, the chemical composition (i.e. carbohydrates, proteins), carbohydrates/proteins ratio, as well as the sodium and heavy metal concentrations have been reported to affect the performance of the DF. Microalgae are made of a high percentage of carbohydrates and proteins which composition is dependent on growth conditions and algal species, as previously mentioned (see Section 2.2). However, algal feedstocks with high concentration of carbohydrates are more suitable for dark fermentation process, as HPB can hydrolyse sugar faster than protein [88]. Xiao et al. [89] demonstrated that hydrogen yield of glucose (0.14 mL H_2 mg^{-1}), was 18 times higher than the yield of protein (0.077 mL H_2 mg^{-1}) in batch systems inoculated with thermally treated activated sludge and glucose or peptone as sole source of carbon [89]. Xiao et al. [89] highlighted also different metabolites routes and performed preliminary analysis on the diversity of communities selected during the degradation of carbohydrates and proteins.

Even if proteins have lower biohydrogen potentials than carbohydrates, they are necessary to balance the *C*/*N* ratio for the HPB growth. Optimal *C*/*N* ratio is usually reported to range between 10 and 90, though high H_2 production was obtained also at rate equal to 200 [90,91]. Comparison of batch experiments of a mixture of 60% glucose and 40% peptone and a control containing only glucose demonstrated that mixed substrate enhanced the biohydrogen potential [88]. In this case, most of the hydrogen was produced from the fermentation of glucose and not peptone. However, the protein seems to stimulate the HPB growth as the ammonia, which is produced from the degradation of peptone, neutralizes the organic acids preventing the acidification and failure of the system [88].

Nevertheless, excessive proteins content of algal biomass can lead to high ammonium release and toxic free ammonia which decrease the intracellular pH of hydrogen-producing bacteria, increase the energy demand of cell maintenance, inhibit requirement for hydrogen-producing bacteria or inhibit specific enzymes related to fermentative hydrogen production [92–95]. Salerno et al. [93] demonstrated that biohydrogen yield and production decrease of

42.8% and 38.8%, respectively; with the increase of ammonia concentration from 0.8 g N L^{-1} to 7.8 g N L^{-1} in continuous flow reactors inoculated with heat-treated agricultural soil. The concentration of ammonia in the system determined the composition of the dominant bacteria, thus the quantity and type of intermediate metabolites depending on the metabolic pathways [94].

Similarly to ammonia, also salinity influences the activity of HPB and dark fermentation efficiency. Marine macroalgae can accumulate substantial level of Na^+ so it must be taken into consideration when used as a feedstock. Batch experiments inoculated with acid-treated anaerobic sludge and sodium concentrations raging between 0 and 8 mg L^{-1} revealed that the biohydrogen production started to decrease at Na+ concentration higher than 2 mg L^{-1} [96]. A more detailed study on a pure culture of *Clostridium butyricum* attributed that to the shift from the butyric to the acetate pathway as the butyrate/acetate ratio decreased linearly from 2.5 at 0 g-Na+ L^{-1} to 0.73 with 12 g-Na+ L^{-1} [96]. High production of acetate was also reported in batch reactors inoculated with mariculture organic waste (MOW) in mesophilic condition with percentages of salinity ranging from 1.5% to 3.5% [97]. Adaptation of HPB to salty environments was elegantly showed by Pierra et al. [98] in batch reactors inoculated with a saline sediment from a lagoon collecting salt factory wastewaters and operated with glucose as substrate (5 g L^{-1}) and increasing concentrations of NaCl (from 9 to 75 g NaCl L^{-1}). Salt concentration exerted a strong selective pressure on the structure of the bacterial communities, which was characterized by the emergence of a new species belonged to the family of *Vibrionaceae* [98].

The potential of micro and macro-algae is maximized when they are integrated into a biorefinery approach. In this context, biohydrogen should be produced from residues of algae after the extraction of lipid and/or high-value compounds. The general thought that the large amount of toxic solvents used in classical extraction methods inhibits bacterial activity was rejected by Nobre et al. [99], that compared the hydrogen producing yield of batch reactors containing pure cultures of *Enterobacter aerogenes* and *Nannochloropsis sp.* (microalga) before and after conventional toxic and supercritical fluid (natural) extraction processes. Similar yields were achieved with the microalgal biomass before and after oil extraction, demonstrating the high versatility of this bacterium.

To make the process even more feasible, microalgae used as feedstock for biohydrogen production should be grown in wastewater effluents or in polluted environments that usually contain high levels of heavy metals (i.e. cadmium, chromium, copper, iron, lead, nickel and zinc). Algae can accumulate relatively high concentration of heavy metals that, depending on type and ratio, can have beneficial or detrimental effect on the biochemical pathways of DF. Lin and Shei [100] reported that the activity of hydrogen producing mixed natural microflora decreased of 50% at concentrations of 4.5 mg Zn L^{-1}, 6.5 mg Cu L^{-1} and 60 mg Cr L^{-1}, though level of Cu and Cr lower than 3 mg L^{-1} and 15 mg L^{-1} respectively have stimulatory effect. Nevertheless, at relatively low concentration, metal ions are required for the metabolism of the HPB. Nickel and iron are the active core of the [FeFe]-hydrogenases and [NiFe]-hydrogenase which catalyse the reversible oxydation $H_2 \leftrightarrows 2H^+ + 2e^-$ [101]. While other metal, such as magnesium, sodium or zinc are involved in transport across cell membrane, or play a role as cofactors of other enzymes [87].

4. Biohydrogen potentials of algae

Table 1 reports the biohydrogen production yields obtained from various micro and macroalgae. Jung et al. [102] investigated the biohydrogen potentials of seven macroalgae (red, brown and green) and reported hydrogen potentials from 10.3 mL H_2 g^{-1}TS (*Huzikia fusiforme sp.*) to 67 mL H_2 g^{-1}TS (*L. Japonica sp.*).

Among microalgae strains, *Chlorella sp.* has been one of the most studied with hydrogen potentials varying from 6.1 to 31.2 [103–106], though *Dunaliella tertiolecta* and *Nannochloropsis sp.* with hydrogen potentials of 13 and 48 mL H_2 g^{-1}alga [99,106], respectively, received substantial attention.

With regard to algal biomasses after lipid extraction, Yang et al. [67] reported a biohydrogen potential of 21 mL g^{-1} TS for lipid extracted *Scenedesmus sp.* microalgae. Similarly, Nobre et al. [99] investigated biohydrogen production from lipid and pigment extracted *Nannochloropsis sp.* (microalgae) using a pure strain of *E. aerogenes*. A biohydrogen potential of 50.5 mL H_2 g^{-1} algae was noticed after lipids extraction by soxhlet method, while 49.5–60.6 mL H_2 g^{-1} algae were found after supercritical extraction [99]. However, biohydrogen potentials among studies are difficult to compare due to the high variability of the DF operational conditions (i.e. pH, temperature, substrate/inoculum ratio, etc.), type of inoculum (pure or mixed cultures) and inoculum pretreatment.

Assuming that biohydrogen is mainly produced from carbohydrates, theoretical biohydrogen potentials (see Table 2) were calculated according to Eq. (1) considering that all carbohydrates are consumed with a rate of 2.5 mol H_2 mol^{-1} hexose (311 NL H_2 g^{-1} hexose), using mixed cultures [71].

$$\text{BHP th (mL } H_2 \text{ g}^{-1} \text{ TS)} = \text{Carb(\%TS)}311 \quad (1)$$

Then, the biodegradability of algae biomass during dark fermentation process was determined using equation (Eq. (2)).

$$\text{Biodegradability (\%)} = [\text{BHP exp/BHP th}]100 \quad (2)$$

Table 2
Comparison of experimental and theoretical biohydrogen potentials of various algae strains.

Feedstocks	Carb. (g 100 g^{-1} TS)	BHP exp. (mL H_2 g^{-1} TS)	BHP th. (mL H_2 g^{-1} TS)	Biodegradability (%)	Ref.
Green macroalga *Codium fragile*	32.3	24.4	100	24	[102]
Red macroalga *Gelidium amansii*	61	43.1	190	23	[102]
Red macroalga *Porphyra tenera*	35.9	15.4	112	14	[102]
Red macroalga *Gracilaria verrucosa*	33.5	26.7	104	26	[102]
Brown macroalga *Hizikia fusiforme*	28.6	10.3	90	11	[102]
Brown macroalga *Laminaria Japonica*	59.7	67	186	36	[102,109]
Brown macroalga *Ecklonia stolonifera*	48.6	26.6	151	18	[102]
Brown macroalga *Undaria pinnatifida*	40.1	20.4	126	16	[102]
Brown macroalga *Laminaria Japonica*	56.4	71.4	175	32	[110]
Microalga *Chlorella Pyrenoidosa sp.*	33.7	8.8	105	8	[27]
Microalga *Nannochloropsis Oceanica sp.*	32.7	2	102	2	[113]
Microalga *Chlorella sp.*	26	6.3	81	8	[105]
Microalga *Chlorella vulgaris sp.*	39.5	31.2	123	25	[104]
Lipid extracted Microalga *Scenedesmus sp.*	24.7	17	77	22	[67]

Interestingly, experimental algal biodegradability was found to be less than 36% (Table 2), suggesting that micro and macroalgae have chemical and/or structural barriers limiting their degradation during dark fermentation process. Besides the intrinsic parameters previously described, others factors can affect the biodegradability of algae strains. The nature and structure of carbohydrates (cellulose, laminarin, alginate, agar, carrageenan) seem to be a key point during the dark fermentation process [102,109]. The biodegradability of each macroalgal component has not yet fully investigated, though laminarin and carrageenan are known to be rather biodegradable materials, while agar is not. Jung et al. [102], compared H_2 productivity of various polysaccharides, including alginate, laminarin, agar, and carrageenan. They found that alginate and laminarin possessed the higher yields of 8.28 mL H_2 g^{-1} substrate and 4.62 mL H_2 g^{-1} substrate, respectively.

The nature and structure of polysaccharides are not the only factors limiting DF. Several studies demonstrated that the biohydrogen potentials is directly correlated to the amount of soluble sugars, thus on the performance of polysaccharide hydrolysis [16,17]. For some algal strains, the hydrolysis performance can be limited by the rigid nature of their cell wall. In order to make the intracellular components more biodegradable pretreatment step is generally necessary [114,115].

4.1. Pretreatments of macro and microalgae strains

Biohydrogen production from algae is reported to be limited by the hydrolysis of carbohydrates which compose algae [17,102,109]. Even if hydrolytic enzymes are generally produced by HPB, their concentrations are quite low, especially if compared to pure bacterial or fungi cultures [17,116,117]. Indeed, a low cellulose activity of 0.08–0.19 FPU/mL of an anaerobic sludge treated at 90 °C for 20 min was reported during dark fermentation of macroalgae *L. Japonica* sp. [17]. Thus, to achieve high carbohydrates hydrolysis and thereafter high biohydrogen yields, a pretreatment step of algae is often recommended [7,111]. Generally, to be effective a pretreatment must enhance the conversion of complex carbohydrates into simple sugars, avoiding the degradation of carbohydrate and the formation of inhibitory by-products to the subsequent hydrolysis and fermentation processes. Moreover, to be sustainable, a pretreatment must be cost-effective [118]. Nevertheless, to date, biomass pretreatment is always considered one of the most crucial and expensive step in the biomass conversion to fermentable sugars [119–121]. Indeed, it is estimated to account for 33% of the total cost equipment in a lignocellulosic biorefinery chain [120]. However, the absence of lignin makes pretreatments of algae easier and less expensive than that of lignocellulosic biomasses.

So far, the most common pretreatments used to enhance the hydrolysis and thus biohydrogen production, of micro and macroalgae are physical (i.e. milling, ultrasonic, microwave), thermal (i.e. LHW, steam explosion,) and thermo-chemical (i.e. diluted acid) pretreatments.

4.2. Effect of pretreatment on chemical composition of algae biomass

Literature studies concerning biohydrogen potentials from both micro and macroalgae strains demonstrated that all pretreatment categories tested (i.e. milling, acidic, alkaline, ultrasonic, microwave, thermal, enzymatic and their combinations) led to a hydrolysis of carbohydrates, with more or less success.

Microwave-assisted H_2SO_4 pretreatment was found efficient in destroying the protein-based cell wall of *Arthrospira platensis*, to expose more carbohydrates to glucoamylase enzyme and to degrade some of the high-molecular-weight carbohydrates into low molecular-weight ones [27]. In the same study, glucoamylase enzyme, applied after the microwave pretreatment, was found efficient in catalysing the hydrolysis of α-1,4- and α-1,6-glycosidic bonds of carbohydrates (mainly glycogen). Thus, most carbohydrates were converted into reducing sugars, which were more easily consumed by HPB for hydrogen production [27]. Cheng et al. [27] observed a reduced sugar yield of 42.7 g 100 g^{-1} TS when wet microalgal biomass (*A. platensis*) was pretreated by microwave heating for 15 min at 140 °C in 1% H_2SO_4 solution and then enzymatically hydrolysed for 12 h.

Xia et al. [122] observed that steam heating, microwave heating, and ultrasonication of *Chlorella pyrenoidosa* biomass were not efficient for the release and hydrolysis of carbohydrates (0.002 to 0.007 g g^{-1} VS). This is because the carbohydrates of *C. pyrenoidosa* biomass are mainly composed of glucan with a high molecular weight and are tightly surrounded by the cell structure [103,123,124]. The reduced sugar yields significantly increased (0.303 ± 0.005 g g^{-1} VS) when *C. pyrenoidosa* biomass was pretreated by steam heating with diluted acid and by microwave heating with diluted acid. This confirms the findings of Cheng et al. [27] that reported that heating with diluted acid could remarkably enhance hydrolysis and saccharification of *C. pyrenoidosa* biomass [122].

Thermal (at 100 and 121 °C) and thermo-alkaline (at 100 and 121 °C with 20% of NaOH dosage) pretreatments were also found to be effective in solubilising both proteins and carbohydrates from lipid-extracted *Scenedesmus* [66,67]. Specifically, the carbohydrate solubilization was 22% for the control, 36–38% after the thermal pretreatment and 38–49% after the thermo-alkaline pretreatment. The proteins solubilization was 2% for the control, 10–18% after the thermal pretreatment, and 28–58% after the thermo-alkaline pretreatment. It should be noted that the lipid-extracted *Scenedesmus* samples were already strongly solubilized after 1 h of thermo-alkaline pretreatment.

Bead milling was found suitable to disrupt some cyanobacterial cells leading to the release of carbohydrates and proteins to be utilized by hydrogenogens for hydrogen production [27]. However, lower sugar yields were obtained than the application of ultrasonic pretreatments, because bead milling is a blander physical method [27].

In the case of macroalgae, diluted acid and thermal pretreatments are the most common methods. Reaction parameters, such as pretreatment temperature, contact time and acidic concentration, affect both COD solubilisation and the total sugars yield. For instance, Park et al. [108] studied the application of a thermal pretreatment on the brown macroalgae *L. Japonica*. They found a COD solubilisation of 70% and 87% by treating *L. Japonica* at 60 °C and 120 °C for 20 min, respectively. The fact that the COD solubilisation increased with pretreatment temperature and contact time was confirmed also by Jung et al. [109], who reported a COD solubilisation (between 57–69%) of the brown alga *L. Japonica* after thermal pre-treatment (T=150–180 °C, t=5–40 min, S/L ratio=1/12). Similar results were also obtained by Park et al. [125], who reported a hydrolysis efficiency of carbohydrates from *Gelidium Amansii* (red microalgae) varying between 2% and 67.7% after thermo-acidic pretreatment (0.5–1.5% w w^{-1} of H_2SO_4, T=120–180 °C, t=15 min, 5–15% w w^{-1} of S/L).

4.3. Impact of by-products (i.e. furans, phenols) on biohydrogen performances

Thermal and thermo-acid pretreatments applied for the improvement of the hydrolysis of algal carbohydrates have the drawback of generating toxic by-products (i.e. 5-HMF, furfural compounds) that may affect significantly the biohydrogen production by dark fermentation [19,71,126]. Furfural and 5-HMF compounds are originated from the dehydration of pentoses and hexoses, respectively. The level of these by-products in the hydrolyzate depends on several parameters such as the nature of

biomass (i.e. structure and nature of carbohydrates) and pretreatment conditions (i.e. solid loading, time, pressure, temperature and chemicals concentrations) [127–129]. For instance, Jung et al. [102] observed furfural concentrations varying from 1.79 g L^{-1} to 4.84 g L^{-1} after thermal pretreatment of macroalgae *L. Japonica* (170 °C, *S/L*: 1/12) and time increasing from 5 min to 40 min. Similarly, 5-HMF were also found in the slurry of macroalgal biomass (*L. Japonica*, *G. amansii*) and microalgae (*Chlorella Vulgaris*) after thermal or dilute acid pretreatment [83,109,125]. Jung et al. [109] reported a negative correlation between H_2 yield and the produced 5-HMF in the hydrolyzate. The inhibition of biohydrogen production of 69% and 75% in presence of 1 g L^{-1} of furfural and 5-HMF, respectively, was also confirmed by Quéméneur et al. [19] in batch systems operated with mixed cultures and glucose. Similarly, Park et al. [126] highlighted that concentration higher or equal than 1.5 g L^{-1} of 5-HMF inhibited totally the hydrogen production from a control with galactose. Interestingly, in most cases, biohydrogen inhibition was not necessary correlated to the absence of bacterial activity as carbohydrates were found to be degraded and converted by non-hydrogen-producing pathways [19,126,130]. Park et al. [126] reported on a galactose solution supplemented with 1.5 g L^{-1} of 5-HMF a total biohydrogen inhibition, but galactose was degraded by propionate and lactate pathways, which are considered competing H_2 pathways [126]. Similarly, Monlau et al., [130] noticed a decrease in biohydrogen production from a glucose control solution supplemented with increasing volumes of dilute-acid sunflower stalk hydrolyzates containing furans compounds. For concentrations lower than 7.5% (v/v), no hydrogen inhibition was noticed, and hydrogen production was concomitant to acetate and butyrate formation. However, for a concentration higher or equal to 15% (v/v), hydrogen production was completely inhibited with a shift from hydrogen-producing pathways (i.e. acetate/butyrate) to non-hydrogen-producing pathways (i.e. lactate/ethanol) carried out by *Sporolactobacillus sp.*

Furthermore, phenolic compounds (i.e. phlorotannins) in macroalgae can also affect mixed anaerobic cultures [131]. Tai et al. [132] investigated the biological hydrogen production from phenol-containing wastewater using pure culture of *C. butyricum* and reported that phenol concentration higher than 800 mg L^{-1} limited the cell growth and the degradation of glucose. Similarly, Quéméneur et al. [19] reported that phenol (1 g L^{-1}) addition in a control with glucose (5 g L^{-1}) during dark fermentation operated with mixed culture affected the performances with less intensity than when furans compounds were added at the same concentration. Nevertheless, the effect of phlorotannins in dark fermentation with algal biomass on dark fermentation has not been investigated and further studies are required to confirm the negative impact.

4.4. Effect of pretreatment on biohydrogen potential

As mentioned before, macroalgae and microalgae generally do not contain lignin. Thus, sugars for the biological production of H_2 can be obtained without expensive pretreatments to remove lignin [125]. Research on pretreatments of seaweeds and microalgae used as feedstock for enhancing hydrogen production has just started and the main findings are summarized in Table 3.

According to authors' knowledge, most of the studies in literature are related to the application of pretreatments to enhance biohydrogen production of both microalgae and lipid-extracted microalgae (i.e. *C. pyrenoidosa, C. vulgaris, Lipid-extracted Scenedesmus sp., Scenedesmus obliquus, Chlamydomonas reinhardtii and Arthrospira maxima*). The best H_2 production increase (up to 847%), compared to untreated sample, was achieved by pretreating *C. pyrenoidosa* by steam and microwave pretreatments combined with a diluted acid pretreatment [122]. Satisfactory results were also obtained by treating the lipid-extracted microalga *Scenedesmus sp.* by thermal and thermo-alkaline pretreatment [66,67]. Yang et al. [66,67] found that the application of a thermal pretreatment at both 100 and 121 °C for 4–8 h increased the H_2 production up to 109%. Additional alkaline pretreatment enhanced the H_2 production up to 168%. In another study, Cheng et al. [27] obtained a H_2 production increase (up to 412%) by pretreating the wet microalga *Arthrospira maxima* by thermal (boiling), mechanical (bead milling), ultrasonic and combined ultrasonic and enzymatic pretreatment (Table 3).

So far, only few studies investigated the effect of pretreatments on biohydrogen production from macroalgae strains. *G. amansii* and *L. Japonica* pretreated with thermal (150–180 °C, 5–40 min, S/L ratio of 1:12) and thermo-acidic (60–180 °C, 6–12% (w/w) of H_2SO_4 or HCl, 5–40 min) methods were the most studied [102,109,125]. Jung et al. [109] studied a combined (acid - thermal) pretreatment to enhance fermentative H_2 production (FHP) from *L. japonica*. Various pretreatment conditions including HCl concentrations (i.e. 0%, 6% and 12% w/w), heating temperatures (i.e. 60, 110 and 160 °C), and reaction times (i.e. 5, 22.5 and 40 min) were optimized via response surface methodology (RSM) with a Box–Behnken design (BBD). They found that the most significant factor affecting biohydrogen enhancement was the HCl concentration, and optimal conditions were HCl concentration of 4.8%, heating temperature of 93 °C, and reaction time of 23 min, to obtain a biohydrogen increase of almost 140%, compared to that of untreated biomass. Recently, Park et al. [125] attempted at the optimization of the control variables (i.e. temperature, H_2SO_4 concentration and solid to liquid (*S/L*) ratio) of a thermo-acidic pretreatment on the red macroalga *G. amansii.* Various pretreatment conditions, including H_2SO_4 concentrations (i.e. 0.5%, 1% and 1.5% w/w), heating temperatures (i.e. 120,150 and 180 °C), and solid to liquid ratios (i.e. 5%, 10% and 15% w/v) were evaluated. Contrarily to the previous study, they found that among the three control variables, temperature resulted the most significant to increase the biohydrogen potential of the red alga.

The comparisons between literature results suggest that the impact of thermo-acidic pretreatment on biohydrogen production of seaweeds depends both on the pretreatment conditions (i.e. temperatures, contact times, solid to liquid ratios and chemical reagent use) and on the type of biomass used (*G. Amansii* and *L. Japonica*) [102,125]. Thermo-acidic (with HCl) pretreatment seems to give the highest biohydrogen increase (up to 143%). However, thermo-acid pretreatment at high temperature and acidic concentrations led in some cases to a decrease of biohydrogen potential (up to −86%) because of inhibitory compounds production (i.e. furans) [102].

5. Dark fermentation effluents integrated in a bio-refinery concept

To make the overall process economically viable, it is important to valorize the outlets of the dark fermentation, as a large part of organic matter cannot be converted into biohydrogen and remains inside the process. The remaining organic matter from DF can be separated into a liquid fraction (rich in VFAs, lactate, ethanol) and a solid fraction (i.e. solid compounds recalcitrant to pretreatment or microbial degradation). According to Cooney et al. [136], in absence of a pretreatment step, only about 10–20% of the energy potential of an organic substrate is obtained through dark biohydrogen fermentation and thus valorization of the side streams in a biorefinery approach has to be highly considered.

The integration of dark fermentation (DF) with photofermentation (PF) or anaerobic digestion process (AD) in DF/PF, DF/AD or DF/PF/AD biorefinery approaches or the conversion of the residual liquid fraction rich in VFAs into valuable biomolecules (i.e. polyhydroxyalkanoates), have been mostly proposed (Table 4).

Table 3
Biohydrogen potentials of pretreated macroalgae and microalgae.

Pretreatment method	Algal biomass	Pretreatment condition	Inoculum	H_2 yields after pretreatment	H_2 increase	References
Macroalgae						
Thermal	*Laminaria japonica*	T=150–180 °C, t=5–40 min, S/L ratio=1:12	Anaerobic digested sludge from WWTP	58.6–107.5 mL H_2 g^{-1} dry alga	From −13% to +60%	[102]
Thermo-acidic (HCl)	*Laminaria japonica*	T=60, 110, 160 °C, t=5, 22.5, 40 min, S/L ratio=1:12, HCl dosage=0, 6, 12%(w/w)	Anaerobic digested sludge from WWTP	9.5–163.1 mLH_2 g^{-1} dry alga	From −86% to +143%	[109]
Thermo-acidic (H_2SO_4)	*G. Amansii*	T=120, 150, 180 °C; t=15 min; H_2SO_4 dosage=0.5, 1, 1.5%(w/w); S/L ratio=5, 10, 15% (w/w)	Anaerobic digested sludge from WWTP	0–37 mL H_2 g^{-1} dry alga	n.d	[125]
Microalgae						
Ultrasonic (wet alga)	*Chlorella vulgaris*	t=n.d, F=20 kHz, P=150 W, SEI levels=10,000–100,000 kJ/kg, S/L ratio=1:10 (w/w)	Anaerobic digested sludge from WWTP	31.9–37.9 mL H_2 g^{-1} dry alga	From +3% to +22%	[83]
Ultrasonication (centrifuged and spry dried)	*C. pyrenoidosa*	time=15 min, S/L ratio=5% (w/v)	Anaerobic digested sludge	16.3 mL H_2 g^{-1} dry alga	44%	[122]
Ultrasonication	*Arthrospira platensis* (wet)	t=20 min, P=200 W	Anaerobic digested sludge from WWTP	55.9 mLH_2 g^{-1} dry alga	247%	[27]
Sonication	*Chlamydomonas reinhardtii*	P=130 W, t=10 min	Termotoga neapolitana	860 mL H_2 mL^{-1} culture	n.d	[107]
Microwave heating (centrifuged and spry dried)	*C. pyrenoidosa*	T=140 °C, t=15 min, S/L ratio=5% (w/v)	Anaerobic digested sludge	12.6 mL H_2 g^{-1} dry alga	11%	[113]
Acidic HCl (wet alga)	*Chlorella vulgaris*	T=n.d, t=10, 35, 60 min, S/L ratio=1:10 (w/w), HCl dosage=0.1, 1.6, 3%(v/w)	Anaerobic digested sludge from WWTP	13.6–36.5 mL H_2 g^{-1} dry alga	From −56% to +17%	[83]
Acidic HCl+ultrasonic (wet alga)	*Chlorella vulgaris*	t=30 min, F=20 kHz, P=150 W, SEI levels=10,000, 55,000, 100,000 KJ/kg, S/L ratio=1:10 (w/w), HCl=0.10, 1.6, 3% (v/w)	Anaerobic digested sludge from WWTP	24.2–41.6 mL H_2 g^{-1} dry alga	From −22% to +34%	[83]
Boiling	*Arthrospira platensis* (wet)	T=100 °C, t=5 min, S/L ratio=50 g/L	Anaerobic digested sludge from WWTP	38.5 mL H_2 g^{-1} dry alga	139%	[27]
Thermal	Lipid-extracted Scenedesmus	T=100, 121 °C, t=4, 8 h, S/L ratio=n.d	Anaerobic digested sludge from WWTP	31.7–31.8 mL H_2 g^{-1} dry alga	From +108% to +109%	[66,67]
Thermal (autoclave)	*Scenedesmus obliquus*	T=121 °C, t=15 min	Clostridium butyricum	90.3 mL H_2 g^{-1} dry alga	n.d	[133]
Thermal (autoclave)	*Scenedesmus obliquus* (wet)	T=121 °C, t=15 min	Enterobacter aerogenes	45.1 mL H_2 g^{-1} dry alga	n.d	[133]
Thermal (autoclave)	*Scenedesmus obliquus*	T=121 °C, t=15 min	Clostridium butyricum	2.9 mol mol^{-1} total sugars	n.d	[134]
Steam heating (centrifuged and spry dried)	*C. pyrenoidosa*	T=135 °C, t=15 min, S/L ratio=5% (w/v)	Anaerobic digested sludge	13.1 mL H_2 g^{-1} dry alga	16%	[122]
Thermo-alkaline	Lipid-extracted Scenedesmus	T=100, 121 °C, t=4, 8 h, S/L ratio=n.d, NaOHdosage=20%(w/w)	Anaerobic digested sludge from WWTP	33.5–40.8 mLH_2 g^{-1} dry alga	From +120% to +168%	[66,67]
Thermal (autoclave)+acid	*Chlamydomonas reinhardtii*	T=121 °C, t=20 min, S/L ratio=30% (g/mL), HCl dosage=0.5–2.5%(w/w)	Termotoga neapolitana	1160 mL H_2 mL^{-1} culture	n.d	[107]
Steam heating with diluted acid (centrifuged and spry dried)	*C. pyrenoidosa*	diluted acid (1% v/v), T=135 °C, t=15 min	Anaerobic digested sludge	97.2 mL H_2 g^{-1} dry alga	759%	[122]
Microwave heating with diluted acid (centrifuged and spry dried)	*C. pyrenoidosa*	diluted acid (1% v/v), T=140 °C, t=15 min	Anaerobic digested sludge	107.1 mL H_2 g^{-1} dry alga	847%	[122]
Methanol	*Chlamydomonas reinhardtii*	Methanol dosage=30% (g DCW/ml), mix time=60 min	Termotoga neapolitana	980 mL H_2 mL^{-1} culture	n.d	[107]
Bead milling	*Arthrospira platensis* (wet)	t=15 min	Anaerobic digested sludge from WWTP	38.5 mL H_2 g^{-1} dry alga	139%	[27]
Enzymatic	*Chlamydomonas reinhardtii*	a-amylase, 90 °C, T=30 min	Termotoga neapolitana	1100 mL H_2 mL^{-1} culture	n.d	[107]
Enzymatic	*Arthrospira maxima* (wet)	Glucoamylase (0.01 g/g TS, pH=4, T=60 °C, t=24 h)	Activated sludge from WWTP domesticated to Arthrospira maxima	78.7 mL H_2 g^{-1} dry alga	22%	[135]
Ultrasonication and enzymatic	Arthrospira platensis (wet)	a-amylase (0.2% of substrate, 60 °C, 2 h)+glucoamylase (0.2% of substrate, 60 °C, 12 h)	Anaerobic digested sludge from WWTP	82.4 mL H_2 g^{-1} dry alga	412%	[27]

Table 4
Integration of dark fermentation (DF) with photo-fermentation (PF) or anaerobic digestion process (AD) in DF/PF, DF/AD or DF/PF/AD biorefinery approaches.

Feedstock characteristics	Biorefinery scheme	Biorefinery characteristic	DF-H_2 yield	Energy yield DF process (KWh t^{-1})[a]	PF-H_2 yield	AD-CH_4 yield	Energy yield biorefinery concept (KWh t^{-1})[a]	Ref
Brown macroalga *Laminaria japonica*, dried at room temperature, ground into 0.5 mm particle size.	DF-AD	**DF**: ASBR, HRT=6 days, $T°$=35 °C, pH=5.5, substrate concentration 20 g Carbo. COD L^{-1} **AD**: UASB (Upflow anaerobic sludge blanket reactor), HRT=2 days, granular sludge from a brewery wastewater treatment plant, feeding at a OLR of 3.5 g COD L^{-1} d^{-1}only with liquid effluents form hydrogen ASBR	58.5 mL H_2 g^{-1} COD	175	–	309 mL CH_4 g^{-1} COD	3265	[110]
Brown macroalga *Laminaria japonica*, knife milled into 0.5 mm particle size, Acid+thermal pretreatments	DF-AD	**DF**: Continuous stirred tank reactors (CSTR), pH=5.5, HRT=2.7 days, substrate concentration 31.1 g COD L^{-1} **AD**: H_2 Fermented Solid State (HFSS) valorized in CH_4-ASBR, seed sludge from anaerobic digester, T=35 °C, HRT=12 days, OLR: 2.5 g COD L^{-1} d^{-1}. H_2 Fermented Liquid State (HFLS) valorized in CH_4-UASBr, granular sludge from a brewery wastewater treatment plant, T=35 °C, HRT=2 days, OLR: 4.5 g COD L^{-1} d^{-1}.	113 mL H_2 g^{-1} TS	339	–	227 mL CH_4 g^{-1} COD (HFSS) 309 mL CH_4 g^{-1} COD (HFLS)	Nd	[137]
Microalga *A. maxima* harvested and dried at 75 °C	DF-AD	**DF**: batch at 35 °C, pH=6 (sludge inoculum was boiled for 30 min and then enriched with hydrogenogens) **AD**: batch at 35 °C, pH=8 (inoculum with methanogens was added to the same reactor of DF)	49.7 mL H_2 g^{-1} alga	149	–	145 mL CH_4 g^{-1} alga	1599	[135]
Lipid extracted microalga *Scenedesmus sp.*; alkaline pretreated	DF-AD	**DF**: batch at 37 °C (sludge inoculum was heated at 95 °C for 30 min), pH=6.3 **D**: batch at 37 °C, pH=7, anaerobic sludge	46 mL H_2 g^{-1} VS	138	–	393 mL CH_4 g^{-1} VS	4068	[138]
Microalga *A. maxima* harvested by suction filtration onto porous filter discs (pore size, 5 μm).	DF-PF	**DF**: batch at 37 °C (sludge inoculum was heated at 95 °C for 30 min), pH=6.3 **AD**: batch at 37 °C, pH=7	97 mL H_2 g^{-1} ^{T}S	291	240 mL H_2 g^{-1} ^{T}S	–	1011	[27]
Microalga *Chlorella pyrenoidosa sp.*, harvested by centrifugation, spray dried; steam heating (135 °C; 15 min), dilute acid (1% v/v).	DF-PF-AD	**DF**: batch at 35 °C, pH=6, Hydrogen-producing bacteria isolated from anaerobic sludge **PF**: batch at 30 °C, pH=7, Photosynthetic bacteria isolated from anaerobic sludge, removal of NH_4+ from DF effluents using zeolite. **AD**: batch at 37 °C, pH=8, anaerobic sludge	76 mL H_2 g^{-1} VS	228	123 mL H_2 g^{-1} VS	DF effluents 61 mL CH_4 g^{-1} TS PF effluents 125 mL CH_4 g^{-1} TS	2457	[122]
Microalga *Nannochloropsis oceanica sp.*, harvested by centrifugation, oven dried, and powdered to 0.02 mm. Microwaves pretreatment (140 °C, 15 min 1%v/v H_2SO_4)	DF-PF-AD	**DF**: batch at 35 °C, pH=6, Hydrogen-producing bacteria isolated from anaerobic sludge. **PF**: batch at 30 °C, pH=7, Photosynthetic bacteria isolated from anaerobic sludge, removal of NH_4+ from DF effluents using zeolite. **AD**: batch at 35 °C, pH=8, anaerobic sludge	39 mL H_2 g^{-1} VS	117	150 mL H_2 g^{-1} VS	DF effluents 96 mL CH_4 g^{-1} TS PF effluents 65 mL CH_4 g^{-1} TS	2177	[113]

These processes will be described in detail within the next paragraph.

5.1. Integration of dark fermentation with anaerobic digestion

Anaerobic digestion involves the degradation and stabilization of organic materials under anaerobic conditions by microbial consortium and it leads to the formation of biogas which consists mainly of CH_4 (55–75%) and CO_2 (25–45%). It involves four degradation steps, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis steps, operated by complex microbial communities.

In the traditional one-stage anaerobic digestion process, H_2 is usually not detected as it is immediately consumed during the methanogenesis to produce CH_4 and CO_2 [138]. The two-stage process, hydrolysis and acidogenic are separated from the acetogenic and methanogenic. In the first hydrolytic–acidogenic process (i.e. DF), organic polymers, such as carbohydrates, proteins, and lipids, are converted to volatile fatty acids (VFAs) and H_2. Then, VFAs (mainly acetate and butyrate) and the remaining solid biomass are then converted into methane during the methanogenic step [138]. It has been proven that the two-stage process offers several advantages, such as enhanced biogas yield and energy recovery, the reduced fermentation time, improved digestion efficiency of the substrate, a good control of microbial community with different functions and improved the stability of the process [138,139].

As recently reviewed by Liu et al. [139], almost 50 journal publications have been published until now on the coproduction of hydrogen and methane, so called biohythane, from biomass by a two-stage process. All of them regard the production of biohythane from sugar-rich substrates (i.e. glucose, sucrose, cassava stillage, Olive pulp, molasse), food/municipal substrates (i.e. food waste, cheese way, potato waste), cellulose-based substrates (i.e. grass silage, cornstalks, wheat straw hydrolysate, water hyacinth leave) and other substrates (i.e. wastewater sludge, swine manure, garbage slurry).

To the authors' knowledge, few research articles considered the use of anaerobic digestion to treat the dark fermentation residues from algae [110,135,137,138]. Yang et al. [138] compared a one-stage with a two-stage DF-AD process from lipid-extracted microalgal biomass residues. They found that two-stage process gave 22% higher methane than one-stage process. Cheng et al. [135] investigated the Energy Conversion Efficiency (ECE) of coupling H_2 and CH_4 from microalgal biomass (*A. maxima*) compared to hydrogen fermentation alone. Results show that the ECE of the cogeneration system is significantly higher (from 2.6% up to 27.7%) than that of hydrogen only fermentation. Finally, Jung et al. [137] showed that in a two stage DF/AD process fed with *L. Japonica*, 7.1% of the initial COD was converted into H_2. After DF, the residual COD derived from both liquid (35.1% of initial COD) and solid (38.7% of initial COD) effluents were further converted into CH_4.

5.2. Integration of dark fermentation with photofermentation

Photofermentation is a fermentative conversion of organic matter to biohydrogen in presence of light by purple non sulphur bacteria. These bacteria utilize the residual organic acids from DF process as electron donors to produce hydrogen during photofermentation. The maximum theoretical yield of hydrogen from photofermentation is 8 mol of hydrogen per mole of hexose. Thus, a combination of dark and light fermentation can theoretically produce the maximum possible yield of 12 mol of hydrogen per mol of hexose.

Until yet, few studies have investigated the combination of DF and PF process exclusively on microalgae [27,112,113,122]. Interestingly, Cheng et al. [27] highlighted that two stages DF/PF process enhance biohydrogen potentials of *A. platensis sp.* microalga from 96.6 mL g^{-1} TS to 337 mL g^{-1} TS compared to DF process used alone. Due to the rich protein content of some microalgae strains, removal of NH4+ produced during DF process is generally necessary as such compounds can significantly inhibit the activity of photosynthetic bacteria [27]. For this purpose, ammonium removal by zeolite have been proposed as an efficient methods [27,113,122].

However, contrary to the DF/AD combination process where both solid and liquid effluents from DF are converted into methane, during the DF/PF process only the rich VFAs liquid effluents are converted into biohydrogen during PF process. For this purpose, the original three-stages DF/PF/AD process has been proposed to valorize all the effluents from DF (Table 4) [113,122]. Xia et al. [122] studied the feasibility of the three-stage DF/PF/AD integrated process on *C. pyrenoidosa* biomass. The overall process consisted in the reuse of the supernatant of the dark fermentation, which contains mainly acetate, butyrate to produce hydrogen during photofermentation, by photosynthetic bacteria (PSB), and to convert the solid residues of dark fermentation (i.e. undigested biomass and HPB cells) and the solution residues of photofermentation (residual SMPs and PSB cells) into methane by AD process [122]. Using such biorefinery approach, they evaluated a total hydrogen yield of 198.3 ml H_2 g^{-1} VS and a methane yield of 186.2 ml CH_4 g^{-1} VS corresponding to an overall energy of 2457 kWh t^{-1} VS (Table 4).

Similarly, Xia et al. [113] investigated the three stage process on *Nannochloropsis Oceanica* microalgae biomass and found that the total energy of the three stage method was 1.7 and 1.3 times higher than that through the two-stage (DF/AD) and single stage AD methods.

5.3. Integration of dark fermentation with biomolecules production or algae growth

Liquid rich VFAs from DF can be converted into economically interesting biomolecules (i.e. polyhydroxyalkanoates) and/or used for microalgae growth [28,31,139]. Yan et al. [31] coupled hydrogen and polyhydroxyalkanoates (PHAs) production from *Taihu blue* algae. PHAs are a group of polyesters totally biodegradable that gain a lot attention as potential alternative to the petro-chemically produced plastics as well as sector like pharmaceuticals and fermentation industries [140,141]. PHAs production from the outlets of DF process of *Taihu blue* algae was carried out using *Balillus cereus* sp. PHAs production of 1.46; 1.83 and 2.26 g L^{-1} were obtained at the flow rates of 30, 60 and 120 L h^{-1} [31]. Rich VFAs liquid effluents can also be used for the growth of microlagae that can serve to biomass for biodiesel or biohydrogen through DF process [30,139]. Furthermore, Lo et al. [28] showed that the biogas produced from DF and PF process can be upgraded into biohydrogen during the growth of microalgae by CO_2 biofixation. In their study, the biogas produced was directly supplied to a microalgal culture of *Chlorella sp.* and the CO_2 was totally consumed [28].

6. Future perspectives for dark fermentation effluents integration

6.1. Thermochemical conversion of solid effluents from DF algae

From an economic point of view, fuel production from algae requires utilization of the complete biomass as efficiently as possible. One promising option is to covert the solid residual biomass from DF process using thermochemical conversion

process. Thermochemical conversion is discussed in this work, having a further insight on pyrolysis, co-pyrolysis and co-gasification with lignocellulosic biomass. Furthermore, the idea of incorporating the utilization of algal waste in existing power installation is part of industrial symbiosis concept in order to add value, reduce costs and improve the environment.

Co-pyrolysis and co-gasification of biomass with algal residues from dark fermentation process has not extensively reported in the literature although the mixture has attracted many interests. Few published studies are related to the co-gasification of land-based biomass and seaweeds [23,142,143]. In particular, the effect of alkali and alkaline in seaweed ash on the gasification of land-based biomass has not been explored although it is found that brown seaweed had a high ash content of alkali and alkaline earth species such as K and Ca than lignocellulosic biomass. Such alkali and alkaline earth species could have great influences on conversion processes because of their catalytic activities on pyrolysis and gasification [23]. Large quantities of alkali and alkaline earth species contained in the brown seaweed may provide a potential source of inexpensive catalysts in the co-processing of lignocellulosic biomass and brown seaweed.

A particular challenge for research in this field is the synergistically interactive co-pyrolysis or co-gasification of algal residues issued from dark fermentation with lignocellulosic biomass.

6.2. Co-pyrolysis of solid effluents from algae DF

Pyrolysis leads to the conversion of biomass into fuels by heating the feedstock material in the absence of air. Pyrolysis products are classified into three categories that could be all used as biofuels: (a) stable (non-condensable) *gases* (b) *liquids* (tar and/or oil), (c) *solid*, mostly solid carbon and ash.

Fast pyrolysis proceeds towards maximization of liquid products due to low residence time that minimizes secondary reactions while slow proceeds towards char production. The obtained results depend on the specific characteristics of the feedstock as well as the operating conditions. Liquids, excluding the aqueous fraction with the hydrosoluble organic compounds, show the highest heating value among all the pyrolysis products and their potential utilization for energy production purposes is recognized [144]. In addition, the gas stream represents a significant proportion of the pyrolysis products with heating value comparable to those of some fuel gases, depending on the process conditions. An optimization of the pyrolysis process would result in gases that could be used to enhance the energy balance of the process or the biorefinery.

Algal wastes from dark fermentation biorefinery, by their addition to lignocellulosic biomass feedstocks can result in enhanced volatiles production and improved gas quality compared to the yields achieved from pure lignocellulosic pyrolysis. The above was shown in previous work by [145], where blends of glycerol with other biomass such as lignite and lignocellulosic biomass were investigated. It was reported that inherent moisture content, during fast pyrolysis with residence times of 0.1–1 s, resulted to enhanced hydrogen production as the steam pyrolysis occurred with steam provided by glycerol dehydration reaction. It was shown that the effect of moisture contained in feedstocks on the H_2 rich gas could be a driver for pyrolysis process [145,146]. The algal waste from DF process is a material with moisture content higher than 30 wt% on dry basis with high ash content and thus can represent an interesting catalyst during co-pyrolysis with lignocellulosic biomass.

6.3. Co-gasification of solid effluents from algae DF

Until yet, very few studies have investigated the conversion of macroalgae to syngas by gasification which can be used directly for energy production [147]. However, if it is the case for algae, the published work related to the post treatment algal residues of DF process in gasification, is inexistent. Compared to land-based biomass which consists of cellulose, hemicelluloses and lignin, the main components in seaweed are carbohydrates, protein and lipids.

Similarly, to fast pyrolysis, co-gasification of biomass with algal residues can be more beneficial than sole biomass gasification due also to another factor, which is the catalytic impact of algal ash in gasification. Seaweed has high ash content and it contains larger amount of alkali and alkaline earth species than land-based biomass [23,142,143]. These alkali and alkaline earth metals remains in the solid residue after dark fermentation of algae and can be greatly enhance H_2 rich syngas production in gasification process.

The alkali impact on syngas quality has been extensively studied only for carboneous biomass [148–151]. Assuming that algae residues can play similar role into co-gasification with earth biomass, it can be predicted co-gasification of algae waste with biomass can result in lower gas yields but improved gas quality regarding hydrogen yield, syngas (H_2+CO) yield, and heating value of the gas. This may be attributed to high moisture content of the algae residues that could generate a steam-rich atmosphere, promoting endothermic reactions between the steam and pyrolysis products as previously investigated by Skoulou and Zabaniotou [146].

6.4. Liquid effluents valorization from algae DF

Liquid streams rich in VFAs can also be used for bioconversion into lipids using oleaginous strains [152] like previously shown for heterotrophic microalgae [30]. Such strains are able to grow in a wide range of substrates, including VFAs, and have the advantage to accumulate lipids within a short period of time [152,153]. Recently, Christophe et al. [153] reviewed the recent developments on using microbial oil to produce biodiesel as an alternative to vegetable oils that compete with human foods. Using the oleaginous bacterial strain *Yarrowia lipolytica*, Fonatanille et al. [152] showed the ability to convert mix of glucose-VFAs or glycerol-VFAs into high biomass concentration of *Y. lipolytica* with lipids concentrations up to 40%.

Hydrogen production can also be linked to bioelectrochemical systems like Microbial Fuel Cells (MFC) and/or Microbial Electrolysis Cells (MEC) to recover valuable energy from liquid effluents rich in VFAs [24]. MFC technologies convert organic wastes directly into electricity [154,155]. In MEC system, bacteria on the anode oxidize organic matter, releasing electrons through the circuit to the cathode where hydrogen can be formed from protons in the water [156]. Such reaction is endothermic, and thus required additional electrical input generally provided by a power source [156].

Such combinations of DF process with MFC process have been already reported on vegetable waste, lignocellulosic biomass and crude glycerol [21,155,156]. Freguia et al. [154] investigated the production of electrical current through MFC using a mix of VFAs at a loading rate of 1.9 g COD L^{-1} d^{-1} and after 30 days of operation, a steady power density of 49 mW L^{-1} was observed. Acetate and propionate were preferred as electron donors, though butyrate, valerate, caproate were also removed but at lower rates [154]. Mohanakrishna et al. [21] investigated the energy conversion of VFA rich effluents from DF of vegetable wastes, using the MFC system. They highlighted that the system was beneficial to improve both energy production and wastewater treatment [21]. Recently, Lalaurette et al. [156] coupled DF/MEC systems with cellobiose and corn stover as feedstock and supplies with an external power source of 0.5 V to produce biohydrogen. The DF effluents were composed of acetic, lactic, succinic formic and ethanol. Such strategy led to an overall hydrogen yield of 9.95 mol mol^{-1} glucose using cellobiose and 16% and 84% were produced from DF and MEC process,

respectively. To avoid the need for an external power source for the MEC system, Wang et al. [157] integrated DF/MFC/MEC systems where electricity requirement for MEC is produced by MFC system. The overall hydrogen production for the integrated system increased by 41% compared with fermentation alone and did not required any external electrical input.

7. Conclusions and future scopes

Algae biomass, but also lipid-extracted microalgae, being rich in carbohydrates, have great potential as feedstock for biohydrogen production. Nonetheless, biohydrogen potentials are low and a pretreatment step is often required to solubilize carbohydrates, enhance microbial accessibility and further biohydrogen production. Among pretreatment technologies, thermal and thermo-chemical pretreatments have been recently investigated to overcome these natural barriers. One major drawbacks of using thermal or thermo-chemical pretreatments is the possible generation of by-products (i.e. furfural, 5-HMF) that can further inhibit the activity of the hydrogen producing bacteria, and thereafter the DF performance. Finally, to make the process economically appealing, the effluents of the DF process must be integrated into a biorefinery approach. Up to date, two-stage dark fermentation/anaerobic digestion, two-stage dark fermentation/photo fermentation and three-stage dark fermentation/photo fermentation/anaerobic digestion were developed and investigated.

Although biohydrogen seems to be an ideal candidate for future biofuels production integrated in a biorefinery, several research challenges must be addressed to optimize its efficiency for the implementation of sustainable technologies.

- Firstly, information on physico-chemical barriers limiting the accessibility of the HPB to produce biohydrogen are still scarce. Such data are compulsory for the definition of more efficient pretreatment strategies.
- Thermal or thermo-chemical pretreatments on algae are well documented in literature, while biological pretreatment received scarce attention. Classical enzymatic cocktail generally used for lignocellulosic substrates could be adapted for microalgae. However, carbohydrates composition of algae is quite different from terrestrial plants and more research is needed to develop an adequate enzymatic cocktail for algal substrates.
- Further research is needed to investigate the impact of by-products (furfural, 5-HMF), generated during thermal or thermo-chemical pretreatments of algae, on HPB. In particular, it is still not clear if the inhibition level depends on the inoculum type (i.e. pure or mixed cultures), inoculum origin, as well as if there are synergetic effects of such by-products on the DF process performances.
- Integration of dark fermentation into a biorefinery, where DF effluents are valorized, is highly recommended to make the overall process viable. For this purpose, co-pyrolysis or co-gasification with lignocellulosic biomass, as well as MFC and/or MEC of liquid effluents seem to be interesting solutions.
- Finally, energetic, environmental and economic assessments of various biorefinery approaches must to be taken into consideration in future research activities, to define the best biorefinery strategy.

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