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DARK FERMENTATION AND MICROALGAE CULTIVATION COUPLED SYSTEMS: OUTLOOK AND CHALLENGES

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KEYWORDS

Dark fermentation; Hydrogen; Microalgal growth; Volatile fatty acids; Biorefinery; Waste valorization.

ABSTRACT

The implementation of a sustainable bio-based economy is considered a top priority today. There is no doubt about the necessity to produce renewable bioenergy and bio-sourced chemicals to replace fossil-derived compounds. Under this scenario, strong efforts have been devoted to efficiently use organic waste as feedstock for biohydrogen production via dark fermentation. However, the technoeconomic viability of this process needs to be enhanced by the valorization of the residual streams generated. The use of dark fermentation effluents as low-cost carbon source for microalgae cultivation arises as an innovative approach for bioproducts generation (*e.g.*, biodiesel, bioactive compounds, pigments) that maximizes the carbon recovery. In a biorefinery context, after value-added product extraction, the spent microalgae biomass can be further valorised as feedstock for biohydrogen production. This integrated process would play a key role in the transition toward a circular economy.

This review covers recent advances in microalgal cultivation on dark fermentation effluents (DFE). BioH_2 via dark fermentation processes and the involved metabolic pathways are detailed with a special focus on the main aspects affecting the effluent composition. Interesting traits of microalgae and current approaches to solve the challenges associated to the integration of dark fermentation and microalgae cultivation are also discussed.

1. Introduction

Environmental damage and the finite petroleum supplies are two main global concerns of the 21st century. To face those challenges, there is no doubt about the necessity to implement sustainable process to produce energy and products from non-fossil sources.

Hydrogen gas (H_2) is considered as the most promising green fuel due to its high energy content (122 MJ/kg) and the lack of carbon dioxide (CO_2) released during its combustion, which makes this technology a key-player to reach a carbon neutral economy (Balachandar et al., 2020). That is why a growing interest in H_2 production and storage has recently emerged globally. Analysts estimate that green H_2 could meet 24 % of energy world demand by 2050, with annual sales in the range of 630 billion € (BNEF, 2020). For its part, the European Commission has recently launched a Hydrogen Strategy targeting the promotion of H_2 technologies in order to address the Green Deal and Europe's clean energy transition. This strategy includes massive investments in the H_2 sector accounting for 180-470 cumulative billion € in the European Union by 2050 (European Commission, 2020).

So far, most of the H_2 is produced from traditional fossil sources. However, strong efforts are being made to develop cleaner H_2 productions such as water electrolysis from renewable electricity sources and thermo-chemical or biological processes (El-Emam and Özcan, 2019). Among the green H_2-producing technologies, dark fermentation (DF) has emerged as one of the most sustainable alternatives. Dincer and Acar (2014) assessed 19 technologies for H_2 production, including environmental, technical, financial, and social impacts. They concluded that DF is the most economical method while exhibiting the lowest global warming potential (< 1 $kgCO_2/kgH_2$).

DF is a well-known technology where biodegradation of organic matter takes place leading to the production of gases (H_2 and CO_2) and other soluble metabolites. One of the main advantages of this technology is the wide variety of feedstocks which can be employed such as activated

sludge, lignocellulosic biomass, food waste or microalgae. This process offers the dual advantage of generating bioproducts while valorising wastes that otherwise should be treated, clearly contributing to a circular economy. However, some concerns associated to BioH_2 production via DF should be tackled. H_2 is necessarily coproduced with CO_2 during DF, which results in a net loss of carbon in the gaseous fraction and therefore, one of the major challenges associated to the biological production of H_2 is the need of purification. This CO_2 contained in the gaseous fraction can finally be valorised as a synthon to produce chemicals such as polycarbonates, carbamates or polyurethanes either through chemical or biological routes, or directly used a substrate for autotrophic micro-organisms (Heffernan et al., 2023; Romans-Casas et al., 2021).

Additionally, the organic matter conversion into H_2 during this bioprocess is uncomplete leading to limited H_2 yields and organic matter-rich effluents. DF must therefore be associated with other processes to reduce the effluent organic matter content before disposal. On one hand, the solid fraction of the dark fermentation effluents contains the more recalcitrant organic matter that is not degraded by the microbial. This solid fraction can be valorized via conventional anaerobic digestion (AD) for biogas production (Llamas et al., 2021). On the other hand, the liquid supernatant is rich on metabolites with commercial value such as VFAs, lactate or ethanol (Dahiya et al., 2015). Those molecules represent an opportunity for the bioeconomy, as they could be a product by themselves or can be used as precursors in other processes (Bundhoo, 2017).

Despite the wide range of applications of those compounds, the major challenge associated with their use is the need of extraction or even purification which entail high costs and technological limitations. Therefore, the search of alternative applications with a direct use of those metabolites avoiding extraction/purification could present a major advantage for the development of feasible processes that serve the circular economy. Some of the processes that

use these organic acids-rich effluents directly include AD, photofermentation and bioelectrochemical systems for energy production **(Figure 1).**

Recently, the novel idea of coupling DF with microalgae culture has been suggested as an effective way to treat DFE and provide cheap substrates for heterotrophic or mixotrophic micro-algae production while maximizing the carbon recovery. Under this innovative biorefinery concept, microalgae could be employed as cell factories for the production of not only third-generation biofuels (biogas, bioethanol, biodiesel, bioH_2) but also high value-added chemicals (i.e. cosmetics, pharmaceuticals, nutraceuticals, biofertilizers, pigments) (Dourou et al., 2021; Siddiki et al., 2022). Although microalgae exhibit lower cell densities and longer cultivation period than other microorganisms such as bacteria or yeast, the use of microalgae technologies presents crucial environmental advantages: CO_2 capture during their photosynthetic activity and shorter production periods than plants. Besides these interesting traits, microalgae present the ability to grow in a residual environment. Considering that the cost of the substrate is claimed to be a key issue for attaining economically competitive bioprocesses, the utilization of renewable waste streams as low-cost substrates for bioproducts generation arises as an attractive option. Therefore, the coupling of DF and microalgae cultivation supports not only DFE treatment but also carbon recovery maximization through an efficient multi-product generation (H_2 and high-value products) (Chong et al., 2022; Scarponi et al., 2021). Many studies have focused on DF coupled to other processes (*i.e.* AD, electrofermentation, photofermentation), but the integration with microalgae cultivation has seldom been reported. To cover this gap of knowledge, this review aims at evaluating the coupling of DF and microalgae cultivation in a biorefinery context, describing recent approaches and associated challenges that need to be faced to reach a viable industrial application.

2. The basis of Dark Fermentation process

DF is a promising technology designated to obtain bioenergy from organic substrates in the form of bioH_2. This bioprocess corresponds to the intermediate fermentative steps of the AD process which ultimately leads to the production of methane (CH_4). During DF, simple monomers (carbohydrates, proteins and lipids) are generated from the hydrolysis of complex organic matter. Subsequently, those monomers are converted into H_2 and CO_2 due to the activity of an anaerobic bacterial consortium.

One of the main drawbacks of H_2 production via DF is the economic viability of the process. The fermentative H_2 production costs (2.5 €/kg H_2) need to be reduced to be competitive with fossil fuel technologies (<1€/kg H_2) (Bundhoo, 2017). In addition, H_2 production by fermentative bacteria is limited by their metabolic constraints: the degradation of organic matter into H_2 is incomplete with a theoretical maximum yield of 33% of the initial organic matter. The rest is retrieved in the form of soluble metabolites in the DFE (Sharma et al., 2020). Those organic matter-rich DFE can serve as feedstock to other processes, allowing the valorization of wastes that otherwise should be treated before disposal.

The physico-chemical properties of the DFE may affect the downstream bioprocess that is chosen to be integrated in the biorefinery scheme as highlighted in **Figure 1**. For instance, when DF is coupled to AD, the effluent composition is not crucial, as long as the total acids concentration remain below the methanogenic bacterial inhibition thresholds. However, for other potential coupled processes such as photofermentation, bioelectrosystems or microbial cultivation, acetate enriched DFE should always be favoured since this molecule is the easiest assimilable metabolites among other ones that are produced.

The composition of the DFE mainly depends on the substrate and inoculum employed as well as the operating parameters applied. These factors strongly affect the bacterial communities

involved and the related fermentative metabolic pathways, which ultimately determine the fate of the organic matter, and therefore the H_2 yields and metabolite profiles (Greses et al., 2020).

BioH_2 generation takes place through sequential biochemical reactions as depicted in **Figure 2**. The DF bioprocess is carried out by anaerobic hydrogen producing bacteria (so- called HPB), that are usually members of the genus *Clostridium*. Other well described HPB genera include members of *Enterobacter* sp., *Escherichia-Shigella*, *Bacillus* sp., *Ethanoligenens* sp., *Megasphera* sp. or *Prevotella* sp. The relative abundance of those species in the bioprocess depends on the organic substrate employed and on the initial inoculum (Cabrol et al., 2017; Etchebehere et al., 2016b).

Metabolically, H_2 production pathways start with the conversion of glucose to pyruvate, which is further oxidized into acetyl-CoA via two different routes: the pyruvate ferredoxin oxydo-reductase (PFOR) and pyruvate formate lyase (PFL) pathways, depending on the bacterial species involved **(Figure 2).** In both cases, excess electrons resulting from these oxidation steps are used to produce H_2 through hydrogenases. The remaining acetyl-CoA can be converted into acetate for ATP generation.

The PFOR pathway is mainly followed by sporulating strict anaerobes related to *Clostridium* sp. (Hallenbeck and Benemann, 2002). Through this pathway, a maximum theoretical yield of 4 mol H_2/mol glucose can be obtained (**Equation 1**, **Table 1**). Together with H_2 and energy production, the purpose of the fermentation process is to regenerate oxidative power (NAD^+). However, the NAD^+ generation in the PFOR pathway is not energetically favoured and thus, this should be alternative regenerated via fermentative metabolites production such as butyrate. Butyrate production pathway is the most thermodynamically favoured pathway for both energy and oxidative power production, generating 2 mol H_2/mol glucose **(Equation 2, Table 1).**

In contrast, facultative anaerobic microorganisms, such as *Enterobacter sp.*, *E. coli* or *Ethanoligenens sp.*, preferentially follow the PFL pathway (Hallenbeck and Benemann, 2002). During PFL pathway the ATP production route must be derived to regenerate NAD^+ mainly by converting the acetyl-CoA into ethanol. In this case, 2 mol H_2/mol glucose can also be obtained alongside with an equimolar mixture of acetate and ethanol **(Equation 3, Table 1)**. In mixed cultures, substrate competition between HPB and other non-H_2 producer microorganisms can occur. Those bacteria are mainly propionate producers such as *Clostridium propionicum* and lactic acid bacteria such as *Lactobacillus* sp. or *Lactococcus sp*. The first ones can convert glucose into propionate according to **Equation 5** while the latter ones convert glucose into lactate according to **Equation 6 or 7**. Lactic acid bacteria may however impact DF processes positively since they can further convert lactate and acetate into butyrate and H_2 **(Equation 8**). Other metabolites such as succinate, caproate or valerate can sometimes also be found in DFE. However, those acids are produced from auxiliary metabolic pathways resulting generally in minor concentrations.

In practice, the H_2 yields obtained using mixed cultures range between 1 and 2.5 mol H_2/mol glucose. Apart from H_2, this process generates effluents rich in compounds including acetate, butyrate and ethanol. The profiles and concentration of those metabolites in DFE are very variable according to the fermentation pattern followed by the bacteria and organic substrate nature and concentration. A compilation of H_2 yields and metabolites profile generated during DF of different substrates are summarized in **Table 2**.

Moscoviz et al. (2018) distinguished two main fermentation clusters corresponding broadly to the aforementioned metabolic equations. The first group, corresponding to an average mixed culture typically found in DF, led to a balance between acetate and butyrate pathways (**Equation 4, Table 1**). Following this stoichiometry, a theoretical yield of 2.5 mol H_2 /mol glucose can be obtained, associated to a molar ratio butyrate/acetate of 0.66. This profile is the

most commonly found in literature, probably because fermentation conditions are mainly favouring it. The second group corresponds to fermentations following the PFL pathway, leading to concomitant acetate and ethanol formation. This kind of profile is usually observed when DF conditions favours the emergence of *Enterobacteriales* (Palomo-Briones et al., 2017). Interestingly, ethanol production can also be observed with *Clostridiales* when grown under stressful conditions, shifting from H_2 production to solventogenesis (Dauptain et al., 2021). Finally, a third group of fermentation patterns can be described that are characterized by low H_2 production with various amounts of lactate, formate or propionate.

All this considered, it seems apparent that fine-tuning of process parameters can be used to promote the activity of the microorganisms involved in a specific metabolic pathway. Therefore, the knowledge about substrate-microbiome-products relationship can be employed as a tool to optimize the generation of a targeted product.

3. Cultivation of microalgae on Dark Fermentation Effluents

Among all the potential microorganisms that can be employed in bioprocesses, microalgae are considered as one of the most promising for bioproducts generation. Indeed, they can be used as cell factories to produce not only third-generation biofuels (bioethanol, biodiesel, bioH_2) but also higher value-added bioproducts (*i.e.*, cosmetics, pharmaceuticals, nutraceuticals, biofertilizers, pigments) (Dourou et al., 2021; Patel et al., 2017). The microalgae group gathers unicellular or multicellular organisms usually able to perform photosynthesis, converting CO_2 into organic matter using light energy. They can be broadly classified into prokaryotic (cyanobacteria) and eukaryotic microalgae such as green algae (chlorophyta), red algae (rhodophyta) and diatoms (Brennan and Owende, 2010). Some microalgae species can use

multiple metabolic pathways for growth, depending on the environmental conditions, substrate, and light availability. This metabolic flexibility enables different cultivation methods, *i.e.*, phototrophic, heterotrophic and mixotrophic culture.

Under phototrophic conditions, microalgae absorb light energy while fixing inorganic carbon for biosynthesis. Photoautotrophic microalgae cultivation is however usually more expensive than plant crops because the growth of microalgae requires appropriate light, mixing, pH and CO_2 and inorganic salt concentration (Yew et al., 2019). Furthermore, light availability and seasonality, self-shading effects and more generally photosynthetic constraints limit final biomass concentration and thus their productivity and commercial potential (Gouveia et al., 2016; Kenny and Flynn, 2017). For this reason, the use of organic carbon sources (under heterotrophic or mixotrophic cultivation modes) has been suggested to circumvent all those drawbacks (Hu et al., 2018). As a result, light is less essential while high cell concentrations and high volumetric productions can be achieved.

With all this in mind, VFAs-rich DFE arise as a potential carbon source for microalgae cultivation in heterotrophy or mixotrophy. Aside from VFAs, DFE also contain mineral nutrients such as ammonium and orthophosphate which can sustain microalgae growth (Turon et al., 2016). Therefore, coupling DF to microalgae cultivation would allow the conversion of mixed VFA from the effluents into valuable biomass, effectively maximizing the carbon recovery from wastes **(Figure 3)**.

3.1. Heterotrophic growth on Dark Fermentation Effluent compounds

When microalgae are cultivated under heterotrophic conditions, organic compounds provide both carbon and energy sources to support the microbial growth. However, consumption rates

and biomass yields achieved heavily depend on the substrates employed as well as the considered species **(Table 3).**

3.1.1. Acetate: the favoured substrate

Acetate has been consistently reported as the most easily assimilable substrate among the VFAs found in DFE, given its simple structure and low electron content (Fei et al., 2015; Turon et al., 2015a; Venkata Mohan and Prathima Devi, 2012). Acetate metabolisation requires thus only a few enzymatic steps which have been extensively described in detail by several authors (Johnson and Alric, 2013; Perez-Garcia and Escalante, 2011). However, its uptake and transport into the cell is not entirely clear. Acids in solution exists under two forms: the undissociated acid (RH) and the ionic acid form (R^-). The undissociated acetic acid form (AcH) is liposoluble and can thus diffuse passively into the cells, without any active transport requirement. The anionic acetic acid form (Ac^-), however, is thought to be actively imported via a monocarboxylate/proton (MCT) transporter as described in other eukaryotes (Perez-Garcia and Escalante, 2011), but no clear evidence can be found in literature. After transport into the cell, acetate is converted to acetyl-CoA, a central metabolite which can serve as precursor for major metabolic pathways such as the glyoxylate cycle and the Krebs cycle, which produce metabolites for further synthesis of amino acids, fatty acids and sugars. Heterotrophic acetate consumption has been widely evidenced for several microalgae strains including *Chlamydomonas reinhardtii* (Moon et al., 2013), *Chlorella vulgaris* (Shen et al., 2016), *C. sorokiniana* (Abiusi et al., 2020), *Scenedesmus sp.* (Ren et al., 2018), *Crypthecodinium cohnii* (Chalima et al., 2019), *Auxenochlorella protothecoïdes* (Fei et al., 2015) or *Euglena gracilis* (Nakazawa, 2017) In fact, growth rates and biomass yields obtained when using acetate as carbon source are generally higher than in pure autotrophy (Perez-Garcia and Bashan, 2015).

3.1.2. Other substrates

Generally, other substrates will not be as easily consumed as acetate, mainly due to the need of additional enzymatic steps before complete assimilation. Given that most of the research carried out on carbon metabolism in microalgae focused on acetate, limited information is available about mechanisms of other carbon compounds transportation and metabolism.

Only few studies investigated the effect of butyrate on microalgae growth in well-controlled conditions, *i.e.*, single strain, axenic conditions and butyrate as single substrate. In human colonocytes, butyrate is transported via a MCT (Cuff et al., 2005). Butyrate is then imported in the mitochondrion where it is oxidized into acetyl-CoA (Donohoe et al., 2011). In microalgae, butyrate metabolism is probably related to butyrate oxidation in the glyoxysomes into acetyl-CoA via a β-oxidation pathway (Baroukh et al., 2017). This pathway has been mostly deciphered in the heterotrophic alga *Polytomella* sp. (Lacroux et al., 2022) but remain to be confirmed in phototrophic algae. Heterotrophic butyrate consumption has been evidenced for few strains such as *C. sorokiniana* (Patel et al., 2021; Turon et al., 2015a), *Auxenochlorella protothecoïdes* (Patel et al., 2021) and the heterotrophic dinoflagellate *C. cohnii* (Chalima et al., 2019). Biomass productivity on butyrate is nevertheless about 5- to 10-fold lower than the one obtained on acetate as carbon source, within a range of 0.1 – 0.29 g/Ld (Lacroux et al., 2020; Turon et al., 2015a). The only known exception is the heterotrophic alga *Polytomella* sp., which exhibits highly efficient butyrate assimilation (Lacroux et al., 2022). Biomass yields (in grams of biomass per gram of substrate) are, nevertheless, higher on butyrate than acetate since butyrate contains twice more carbon than acetate.

Similarly, little information on lactate metabolism in microalgae is available. Lactate is likely first converted into pyruvate via the enzyme lactate dehydrogenase (LDH), as found in *C. pyrenoidosa* (*C. sorokiniana*) and *C. reinhardtii* (Gruber et al., 1974; Husic and Tolbert, 1985). This enzyme can either produce (or oxidize) D-lactate from (or into) pyruvate and it is thought

to participate in NADH recycling notably in anaerobic growth (Burgess et al., 2016). However, several authors evidenced that external lactate is not consumed by *Chlorella* species either in heterotrophy (Turon et al., 2015a) or mixotrophy (Liu et al., 2013). By contrast, heterotrophic lactate consumption was reported by some other species such as *Euglena gracilis* (Fujita et al., 2008), and *Scenedesmus abundans* (Lin et al., 2020).

Finally, ethanol consumption also seems to be highly strain-specific even among the same genus. The ethanol molecule can diffuse passively through the cell membrane and is then oxidized in acetyl-CoA by an alcohol/aldehyde dehydrogenase (ADH) enzyme (Atteia et al., 2003). However, *C. reinhardtii* can neither consume ethanol nor butanol even in presence of the required enzymes (Catalanotti et al., 2013; Jiang et al., 2017). Ren et al. (2018b) cultivated an isolated *Scenedesmus* sp. in heterotrophic conditions on an effluent containing several substrates (acetate, butyrate, propionate, valerate, ethanol) resulting in the exhaustion of all substrates but not ethanol, even at the lowest concentration of 0.15 g/L. By opposite, other species such as *Polytomella* sp. has been shown to grow on ethanol as concentrated as 40 mM (Atteia et al., 2000).

3.1.3. Behaviour in mixtures

The profile and concentration of all these compounds may differ among DFE. In the presence of multiple carbon sources, microorganisms tend to use the simplest one first and then the more complex ones. The phenomenon is known as carbon catabolite repression and has been extensively discussed for bacteria (Görke and Stülke, 2008). In the case of microalgae, a diauxic growth behaviour, where acetate is always consumed before butyrate, was evidenced for *C. sorokiniana* and *Auxenochlorella protothecoïdes* growing in heterotrophy (Turon et al., 2015a) or mixotrophy (Lacroux et al., 2021a). Considering the easy acetate assimilation, the presence of this acid has always been reported to improve biomass productivities (Fei et al., 2015; Turon et al., 2015a, 2015c; Venkata Mohan and Prathima Devi, 2012). Diauxic behaviour was

evidenced by Fei et al. (2015) as well, who investigated the effect of VFA ratio on the growth of *A. protothecoïdes* in synthetic mixtures. An acetic:propionic:butyric acid mixture at an ratio 8:1:1 was the most beneficial for growth, nearly doubling final biomass as compared to a 4:3:3 ratio. Similarly, Kim et al. (2019) reported the maximal growth of *C. vulgaris* on a medium containing a 6:1:3 mixture ratio, concluding that the presence of acetate as major component also boosted butyrate consumption two-fold. Patel et al. (2022) recently cultivated *A. protothecoïdes* and *C. sorokiniana* on VFAs from acidogenic fermentation of waste lignocellulosic biomass from brewers' spent grain, initially containing high amounts of acetate, propionate and butyrate (10.07, 0.81 and 1.24 g/L respectively). Interestingly, they obtained high biomass and lipid productivities in comparison with other studies using similar mixtures **(Table 3)**, probably owing to the high acetate concentration in the medium. A sequential VFAs assimilation was also observed by the authors, acetate being consumed first followed by butyrate and propionate.

3.1.4. Mechanisms of substrate inhibition

The major DF compounds (*e.g.*, VFAs, ethanol) can exert an inhibitory effect on microalgae at high concentrations. The ethanol toxicity on microalgae highly depends on concentration and the species studied. Few strains such as *Dunaliella tertiolecta*, *Isochrysis galbana*, *Monodus subterraneus* or *Spirulina platensis* show a relatively high tolerance to ethanol concentrations above 15 g/L (Miazek et al., 2017). *C. reinhardtii* was not able to grow on 23.6 g/L ethanol and 4 g/L butanol and exhibited a 50% growth reduction when cultivated in presence of 14.2 g/L ethanol or 2.4 g/L butanol. It should be mentioning that these ethanol concentrations are not commonly attained during DF. According to Moscoviz et al., unless operating under specific conditions, an average ethanol concentration of 0.5 g/L has been commonly found in DFE (Moscoviz et al., 2018).

Regarding acetate, Chen and Johns, (1994) reported for the first time a reduction of *C. reinhardtii* maximal heterotrophic growth rate on acetate at concentrations above 0.5 g/L. They concluded that acetate was an inhibitory substrate. Similarly, VFA mixtures over 8 g/L were found to be inhibitory for *A. protothecoïdes* and an increase in lag phase duration was observed when increasing VFAs concentration from 2 g/L to 4 g/L (Fei et al., 2015). Turon et al. (2015a) stated that *C. sorokiniana* and *A. prothotecoïdes* heterotrophic growth was negatively affected when using VFAs mixtures over 2.5 g/L acetate or 0.4 g/L butyrate. The sensitivity to VFAs is besides highly strain dependant. For example, Cheng et al. (2021) found that growth rate of *Scenedesmus obliquus* was increasingly lowered with increasing concentration of acetate as low as 50 mg/L. As a result, microalgae cultivation on these substrates has been generally performed at low total VFA concentrations (<4 g/L).

The acid inhibitory effect is caused by the undissociated form of the acid (RH) at high concentrations. Indeed, this undissociated form is liposoluble and can cross the cell membrane into the cell, where it will dissociate causing cytosolic acidification. The export of this internal dissociated form out of the cell is energy consuming, resulting in ATP depletion when RH concentration is in excess. As a result, a lack of energy available for division causes the cells to stop growing (Russell, 1992). RH concentration in solution directly depends on the acid concentration and the pH of the medium and it can be calculated by the Henderson-Hasselbalch equation (Eq.1) where C_t refers to the total acid concentration (g/L).

$$[RH] = \frac{Ct}{1+10^{pH-pKa}} \quad \text{(Eq.1)}$$

The detrimental effects of RH on bacterial or yeast cells have been well documented (Giannattasio et al., 2013). In contrast, the literature on microalgae is scarce, especially for molecules other than acetate. Lacroux et al. (2020) determined the acetate and butyrate inhibition threshold of four chlorophyte strains (*i.e.*, *Acutodesmus obliquus*, *Auxenochlorella*

protothecoïdes, *Chlamydomonas reinhardtii* and *Chlorella sorokiniana*). The growth of those strains was found to be inhibited at concentration ranges of 47 to 207 mg/L acetic acid and 12.5 to 50 mg/L butyric acid depending on the studied strain.

Therefore, knowing the pH of the fermentation effluent and the metabolite concentration, RH concentration in DFE can be calculated. According to the determined concentration and the strain tolerance to RH, an accurate pH control or effluent dilution needs to be employed to avoid the inhibitory effect exerted by acids. For instance, the concentration of acetic acid and butyric acid in the experiments conducted by Fei et al. (2015) were of 147 mg/L and 73 mg/L respectively (pH of 6.3; total concentration of acetate 4.8 g/L and butyrate 2.4 g/L). These concentrations were in the range of the inhibitory thresholds given above, which could explain while inhibition occurred. Besides, the fact that these substrates were mixed could have further aggravated their toxic effects.

3.2. Tuning the mixotrophic process

Besides heterotrophy, microalgae can also be cultivated on organic substrate under mixotrophic conditions. Mixotrophic growth combines phototrophic and heterotrophic modes and therefore a simultaneous consumption of organic and inorganic carbon sources occurs in presence of light to obtain both carbon and energy (Perez-Garcia and Bashan, 2015). Mixotrophy usually results in enhanced growth rates and biomass yields when compared to purely autotrophic or heterotrophic conditions, as these two latter processes may occur non-competitively and simultaneously (Pang et al., 2019). For instance, when growing *C. sorokiniana* on glucose under mixotrophy, 4.57 g/L biomass was obtained while 1.7 g/L and 2.78 g/L biomass were reached under autotrophic and heterotrophic conditions, respectively (Li et al., 2016). However, care

should be taken when interpreting and extrapolating mixotrophic results, mainly due to the complex interactions occurring between the heterotrophic and autotrophic metabolisms.

In some instances, mixotrophic growth rates can indeed be higher to the sum of autotrophic and heterotrophic growth rates since synergic effects occur between the two metabolisms, allowing a more efficient use of carbon and energy (Zhang et al., 2017). Regarding CO_2 fixation, internal recirculation of the CO_2 coming from cellular respiration can occur in mixotrophic cultivation. This allows to reach biomass yields up to 1 gC_X/gC_S (Abiusi et al., 2020) while reducing net CO_2 emissions compared to heterotrophic cultures (Smith et al., 2015). This increased internal CO_2 concentration was evidenced on the gene expression level by Cecchin et al. (2018) who found that acetate presence caused upregulation of the phosphoenolpyruvate carboxylase enzyme. Aside from maximizing the carbon recovery, environmental assessments advocate mixotrophic cultivations where CO_2 is fixed contributing in the reduction of CO_2 emissions, instead of heterotrophy (Hu et al., 2018). Regarding light, microalgae cultivated in mixotrophy can withstand higher light intensities compared to autotrophy. Indeed, the presence of acetate reduces the photo-inhibition by interacting with the photosystem PSII, which reduces production of oxygen radicals (Roach et al., 2013). For instance, when *C. sorokiniana* was cultivated on acetate under mixotrophy, Xie et al. (2016) showed that high light intensity (up to 800 $\mu E/m^2/s$) resulted in a positive effect on acetate assimilation and thus, in the microalgae growth rate. In contrast, autotrophic growth rate decreased by 20% under high light intensities (800 $\mu E/m^2/s$) compared to the low intensity tested (90 $\mu E/m^2/s$). In a specific light condition, growth rates in mixotrophy can thus be higher than expected (Xie et al., 2016). Finally, organic carbon uptake by some strains such as *C. sorokiniana* or *A. protothecoïdes*, especially butyrate, has been demonstrated to be light-dependent (Turon et al., 2015c). Although the reasons are not entirely deciphered, it is probable that light provides the necessary energy to metabolize

this organic substrate. Consequently, a null growth in heterotrophy could still result in a higher-than-expected growth rate in mixotrophy due to specific interactions.

On the contrary, mixotrophic growth rates and yields can also be lower than the sum of the autotrophic and heterotrophic one. For example, high level of easily assimilable substrates such as acetate may reduce photosynthetic activity. Heifetz et al. (2000) showed that the presence of 29.4 mM acetate reduced external CO_2 fixation to 66% compared to the autotrophic control while not affecting the growth rate, indicating that organic carbon assimilation was favoured over inorganic carbon capture. This is probably due to the binding of acetate to PSII, which reduced its activity (Roach et al., 2013). Similarly, the influence of inorganic carbon on the organic carbon uptake is not completely elucidated. Liu et al (2013) also showed that supplementing a medium containing 2.72 g/L bicarbonate with several concentrations of butyrate (from 0.5 to 1.8 g/L), resulted in a concomitant increase in *C. vulgaris ESP-6* biomass yield. These studies suggest that organic carbon assimilation always occurs compared to autotrophic growth when possible, irrespective of CO_2 or bicarbonate concentration. However, contradictory results found by other authors indicate that inorganic carbon does influence the mixotrophic process. For example, Liu et al. (2013) showed a microalgae growth inhibition on butyrate (0.5 g/L) when bicarbonate concentration is above 2.72 g/L. Sforza et al. (2012) showed that when bubbling 5% CO_2, glycerol consumption by *Chlorella protothecoïdes* was reduced by 3.8-fold compared to cells grown under atmospheric CO_2 concentration (0.04% CO_2). Similarly, in the case of *Nannochloropsis salina*, switching from atmospheric CO_2 concentration to 5% CO_2 conditions, almost a complete inhibition of the glycerol consumption was reported. Thus, the influence of inorganic carbon in the mixotrophic process should be still elucidated. More specifically, the ratio of inorganic/organic carbon regarding light availability and organic substrate nature, should be investigated to unravel the interactions between heterotrophic and autotrophic metabolisms.

4. Approaches to enhance microalgae cultivation on Dark Fermentation Effluents

Two major challenges should be faced in order to reach an optimal integration of DF and microalgae cultivation and attaining sustainable and economically competitive combination of these two bioprocesses. Firstly, microalgal biomass productivity is relatively low, and the VFA assimilation rate by microalgae is projected to be lower than the VFA production rate by DF. Indeed, between 6-15 days are usually needed for the algae to grow and completely remove VFAs (Lacroux et al., 2021a), while DF is usually carried out with HRT around 12 hours (Ren et al., 2018). Secondly, high concentrations of the major DF compounds (e.g., acetate, butyrate, ethanol, lactate) can inhibit proper microalgae growth. This limits microalgae cultivation to low VFA concentration, usually in the range of 1-2 g/L (Li et al., 2020). The following sections aim to describe the causes of these limitations and the proposed solutions to overcome these challenges.

4.1. Dark Fermentation strategies

DFE often present higher concentration of VFA with chains longer than acetate (Moscoviz et al., 2018). Therefore, to optimize the coupling between DF and microalgae cultivation, special attention must be paid to the factors affecting the microbial activity during DF (as previously explained in Section 2) which ultimately determines the fate of the organic matter and the acid concentration and profile. However, those factors (*i.e.* feedstock and inoculum employed or operational parameters applied) should be optimized to drive DF towards suitable metabolites without sacrificing H_2 yields.

4.1.1. Substrate and nutrients

Widely available and low-cost organic waste are potential feedstock sources for DF, such as waste activated sludge, algal biomass, lignocellulosic-based biomass or food waste (Guo et al., 2010). The macromolecular composition of a given substrate affects bioconversion yields due to the different hydrolysis rates of carbohydrates, proteins and lipids (Angelidaki and Sanders, 2004). Although the nature of the organic substrates is complex and their composition diverse, it is well known that H_2 yield is directly correlated to the soluble carbohydrates content (Jarunglumlert et al., 2021) while proteins and lipids contributions are not significant. Besides, it has been demonstrated that the macromolecular substrate composition can significantly affect the metabolite profile obtained. Regueira et al. (2020a) stated that odd-chain acids (especially propionic acid) are mainly associated to protein-rich substrates such as microalgae biomass. By contrast, even-carbon number VFAs (acetic and butyric acid) have been reported to prevail during DF of carbohydrate-rich substrates. A compilation of H_2 yields and metabolites profile generated during DF of different substrates are summarized in **Table 2**.

The C:N ratio has been reported as key parameter in DF as well. Optimal C:N ratios ranging between 5 and 200 have been reported in the literature for DF using different configurations and operational parameters (Elbeshbishy et al., 2017). For instance, after testing several C:N ratios (40-130) using sucrose as substrate and *Clostridium pasteurianum* as inoculum. Lin and Lay (2004) reported an optimum H_2 production (4.8 mol H_2/mol sucrose) applying C:N ratio of 47. Those authors concluded that higher C:N ratios (> 47) lead to a low H_2 production due to nitrogen-limited growth while lower ratios (C:N < 47) lead to potential free ammonia inhibition. Regarding the metabolic profiles, acetate and propionate fractions increased by 75% and 90% when C:N ratio increased from 47 to 130, respectively, suggesting that butyrate fermentation shifted towards acetate fermentation. Conversely, Anzola-Rojas and co-woerkes (2015) studied C:N ratios from 40 to 190, reporting an optimal C:N ratio of 137 for a maximum H_2 yield of 3.5 mol H_2/mol sucrose. These authors did not find a clear influence of the C:N ratio

on the fermentation pathways, since similar metabolic profiles in terms of ethanol, acetic acid and butyric acid were produced.

4.1.2. Anaerobic inoculum

To ensure H_2 production from organic matter, fermenters are often inoculated with HPB issued from various environments (anaerobic sludge, aerobic sludge, sediments) (Etchebehere et al., 2016b). These inocula also host various non-HPB such as lactic acid or propionic bacteria as well as H_2-consuming organisms such as homoacetogenic bacteria and methanogenic archaea, that inevitably reduce the DF yield by either outcompeting for the substrate or consuming the desired product. Therefore, HPB inoculum enrichment methods have been investigated to improve H_2 yields. A way of selecting some HPB relies on their ability to form spores, such as *Clostridium sp.* Thus, inoculum are commonly pre-treated by applying a physical (thermal shock, micro-aeration, irradiation, sonication) or chemical stress (pH, 2-bromoethanesulphonate) (Rafieenia et al., 2018). Recently, Luo et al. (2022) compared different inoculum pretreatment methods aiming to maximize the bioconversion of food waste into H_2. They concluded that the alkali-treated inoculum exhibited the highest H_2 yield (157 mL H_2/VS) corresponding to a 70% improvement in comparison to control experiment.

At this point it is worth to highlight that different microbial populations lead to different distribution of soluble products (as described in Section 2). It has been reported that acetic-butyric acid rich effluents were obtained when using heat-shock and alkaline pre-treated inocula, while acetic and propionic acid were the main products when and acid pre-treated inoculum was employed, and ethanol is produced when aeration pre-treatment is applied (Ren et al., 2008)

4.1.3. Operational parameters

H_2 yields and production rates can be enhanced by optimizing the operational and design parameters of DF bioreactors (*i.e.*, temperature, pH, hydraulic retention time (HRT), organic loading rate (OLR) and partial pressure of H_2) which, in turn, can control the microbial's metabolism.

Several studies have correlated H_2 production performances with the microbial population density. Therefore, different cell retention strategies such as the use of granulated sludge and biofilm in those reactors have been employed (Etchebehere et al., 2016a). Besides the specific advantages of these different bioreactor configurations, HPB selection can also be ensured by taking advantage of the differences in growth of the various microorganisms. In continuous operation, differences in growth rates among the microorganisms enable selection of HPB by shortening the hydraulic retention time (HRT). Short HRT (6-12 h) are indeed more favourable for H_2 producers, while longer HRT (18-24 h) negatively affects the H_2 yields due to microbial community shifts (Palomo-Briones et al., 2017). In the same way, pH and temperature are crucial parameters for H_2 synthesis. Acidic pH values (around 5.5) inhibits the methanogenic archaea activity while allowing maximum H_2 production (Liu et al., 2008). However, pH lower than 4.5 tends to cause metabolic shifts in *Clostridium* sp. towards solventogenesis (*i.e.*, acetone-butanol-ethanol) due to the accumulation of undissociated VFAs (Van Ginkel and Logan, 2005). Operational pH affects the metabolic by-products as well. In most of the studies, neutral pH favours the acetate pathways, while acidic pH conditions favour the butyrate production pathways (Ghimire et al., 2015). Temperature affects not only the microbial activity shifting the DF products, but also the physical state of the organic matter. Several authors reported higher H_2 yields at thermophilic than mesophilic temperatures when using organic wastes as substrate (Shin et al., 2004; Valdez-Vazquez et al., 2005). In fact, it is known that inhibition of the H_2-consuming homoacetogenic activity can be achieved under thermophilic conditions (Luo et al., 2011). In terms of VFAs production, acetic acid was reported as a

dominant by-product in thermophilic digestion, whereas butyrate is mainly formed in mesophilic conditions (Liu et al., 2008).

Butyrate inhibition can be reduced by increasing the acetate content in the medium (Baroukh et al., 2017; Fei et al., 2015). In this way, microalgae can quickly build up biomass from acetate leading to a decrease in the butyrate to biomass ratio. Nevertheless, the potential inhibition by acetate should not be neglected when using this strategy. During the DF process, acetate can be also produced via homoacetogenesis (Pavlostathis and Giraldo-Gomez, 1991). Therefore, maximizing acetate concentration in DFE could be done by applying the proper favourable conditions to ensure homoacetogenic bacteria activity (i.e. increasing HRT) (Siriwongrungson et al., 2007). However, this strategy likely decreases the H_2 yield obtained during the process (Saady, 2013).

Kim et al. (2019) investigated the growth of *C. vulgaris* on different effluents produced from the DF of algal biomass. By adjusting DF operational parameters (*e.g.* temperature, pH and HRT), they obtained different fermentation profiles, either propionate- (ratio 5:4:1) or acetate-rich (ratio 6:1:3). Maximum algal biomass was achieved for the latter ratio, mainly because the strain was unable to consume propionate.

Since changes in DF conditions can affect the H_2 productivities, major efforts must be made to optimize both DF and the microalgae cultivation bioprocess.

4.2. Microalgae cultivation strategies

Microalgae cultivation on DFE can be improved by optimizing the reactor design, operational parameters or product-recovery techniques. Additionally, the use of newly isolated or developed microalgae strains resistant to the metabolites usually found in DFE as well as co-

cultures with heterotrophic organisms, can also be promising approaches to circumvent challenges associated with DFEs.

4.2.1. pH control

Although an effluent can be simply diluted to decrease the total acid concentration and avoid inhibition, the resulting lower final biomass concentration increases the biomass harvesting costs. Lowering RH to below toxicity levels can also be achieved by increasing the initial pH. For example, growth of *C. sorokiniana* was completely inhibited at pH 6.0 by 0.8 g/L butyrate, while increasing the pH to 7 allowed growth at the same total butyrate concentration (Lacroux et al., 2020). When further raising the pH to 8.0, Lacroux and co-workers (2021) could cultivate *C. sorokiniana* on 8 g/L butyrate without any inhibition. On the opposite side, when growth and organic acid consumption occur, pH of the medium will inevitably rise (Chalima et al., 2019; Lacroux et al., 2021b). From these studies, the upper pH value tolerated by the algae seems to be around 9, with pH values around 10 causing the complete inhibition of acetate or butyrate consumption. pH should thus be tightly controlled to remain around neutral values during growth. For example, acetate removal by *C. sorokiniana* was found to decrease by 70% when increasing the pH from 8 to 9 (Lacroux et al., 2020). Lacroux et al. (2021) almost doubled both biomass production and substrate consumption during batch cultivation of *C. sorokiniana* on 2.5 g/L acetate using buffer mediated pH control (0 mM to 100 mM). For the cultivation of *C. cohnii*, a fed-batch pH-auxostat strategy was adopted since the pH was found to increase up to 8.9 in spite the presence of buffer (Chalima et al., 2019). Since VFAs-rich effluent is acidic, pH could be lowered through VFA addition. Using a similar strategy, a final biomass of 22 g/L was obtained using acetate in fed-batch, compared to a maximum of around 6 g/L achieved in batch (Chalima et al., 2019). Cho et al. (2015a) found that the biomass production rate of *C. vulgaris* on a concentrated effluent (13.7 gCOD/L as VFA) improved from 296 to 433 mg/L/d when maintaining the pH between 7 and 8.5.

4.2.2. Initial microalgae inoculum

The inhibitory effect of the acids can also be reduced by increasing initial microalgae density of the inoculum or by decreasing the substrate to biomass ratio S/X. As an illustration, *C. vulgaris* butyrate consumption and biomass production rate were increased by respectively 2.5-fold and 2-fold when decreasing S/X from 8.0 to 1.5 (Liu et al., 2013b). Similarly, the growth of *C. reinhardtii* could not be observed on DFE effluent containing more than 2 g/L total VFA when S/X was above 20. Increasing inoculum density 21 times enabled growth on 2.5 g/L total VFA (Radhakrishnan et al., 2021).

4.2.3. Light intensity

Light can provide the necessary energy to deal with inhibitory effects: biomass can grow based on autotrophic metabolism and, once sufficient biomass concentration had been reached, butyrate consumption can start. In fact, light has been shown to alleviate heterotrophic growth inhibition of *C. sorokiniana* on butyrate (Turon et al., 2015c). Butyrate uptake by *C. sorokiniana* was not observed in darkness, while it was promoted under 100 $\mu E/m^2/s$ continuous illumination (Turon et al., 2015c). Similarly, butyrate consumption by *C. vulgaris* increased from 10% to almost 100% when switching from dark conditions to illumination with 150 $\mu E/m^2/s$ (Liu et al., 2013b). It should be highlighted that raw fermentation effluents may present a dark colour as well as a high solid content. These specific characteristics may severely reduce light penetration in the reactor bulk and could thus prevent mixotrophic growth. DFE also likely contains dissolved inorganic carbon as a result of bacterial respiration (Liu et al., 2013a). Therefore, since light presence is essential for the consumption of organic substrates and microalgae growth, DFE pre-treatments or dilution may have to be considered.

Besides light intensity, light wavelength is another important factor to be considered during mixotrophic cultivation. Although the exact wavelength required for photosynthesis depends on the species and its internal pigment composition, the blue (420-470 nm) and red (660 nm) lights usually promotes best autotrophic microalgae growth (Schulze et al., 2014). However, very few studies investigated the influence of light colours on DFE substrates assimilation. In the case of acetate, *S. abundans* final biomass increased to 0.82 g/L using red light as compared to 0.52 g/L using white light (Gupta and Pawar, 2018). Similarly, optimum *Dunaliella salina* biomass productivity was obtained on 4 g/L using a combination of 65% blue and 35% green light (Bredda et al., 2020). As discussed in section 3.2, it remains unclear whether the boost in productivity is only due to the increase of autotrophic activity or to potential positive interactions.

4.2.4. Nutrients requirements (C:N:P balancing)

Alongside carbon, microalgae need mineral nutrients such as nitrogen (N) and phosphorus (P) for the synthesis of various biomolecules such as nucleic acids and amino-acids. Optimum nutrient requirements of microalgae can be determined by estimating their elemental composition under non-limiting conditions in which their maximum growth rate is achieved. A common molar C:N:P given for microalgae is the Redfield ratio 106:16:1 (Redfield, 1958). This ratio is however an average and strong variations can be found among species and cultivation conditions.

Under high C:N:P conditions, growth rates tend to diminish to benefit carbon storage compounds such as lipids and carbohydrates. For example, *C. vulgaris* biomass productivity dropped from 137 to 70 mg/L/d when switched from N- and P- rich conditions to N- and P-limited conditions while a nearly 3-fold increase in lipid yield was attained (Shen et al., 2016). That strain could still assimilate up to 362.8 mg/L acetate even under complete N and P

depletion. When cultivating *A. prothotecoïdes* and *C. sorokiniana* on a mixture of VFAs in heterotrophy, Patel et al. (2021) reported an increase in lipid content from 10% to 30% for both strains when increasing the C:N ratio from 20 to 60. The C:N ratio increment did not affect the acetate, propionate and butyrate consumption rates which were exhausted at the end of the culture. However, longer-chain VFAs (valerate and caproate) could not be further consumed.

Adjusting C:N:P ratio in the medium to the biomass requirements could be done by mixing DFE with other N or P sources with a poor C content (*e.g.* AD effluent or another wastewater). For example, Chiranjeevi and Mohan (2017) produced lipids from microalgae using a dual growth phase strategy, using a nutrient-rich effluent for a growth step followed by a lipid accumulation step on a nutrient-poor effluent. They observed an increase in carbohydrate content during growth phase from 0.15 up to 0.4 g/g. As the stress phase was extended, the amount of carbohydrates later decreased down to 0.3 g/g while microalgae accumulated lipids up to 0.35 g/g.

4.2.4. Isolation and screening of new microalgae

Most of the studies published on the coupling of DF and microalgae cultivation processes focused on photosynthetic strains such as *Chlorella* or *Scenedesmus* species **(Table 3)**. As detailed earlier, these species seem limited in their ability to grow on DFE. Expanding collection screening to other species and especially phyla other than chlorophytes appear necessary. Currently, selecting a microalgal strain for growth on DFE based on metabolic and biochemical traits is rather challenging due to the little data accumulated on the subject and the great diversity in algal phylogeny. However, some species may appear more suitable than others. For example, *Euglena gracilis* and related species could be promising candidates given their ability to consume organic acids and ethanol while presenting photosynthetic properties

and accumulating paramylon, a valuable polysaccharide which structure is similar to starch (Santek et al., 2012). Alternatively, purely heterotrophic species could be more adapted to grow on organic acids. For instance, (Chalima et al., 2019) cultivated the heterotrophic marine microalgae dinoflagellate *C. cohnii* on various single VFA (acetic, propionic and butyric acid) for the production of docosahexaenoic acid (DHA). The strain was able to grow and consume acetic, propionic and butyric acids at 30 g/L, 10 g/L and 15 g/L, respectively. The microalgae performance was further evaluated on a DFE permeate: the strain could remove all organic carbon in only 60 h, which is relatively fast considering the growth rates of green strains. Similarly, when screening for butyrate consuming strains, Lacroux et al, 2022, found that the heterotrophic strain *Polytomella* sp. growed at constant growth rates of 3.8 d^{-1} up to 38 g/L acetate and 2.5 d^{-1} up to 18 g/L butryate. However, the main advantage of using microalgae *i.e.* CO_2 fixation is lost. Secondly, isolation of strains from the environment could be another step in improving the coupling (Lacroux et al., 2022). Ren et al. (2013) could for example isolate a new Scenedesmus strain by screening the lipid content of 88 isolates using a Nile red staining method. They could isolate a lipid accumulating strain able to consume most of the organic compounds except ethanol when cultivated on DFE (Ren et al., 2018). Isolation criteria should not only be based on the type of storage compound but also on the ability of the strain to consume the organic acids present in DFE.

4.2.5. Genetic Modifications/ adaptive evolution

The model strains could be improved through adaptive laboratory evolution experiments (ALE). ALE is a powerful tool enabling the selection of microorganisms with higher fitness to a given environment. Besides, the phenotypic adaptation can be further linked to genotypic changes thanks to omics techniques, allowing to unravel the mechanisms leading to the desired traits (Dragosits and Mattanovich, 2013). This technique has for example been used to generate

various microalgae strains resistant to different environmental stresses (Zhang et al., 2021). In the case of DF coupling, the growth rate of *Auxenochlorella prothotecoïdes* on butyrate improved by nearly 3-fold after three growth cycles on this substrate (Turon et al., 2015a). Although hardly qualifiable as evolution, these results show that adaptation to VFA is possible using the consumption of one of the organic acids as a selection factor.

4.2.6. Microalgae co-cultivation

Finally, microalgae could be co-cultivated with heterotrophic organisms, more adapted to degrade complex organic compounds present in the DFE compared to microalga. The fermentative communities have for example been advantageously used to increase VFA removal rates while reducing the need of sterilization. As an example, Turon et al. (2015b) cultivated *C. sorokiniana* in heterotrophy on a VFA rich effluent (0.74 g/L acetate, 1.25 g/L butyrate) containing the fermentative bacteria. They showed that, in heterotrophy, the algae could outcompete bacteria for acetate due to drastic change from anaerobic to aerobic conditions. Once the aerobic community developed, butyrate was however only consumed by bacteria (Turon et al., 2015a).

Mixotrophic cultivation can further promote microalgae growth, by taking advantage of the synergetic interactions between phototrophic and heterotrophic species. Indeed, under light, the CO_2 produced by heterotrophic respiration can be further photosynthetically fixed by microalgae (Sial et al., 2021). For example, Qi et al., (2018), cultivated *C. sorokiniana* on a synthetic fermentation effluent containing ethanol (0.16 g/L), butanol (0.11 g/L), acetate (0.21 g/L) and butyrate (0.93 g/L) with three different bacterial species (*Exiguobacterium aurantiacum*, *Stenotrophomonas acidaminiphila* and *Chryseobacterium scophthalmus*). The presence of bacteria always improved the final microalgal biomass concentration by around

40% compared to the control. This result was mainly explained by the increased total COD removal rate in presence of bacteria, which simultaneously increased the dissolved CO_2 concentration.

Algal mixotrophy can also be used to design an anaerobic-microaerobic consortium. Indeed, in mixed culture, bacteria and microalgae should compete not only for organic substrates but also for oxygen (Sforza et al., 2018). By controlling the amount of dissolved oxygen produced by microalgae and in absence of external oxygen, simultaneous cultivation of fermentative bacteria and microalgae is possible. This strategy was followed by Ren et al. (2015) who could simultaneously produce hydrogen and lipids from various starch-rich wastewaters using an anaerobic sludge - *Scenedesmus* consortium. The use of the symbiotic consortium always resulted in an improved COD and mineral nutrient removal efficiency by almost 4-fold compared to anaerobic sludge alone. As a result, residual VFA concentration was minimal and total energy conversion efficiency was almost doubled.

5. Microalgal biorefinery

5.1. Microalgae applications

Microalgae have been the focus of a large body of research due to their capacity to produce not only biofuels but also high value-added products (Siddiki et al., 2022). However, when microalgae are cultivated on residual effluents, this biomass cannot be used for human consumption but another type of lower cost product biorefinery can be envisaged.

Microalgae have been successfully cultivated on various DFE, mainly for biolipids production purposes (Sajjadi et al., 2018). The main strains used for such a coupling are *Auxenochlorella*

protothecoïdes, *Chlorella* sp. and *Scenedesmus* sp. either for their high lipid content, their ability to grow in hetero- or mixotrophy on VFA and their overall robustness.

Recent studies on the coupling of DF and microalgae cultivation were focused on the use of synthetic VFA as model substrates or the use of effluent obtained from glucose DF as model effluent. These studies demonstrated the economic potentialities of algae production from DFE (Fei et al., 2015). Since then, microalgae cultivation has been successfully carried out on real waste streams, either from food waste, starch-rich wastewater or lignocellulosic biomass. For example, Ren et al. (2018) compared the performances of the coupled system with three simulated wastewaters (protein, fat, or carbohydrates rich). Overall, starch-rich wastewater was the most appropriate for both H_2 production (134 mg/L substrate) and microalgae cultivation (100% VFA removal, 52.6 mg/L/d lipid productivity). The coupled system improved the global energy conversion efficiency by two-fold compared to DF alone. Similarly, Ren et al. (2019) obtained a 17% increase in energy conversion efficiency (in comparison to DF alone) when applying a two-step process for DF of agricultural waste and further microalgae cultivation on DFE rich in acetate and butyrate, obtaining a co-production of 811 mL H_2/L and 58 mg/L/d of algal lipids. Mu et al. (2020) used duckweed biomass as a DF substrate to produce 170 mL H_2/g substrate. *C. saccharophila* was subsequently cultivated on the acidogenic effluent, effectively removing 70% VFA and all residual nitrogen while producing up to 0.27 g/L lipids. In another study, high starch wastewater was co-digested with poultry manure to produce a maximum of 5.03 mol H_2/kg COD reduced. The liquid effluent was then used to cultivate *C. reinhardtii*, yielding a biomass concentration of up to 1.45 g/L associated with a lipid yield of 0.29 g/L (Radhakrishnan et al., 2021).

Besides biofuel application, microalgae biomass from VFA has been suggested as a source of protein (Patel et al., 2022) or docosahexaenoic acid (DHA) (Chalima et al., 2019). Even though these applications would have higher added value than biolipids, health and safety issues should

first be properly addressed if these products were to be used for human consumption (Vilas-Boas et al., 2021). In addition, carbohydrates obtained from mixotrophic microalgae cultivation can be subsequently employed as feedstock to synthesize more chemicals and energy via DF in a closed loop system as reported by Liu et al. (2013b).

5.2. Microalgae as a substrate for Dark Fermentation

The macromolecular composition of microalgae (carbohydrates up to 65 % DW and protein up to 70% DW depending on the species) along with the lack of lignin, make this biomass a suitable substrate for bioH_2 production (Tyagi, 2017).

In addition, microalgae biomass constitutes a versatile substrate since it can be used as raw biomass, lipid-extracted microalgae biomass as well as residual microalgae biomass after the production of value-added compounds (Nobre et al., 2013) **(Figure 4)**. After lipid extraction, microalgae biomass generates between 60 - 70% of residue (Ghimire et al., 2017), becoming a rich-carbohydrate feedstock. The lipid extraction during that process contributes to the biodegradability of the cellular structure, and therefore facilitates employing this residue as a substrate enhance the accessibility of HPB to intracellular content (Nobre et al., 2013). For instance, *Nannochloropsis* sp. residual biomass after lipids and carotenoids extraction was employed for H_2 production obtaining a H_2 yield 26% higher than the one from raw microalgae biomass (Nobre et al., 2013). Likewise, a high fermentative H_2 production yield (192 mL H_2/g VS) was reported for *Dunaliella* lipid-extracted biomass (Chen et al., 2020).

However, when using microalgae biomass as a potential substrate for DF, diverse limiting factors should be considered.

Firstly, microalgae have a complex layered cell wall structure consisting of an inner and an outer layer, typically formed by polymers such as cellulose, hemicellulose, pectin and starch (Hallenbeck and Benemann, 2002). This structure can hamper bacterial hydrolysis and affect the release of intracellular compounds. To tackle this challenge, different pretreatments have been intensively studied in terms of microalgae disruption and organic matter solubilization for microbial degradation (e.g. thermal, electromagnetic radiation, acid/alkali and enzymatic pretreatments). However, cell disintegration does not necessarily translate into H_2 production and other techniques are necessary for polysaccharide hydrolysis into simple monomers to be available for HPB. For example, the combination of ultrasonication (20 min, 200 W) with enzymatic hydrolysis (α-amylase and glucoamylase) of cyanobacteria *Arthrospira platensis* increased by 47% the fermentative H_2 yield (82.4 mL H_2/g DW) in comparison to ultrasonication as the sole pretreatment (55.9 mL H_2/g DW) (Cheng et al., 2012).

H_2 production yield declines with carbohydrate chain length (Quéméneur et al., 2011). **Table 4** presents the carbohydrate profile that can be obtained from some microalgae species. Similar H_2 yields (1.84-2.2 mol H_2/mol substrate) were reported for monosaccharides such as glucose, arabinose, xylose, and fructose (Masset et al., 2012; Quéméneur et al., 2011; Taguchi et al., 1994) while lower values (1.65-1.67 mol H_2/mol hexose) were obtained for disaccharides such as maltose and sucrose (Quéméneur et al., 2011). When cellulose was used as a substrate, H_2 yield as low as 0.48 mol H_2/mol hexose was reported (Zagrodnik and Seifert, 2020). In contrast, starch fermentation yielded 1.5 mol H_2/mol glucose equivalent when using cultures of the hyperthermophylic bacterium *Thermotoga neapolitana* (Nguyen et al., 2010).

Secondly, the high protein content in microalgae biomass **(Table 4)** causes ammonium release during DF process. Excess of NH_3 can be inhibitory since this unionized form of nitrogen can easily penetrate the microbial cell wall, changing the intracellular pH, increasing the

maintenance energy and finally inhibiting specific enzymes involved in H_2 production inhibiting the HPB activity (Ramos-Suárez and Carreras, 2014). Besides, excess ammonium can lead to an unbalanced C:N ratio.

One alternative to obtain a suitable C:N ratio is to increase the microalgae carbohydrate content via optimization of the environmental conditions during microalgae cultivation (temperature, nutrients starvation, CO_2 concentration) (Brányiková et al., 2011; Izumo et al., 2007; Markou et al., 2012). However, playing on environmental conditions is not always feasible. In this sense, despite the low H_2 potential of proteins (N-rich) in comparison to carbohydrates (C-rich), the co-fermentation of microalgae with other substrates containing a different macromolecular composition can be a strategy that contributes to a balanced carbon to nitrogen (C:N) ratio is essential to optimize H_2 production in a fermentative process (Sun et al., 2018; Xia et al., 2016).

Lastly, sodium inhibition can occur when using marine strains. Despite sodium being an essential trace element for the synthesis and metabolism of anaerobic microorganisms, sodium excess (above 2 mg/L) increases osmotic pressure in the solution, leading to inactivation or death of bacteria (Lee et al., 2012). Some alternatives to address sodium inhibition include the use of salinity-tolerant inoculum (Riffat and Krongthamchat, 2007) or the acclimation of anaerobic microorganisms to gradually higher concentrations of sodium (Lefebvre et al., 2007).

6. **Conclusions**

The effluents derived from organic wastes produced during dark fermentation arise as a potential carbon source for microalgae cultivation that can boost the viability of bioproduct generation. The present review brings to the forefront the efficient multi-product generation (bioH_2, biofuel and bioproducts) from a single waste by integrating different bioprocesses.

The increasing number of research studies in biorefinery approaches during the recent years is indicative of the significant progress and expectations to attain efficient bioproduct generation from low cost carbon sources in the near future. For that purpose, major efforts should be still made to optimize the bioprocesses of dark fermentation and microalgae cultivation and tackle the main challenges associated to their integration.

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CRediT authorship contribution statement

Julien Lacroux: Conceptualization, Writing – original draft; Writing - review & editing. **Mercedes Llamas:** Conceptualization, Writing – original draft, Writing - review & editing. **Kevin Dauptain:** Writing – original draft. **Romina Avila:** Writing – original draft. **Jean-Philippe Steyer:** Conceptualization, Funding acquisition, Supervision. **Robert van Lis:** Conceptualization, Supervision, Funding acquisition. **Eric Trably:** Conceptualization, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this review.

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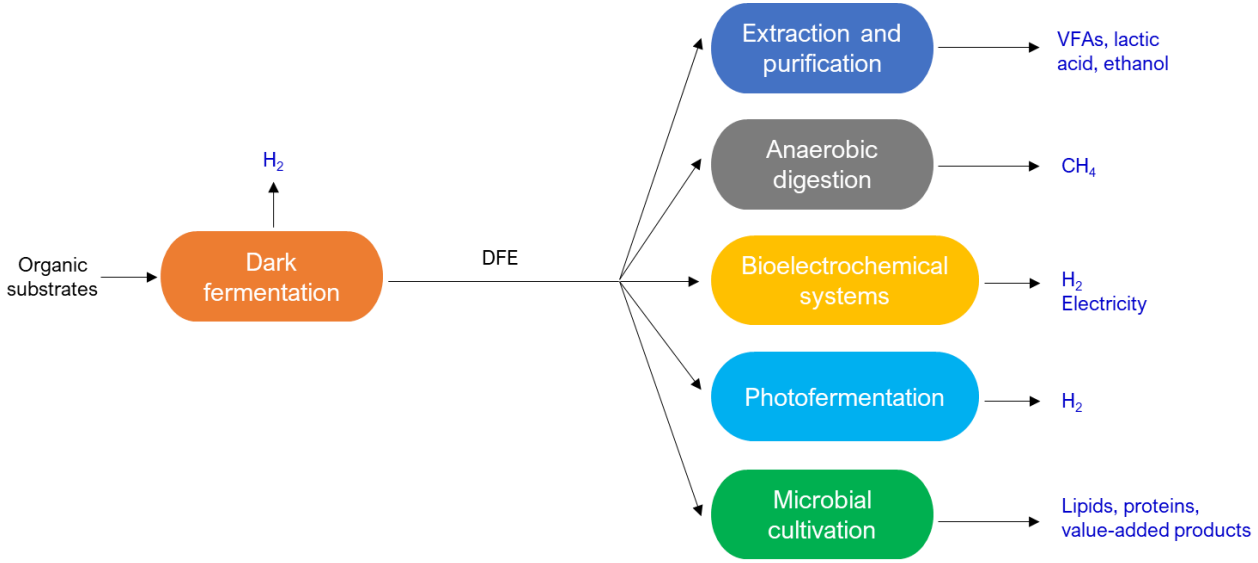

Figure 1. Dark fermentation effluents utilization in coupled technologies under a biorefinery concept.

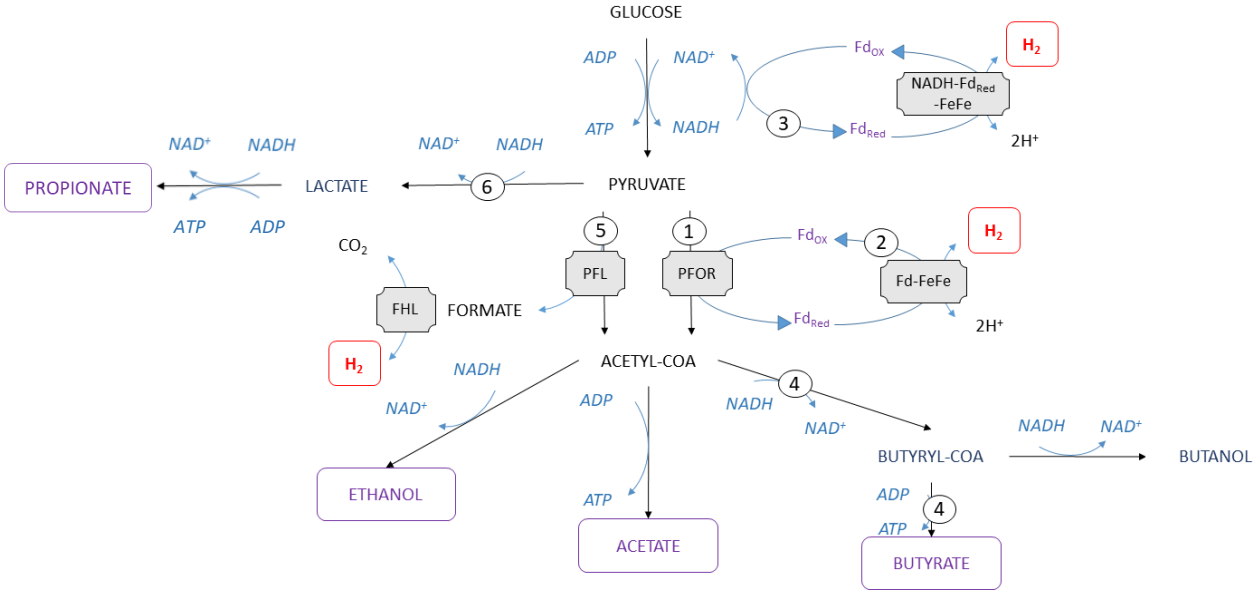

Figure 2. Metabolic pathways in dark fermentation. Pyruvate is the key intermediate of the metabolic pathways. Pyruvate can be converted in acetyl-CoA via the pyruvate ferredoxin oxidoreductase (PFOR) pathway (1), which leads to the production of reduced ferredoxin (Fdred). The oxidation of Fdred into oxidised ferredoxin (Fdox) through Fe-Fe hydrogenases (Fd-FeFe) leads to the production of H_2 (2). Organisms following the PFOR pathway are able to regenerate NAD^+ via NADH by the NADH-ferredoxin oxidoreductase (NADH-Fdred-FeFe) with concomitant H_2 production (3). To regenerate NAD^+, acetyl-CoA can also be converted to butyrate (4). Alternatively, pyruvate can be cleaved into formate and acetyl-CoA via the pyruvate formate lyase (PFL) pathway (5). The formate is subsequently converted into H_2 and CO_2 via the formate hydrogene lyase complex (FHL). Pyruvate can also be converted to lactate via homolactic fermentation (6).

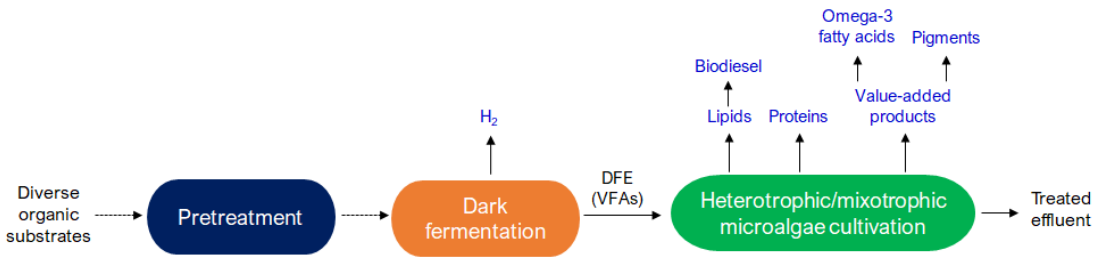

Figure 3. Coupling dark fermentation and microalgae cultivation. Dark fermentation leads to the concomitant production of H_2 and VFAs while treating various organic wastes. These VFAs can serve as low-cost organic substrates for the cultivation of microalgae and production of valuable compounds

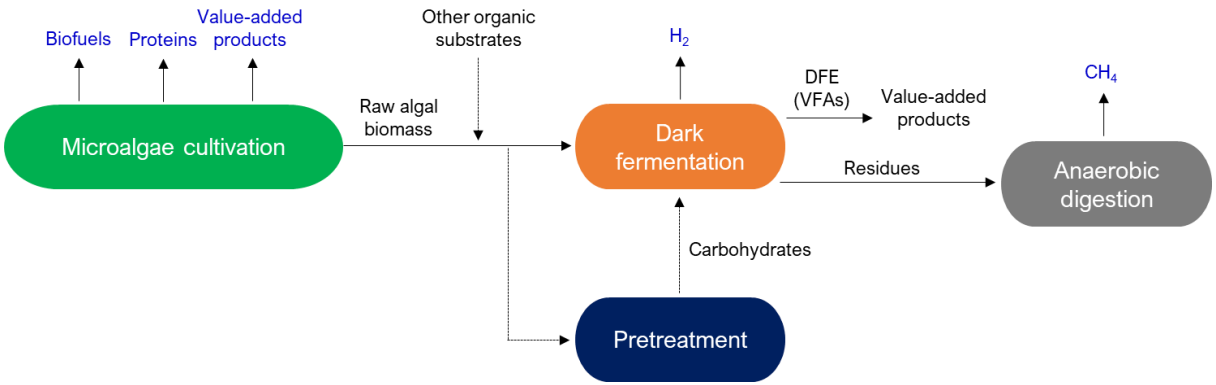

Figure 4. Microalgae as DF substrate.

Table 1. Equations of the main metabolic pathways occurring during dark fermentation. Equations 1 and 2 correspond to the theoretical pathways followed by HPB using the PFOR pathway while equation 3 correspond to the theoretical pathway followed by HPB using the PFL pathway. Equation 4 correspond to the mixed culture assumption equation. Equation 5 is a H_2 consuming pathway followed by propionic acid bacteria. Equation 6 and 7 are respectively the homo- and hetero-lactic fermentation pathways followed by LAB. In some cases, LAB can produce H_2 through equation 8.*Glu: Glucose; AcA: Acetic acid; ProA: Propionic acid; ButA: Butyric acid; Eth: Ethanol; LA: Lactic acid*

Equations	**Nº**
$C_6H_{12}O_6\ (Glu) + 2\ H_2O \rightarrow\ 2CH_3COOH\ (AcA) + 2\ CO_2 + 4\ H_2$	1
$C_6H_{12}O_6\ (Glu) \rightarrow\ CH_3CH_2CH_2COOH(ButA) + 2\ CO_2 + 2\ H_2$	2
$C_6H_{12}O_6\ (Glu) + 2\ H_2O\ \rightarrow CH_3COOH(AcA) +\ CH_3CH_2OH(Eth) +\ 2\ CO_2 + 2\ H_2$	3
$4\ C_6H_{12}O_6\ (Glu) + 2\ H_2O\ \rightarrow 2\ CH_3COOH(AcA) +\ 3\ CH_3CH_2CH_2COOH(ButA) + 8\ CO_2 + 10\ H_2$	4
$C_6H_{12}O_6(Glu) + 2\ H_2\ \rightarrow 2\ CH_3CH_2COOH(ProA) +\ 2\ H_2O$	5
$C_6H_{12}O_6\ (Glu) \rightarrow\ 2\ CH_3CH(OH)COOH(LA)$ $C_6H_{12}O_6\ (Glu) \rightarrow\ CH_3CH(OH)COOH\ \ (LA) +\ CH_3CH_2OH(Eth) +\ CO_2$ $4\ CH_3CH(OH)COOH\ (LA) + 2\ CH_3COOH\ (Ac) \rightarrow\ 3\ CH_3CH_2CH_2COOH(ButA) +\ 4\ CO_2 + 2\ H_2$	6 7 8

Table 2. Fermentation profiles of various substrates. The table provides the H_2 yields (in mmol H_2/molhexose or mmol H_2/gCOD) of simple sugars or complex substrates as well as the main metabolites (g COD/L) obtained at the end of the fermentation.

Fermentation parameters			H_2 yields		Metabolites (g COD/L)					References
Substrate	**Substrate concentration (g COD/L)**	**Inoculum and pretreatment**	**mmol H_2/ mmol hexose**	**mmol H_2/ g COD**	**Acetate**	**Propionate**	**Butyrate**	**Lactate**	**Ethanol**	
Starch	20.0	AS (HT)	0.9	4.7	1.7	1.2	12.7	0.5	-	(Arooj et al., 2008)
Raw cassava starch	10.0	AS (U)	1.7	-	0.35	-	2.7	-	0.33	(Wang et al., 2017)
Lactose	12.3	AS (HT)	2.1	5.4	1.1	-	4.7	0.4	-	(Palomo-Briones et al., 2018)
Glucose	17.1	AS (HT)	2.6	15.7	4.4	0.15	5.5	-	0.13	(Hafez et al., 2010)
Glucose	5.5	WWTPS (U)	-	5.1	0.9	0.3	0.4	-	3.1	(Song et al., 2011)
Cellulose	2.1	AS (HT)	1.1	5.7	0.2	-	0.2	0.1	1.0	Santos-Lopes et al (2020)
Molasses	5.0	WWTPS (AE)	-	8.5	0.75	0.2	0.2	-	1.3	(Ren et al., 2018)
Molasses	8.0	WWTPS (AE)	-	10.7	0.6	0.2	0.9	-	3.8	(Wang et al., 2014)
Food waste	20.0	AS (U)		3.02	5.2	3.9	6.93	-	-	(Micolucci et al., 2020)
Food waste	76.4	AS (U)	-	4.1	6.1	1.94	28.3	-	22.8	(Greses et al., 2022)
Food waste	30.1	AS (HT)	-	0.02	0.2	0.6	0.9	4.9	47.4	(Santiago et al., 2019)
Food waste	12.0	AS (HT)	-	4.3	6.2	0.8	7.5	6.7	3.1	(Moreno-Andrade et al., 2015)

AS: Anaerobic sludge; WWTPS: Wastewater treatment plant sludge; DSS: Domestic sewage sediments; HT: Heat-chock pretreatment; U: untreated; AE: Aerobic pretreatment.

Table 3. Growth characteristics of pure microalgae strains on single VFA (acetate or butyrate).

Strain	Substrate	Substrate concentration (g/L)	Metabolism	Growth rate (d^{-1})	Biomass productivity (g/L/d)	Biomass yield (gX / gS)	Reference
Acutodesmus obliquus	Acetate	0.6	H	0.4	-	-	(Combres et al., 1994)
	Acetate	0.6	M	1.2	-	-	(Combres et al., 1994)
	Acetate	1.25	M	-	0.46	1.06	(Lacroux et al., 2020)
	Butyrate	0.9	M	-	0.11	3.72	(Lacroux et al., 2020)
Auxenochlorella protothecoides	Acetate	0.25 - 2.5	H	2.05	-	0.3	(Turon et al., 2015a)
	Acetate	20.5	M	-	0.54	0.08	(Heredia-Arroyo et al., 2011)
	Acetate	1.25	M	-	0.38	1.46	(Lacroux et al., 2020)
	Butyrate	0.18 - 0.45	H	0.22	-	0.53	(Turon et al., 2015a)
	Butyrate	0.9	M	-	0.1	1.07	(Lacroux et al., 2020)
Crypthecodinium cohnii	Acetate	3.5**	H	-	2.1	0.72	(Chalima et al., 2019)
	Butyrate	2.4**	H	-	2.8	0.29	(Chalima et al., 2019)
Chlamydomonas reinhardtii	Acetate	1.25	M	-	0.56	1.16	(Lacroux et al., 2020)
	Acetate	1	H	0.84	-	0.52	(Boyle and John, 2009)
	Butyrate	0.9	M	-	0.1	3.28	(Lacroux et al., 2020)
Chlorella sorokiniana	Acetate	0.25 - 2.5	H	2.23	-	0.336	(Turon et al., 2015a)
	Acetate	3	H	4.32	-	0.4	(Abiusi et al., 2020)
	Acetate	0.75	M	4.14	-	0.64	(Turon et al., 2015c)
	Acetate	2	M	-	0.789	0.75	(Wang et al., 2016)
	Butyrate	0.18	H	0.16	-	0.62	(Turon et al., 2015a)
	Butyrate	0.55	M	-	0.14	0.952	(Turon et al., 2015c)
	Butyrate	0.9	M	-	0.23	2.11	(Lacroux et al., 2020)
Chlorella vulgaris	Acetate	13.7	M	-	0.3	0.29	(Yeh et al., 2012)
	Acetate	1	M	-	0.4	0.8	(Liu et al., 2013)
	Butyrate	1	M	-	0.29	2.67	(Liu et al., 2013)

*H: heterotrophic metabolism; M; mixotrophic metabolism; *Substrate concentration in the fed-batch feed*

Table 4. Macromolecular composition and carbohydrate profile of some microalgae species. Polysaccharides and monosaccharides content in algal biomass are presented for selected microalgae and cyanobacteria due to their diverse effect on H_2 production.

Microalgae/Cyanobacteria species	Proteins (%)	Lipids (%)	Carbohydrates (%)	Major carbohydrates	Other carbohydrates	Cultivation conditions	References
Chlamydomonas reinhardtii	9.2	-	59.7	43.6% starch 44.7% glucose	2.7% galactose 1.9% arabinose 1.4% mannose 0.9% rhamnose 0.4% fucose	Operation mode: fed-batch (1 M acetic acid). Cultivation time: 4 d.	(Choi et al., 2010)
Scenedesmus obliquus	-	19	51.8	78% glucose	22% xylose + galactose	Operation mode: batch, nitrogen starvation. Cultivation time: 3 d.	(Ho et al., 2012)
Dunaliella tertiolecta	20	15	12.2	85.3% glucose	5.5% rhamnose 4.5% mannose 2% ribose 1.1% galactose 1% xylose 0.65% arabinose	Operation mode: batch, growth media.	(Brown, 1991)
Chlorella vulgaris	51-58	14-22	12-17	42-50% rhamnose 22–30% galactose	5–17% xylose 2–10% mannose 4–9% arabinose 0–4% glucose	Not specified.	(Pieper et al., 2012; Wang and Yin, 2018)
lsochrysis galbana	29	23	12.9	76.5% glucose	19% galactose 5.7% arabinose 3.6% mannose 2.3% xylose 2% ribose	Operation mode: batch, growth media.	(Brown, 1991)
Anabaena variabilis (cyanobacteria)	-	-	46.2	27.6% reducing sugar	11.6% glycogen 2.5% starch 2.1% cellulose 1.2% hemicellulose	Operation mode: BG-11 medium without N source. Cultivation time: 24 d.	(Deb et al., 2019)
Microcystis aeruginosa	-	-	41.1	23.4% reducing sugar	9.7% glycogen 3.1% starch 2.6% cellulose 0.7% hemicellulose	Operation mode: BG-11 medium. Cultivation time: 24 d.	(Deb et al., 2019)
Spirulina platensis (cyanobacteria)	55	-	13.6	54.4% glucose 22.3% rhamnose	9.3% mannose 7% xylose 2.6% galactose	Not specified.	(Shekharam et al., 1987; Soto-Sierra et al., 2018)
Nannochloropsis oculata	35	18	7.8	68.2% glucose	4–8% of rhamnose, mannose, ribose, xylose, fucose, and galactose	Not specified.	(Brown, 1991)