Bioprocesses Coupling for Biohydrogen Production: Applications and Challenges

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ABSTRACT

The decarbonisation of industry based on the sustainable use of resources is one of the main objectives of our current society. To achieve this, rich-carbohydrate residual streams constitute a cost-effective feedstock from which hydrogen can be produced via dark fermentation (DF). In recent years, bench-scale testing has delivered encouraging results. Nonetheless, the low hydrogen productivity obtained still prevents the upscaling of this technology. A possible solution to overcome this technical barrier might be to couple DF with other available bioprocesses. The resulting coupling would enhance substrate exploitation and increase hydrogen productivity. The biohydrogen produced could be used either as an energetic vector or as a platform molecule for added-value compound production. This chapter aims to comprehensively review the existing bioprocesses under investigation coupled with DF as a pivotal technology for biohydrogen production. More specifically, technologies such as microbial electrolysis cells, microalgae cultivation, biomethanation, photofermentation, and lactate production are evaluated. Aspects such as the optimal operational conditions that favour the coupling in each case and the hydrogen yields obtained, are reported. Furthermore, the advantages and disadvantages of the process couplings are also discussed. Finally, current challenges and future perspectives that each hydrogen production platform entails are pointed out to set the way forward in the coming years.

Keywords: Bioeconomy, Biohydrogen, Bioproducts, Coupling processes, Dark Fermentation

Biological hydrogen production through the dark fermentation (DF) process is considered the most promising and viable method among other bioprocesses (*i.e.*, biophotolysis and photofermentation [1–3]. DF is a biological process where biomass can be anaerobically converted into hydrogen-rich biogas and a mixture of fermentative metabolites [4]. Nonetheless, the low hydrogen yield obtained due to a thermodynamic limit of 4 mol H_2 per mol of glucose is the main disadvantage of this technology [5–7]. The metabolites produced mainly comprise volatile fatty acids (VFAs) such as acetate and butyrate, propionate, and other acids such as lactate and ethanol. However, through DF, only 20-25% of the chemical oxygen demand (COD) of the initial organic substrate is converted into bio-H_2, while the remaining 75-80% is obtained in the form of the abovementioned fermentative metabolites [8]. For this reason, an integrated scheme treating the DF effluent with secondary processes is necessary to maximize COD recovery and ensure the economic viability of the process.

DARK FERMENTATION – MICROBIAL ELECTROLYSIS CELLS

One of the most attractive options for the further use of VFAs is microbial electrochemical technologies (METs). [9]. These technologies are based on the ability of the so-called electroactive bacteria (EABs) to perform extracellular electron transfer (EET), which is a type of microbial respiration where electrons are transported through the cell wall to solid external electron donors or acceptors (*e.g.,* metals, electrodes) for energy metabolism [10, 11]. METs consist of a circuit between an anode and a cathode placed in one or two separate compartments, where redox reactions are bio-catalyzed in one or both electrodes. METs can be classified into two major categories according to the spontaneity of the reaction: i) Microbial Fuel Cell (MFC), where the reactions take place spontaneously, and ii) Microbial Electrolysis cell (MEC), where the reaction is not spontaneous, and energy input is required. The extra voltage is achieved by either setting the anode potential with a potentiostat and a reference electrode (three-electrode set-up) or by adding voltage with a Direct Current (DC) power supply [12]. Both MFC and MEC technologies can be potentially used for treating DF effluents. While electrons provided by the oxidation of organic matter at the anode produce electricity in MFCs, hydrogen is produced in MECs at the cathode [3]. Overall, MECs show higher performance efficiencies [7]. Even though energy input is required for hydrogen formation at the cathode, it is minimal (0.2-0.8 V), especially when compared with traditional abiotic water electrolysis (1.23-1.8 V) [7, 13, 14]. Usually, MECs are designed as a two-chamber system (Figure 1). In the anodic chamber, EABs defined as exoelectrogens [15] or anode-respiring microorganisms, develop a biofilm converting organic matter into protons and electrons, the latter cross the electric circuit from the anode to the cathode where protons are reduced into hydrogen. Other than avoiding short-circuiting between the electrodes, the separation between anodic and cathodic compartments, usually with an ion exchange membrane (IEM), keeps the purity of the hydrogen produced at the cathode.

Moreover, cathode colonization by electrotrophs is prevented, as well as hydrogen consumption by hydrogenotrophs such as homoacetogens or methanogens. The main drawbacks when using IEMs are internal resistance increases, substrate crossover from anode

to cathode, biofouling, undesirable ion crossing, and pH splitting [16–18]. In this respect, pH is maybe the main disadvantage for bioanodes as they work more efficiently around neutrality [17]. Indeed, most known EABs are completely inhibited at pH below 6.

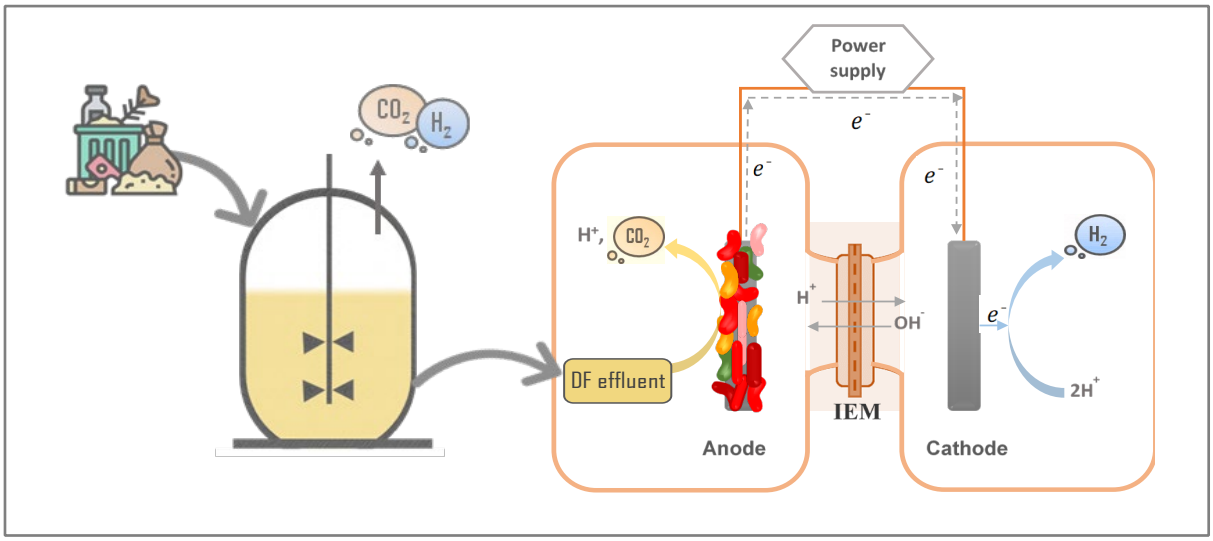

Figure 1. Integrated DF and MEC process. During DF, glucose follows the overall reaction: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$. Acetate produced can be further oxidized at the anode: $CH_3COO^- + 2H_2O \rightarrow 2CO_2 + 7H^+ + 8e^-$, and electrons are delivered at the cathode to produce H_2: following: $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$ With the integrated anode-cathode reactions: $CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$ 4 mol H_2 are obtained per mol of acetate.

Two important parameters to evaluate MECs performances are the Coulomb efficiency (CE) and the current density (CD) [13] (see [19] for calculation). The CE represents to which extent the oxidized substrate is transformed into current (number of electrons delivered to the anode). At the same time, CD (A/m²) indicates the number of electrons delivered per unit of time to the electrode, indicating how fast the substrate is oxidized. Cathodic hydrogen recovery is also routinely reported on MEC studies, indicating the ratio between the amount of hydrogen recovered and the theoretical amount based on the current measured. Ideally, this recovery should approach 100 % when there is no hydrogen recycling, and the MEC is airtight enough to avoid losses. High CE (up to 90 %) could be achieved when a true EAB community predominates in the system. Otherwise, substrate consumption diversion could occur in non-current generating reactions by non-EAB. On the other hand, CE above 100% could indicate MEC dysfunction (*e.g.*, hydrogen recycling).

A wide spectrum of substrates has been used as feedstock for MECs, from simple to complex industrial waste [20]. Even if complex substrates could be directly applied to MECs, performances are far behind those achieved with simpler ones due to the restricted substrate spectrum of EABs, which needs syntrophic partners to completely oxidize the most complex ones [7, 21]. For example, when it comes to VFAs, the model EAB *Geobacter sulfurreducens*, largely found in MECs, can only oxidize acetate. Other EABs, such as *Geobacter metallireducens* have a wider spectrum of substrates. Nevertheless, acetate remains the preferred organic acid as electron donor for MECs [20]. The need for a process like DF for obtaining a VFA-rich feedstock for MECs when treating complex substrates seems evident,

making it a current research topic [3]. Some explicit DF+MEC coupling proposals are shown in Table 1. Certainly, DF well complements MEC as it efficiently breaks down large and complex organic compounds into low-molecular organic acids (*i.e.*, VFAs) that can be used by exoelectrogens [6]. This coupling greatly boosts hydrogen yields with a theoretical output of 12 mol H_2 per mol of glucose [7]. One of the first studies dealing with the coupling was carried out by Lu *et al.,* in 2009 [14], reporting an overall hydrogen recovery of 96% of the maximum theoretical yield (0.125 gH_2/gCOD), with a buffered DF effluent.

The importance of acetate for the maintenance of an efficient anodic community has already been pointed out by Moreno *et al.,* who worked with cheese whey in 2015 [22]. They diluted the DF effluent to reduce the effects of low pH on MEC. However, this resulted in a low CE and the need to add salts (K^+, Cl^-, PO_4^{3-}) and acetate to achieve an optimal acetate/lactate ratio as previously determined with a synthetic medium. They also observed high cathode methane production, probably due to H_2 reconsumption. This was confirmed by the occurrence of *CE* above 100%. Even though the DF-MEC combination can lead to lower energy consumption, a study has shown that the integration of MFC technology is possible as an additional technology to cover the energy demand for MEC. [23]. With respect to this combination, the authors reported an overall hydrogen yield increase of 41% from cellulose. They also observed hydrogen reconsumption when working with a single chamber, as evidenced by an increasing CE of over 175 % and zero hydrogen recovery at the end of the assays.

It has also been stated that the origin of the inoculum plays an important role in MEC performance [9]. However, the wastewater/nutrient influx is also an important issue, because its composition shapes the microbial structure by favouring or disadvantaging the electroactive community. This key feature was outrighted in a recent study where different effluent composition profiles from different substrates after undergoing DF, despite the same operational conditions [24]. These different profiles impacted MEC performances, with *CE* decreasing from 33 to 76 %. It is important to mention that anodic enrichments were carried out under the same conditions, *i.e.*, the same type of inoculum, synthetic medium, and operational conditions.

MECs are usually operated under mesophilic temperature conditions and at neutral pH. Khongkliang et *al.* (2017) [6], demonstrated that MEC operation under thermophilic conditions is also possible. In their study, DF and MEC were fully integrated and operated in continuous mode (up-flow) under thermophilic conditions to treat a complex substrate (cassava starch processing wastewater). DF effluent was directly fed to MEC without pH amendment (pH 6). Interestingly, primary MEC enrichment was done at 55°C and pH 6.5, which certainly favored the establishment of a thermophilic community and the acclimation to mildly acidic pH conditions. Concerning the microbial community composition found in the MEC, several specific representatives reported as thermophilic were observed with predomination of *Brevibacillus* sp., *Caloranaerobacter* sp., and *Geobacillus* sp. species that were very different from those “classically” found in MEC operated under mesophilic conditions.

Table 1. Examples of studies coupling DF+MEC for H_2 production

DF type (substrate)	DF conditions	Major DF metabolites/MEC influent	MEC type/ operation	Anode/ cathode	Added/applied voltage (V)	MEC conditions	CE (%)	H_{2cat} recovery (%)	Total H_2 recovery (%)	Ref
Molasses wastewater	Ethanol-type CSRT continuous ORL 22.8 kg COD/m^3/d	EtOH, C2, C3, C4, C5, residual sugars	Single chamber Batch mode	Grafite brush/Carbon cloth with Pt	0.6 (DC power supply)	Buffered effluent pH 6.7-7.0 25 °C	87	83	96 %	[14]
Cheese whey	Batch/ 35°C	Lactate, C2, C4, C3	Membrane-free polycarbonate plates Continuous (10 h, HRT)	Carbon felt /gas diffusion with Ni particles	1 (DC power supply)	Acetate and salts amended	80	N.M.	N.M.**	[22]
Cellulose	Continuous/60°C	C4, C2, C5, EtOH, C3	Batch membrane-free	Carbon brush/ Carbon cloth with Pt content	0.44 V (MFC supplying)	Buffered pH 7 25 °C	58-175	8.7-92	N.M.**	[23]
FJW, **VB2,** **CW,** **FPW,** **SW,** **PW**	Batch	**FJW:** C4, C2 **VB2:** C4, 1,3-PDO, C2, C3, Succ; C2; **CW:** EtOH, C4, C2, Succ; **FPW:** EtOH, C2, C4; **SW:** C2, C4, EtOH, C3, Succ; **PW:** C4, Succ, C2, EtOH, C3	Double chamber/AEM	Carbon felt/ Pt-Ir mesh	0.44 V (anode potential vs SHE)	37 °C, pH adjusted (7)	76 75 75 80 38 33	101 65 62 53 61 53	115.02* 106.34* 59.84* 53.93* 28.33* 18.36*	[24]
Cassava starch (manioc)	Continuous/UASB 55°C 25.2 kg COD/m^3/d	C4, C2, C3, C5	Continuous up-flow membrane-free	Graphite felt/Cu wire	0.6 V (DC power supply°	55°C pH 6	N.M	N.M	33 %*	[6]

CE = coulombic efficiency; H_{2cat}= cathodic H_2 recovery; *calculated with available data on the paper based on a molar volume (Vm=22.414) at standard temperature and pressure conditions; N.M. = not mentioned; ** not enough data to calculate; **CW =** cheese wey**; FPW =** fruit processing wastewater; **SW =** sugar production wastewater; **FJW =** industrial fruit juice production wastewater; **VB2 =** concentrated vinasse residue**; PW = paper mill wastewater**

Coupling DF and MEC instead of a single process to maximize hydrogen production is primarily advantageous. Mainly because this enables more efficient regulation of the individual processes. [18]. However, further efforts to improve overall hydrogen yields are required to scale up this two-process system, for example, by producing DF effluent with a profile composition favoring EABs. Moreover, studying the microbial community composition and the role of microbial interactions in electroactive biofilms are key aspects to better understand and improve MEC performances.

DARK FERMENTATION – MICROALGAE CULTIVATION

Microalgae cultivation coupled with DF is a promising technology to enhance substrate conversion to hydrogen and other high value-added compounds. Microalgae are unicellular eukaryotic microorganisms ubiquitously present in nature thanks to their metabolic versatility, exhibiting autotrophic, heterotrophic, and mixotrophic metabolisms. For simplicity, in this chapter, the term *microalgae* includes the prokaryotic cyanobacteria (green-blue algae) that share the same bioenergetic metabolism and biotechnological applications. Microalgae have gained attention because of their ability to convert carbon dioxide and organic compounds into high-added value molecules such as lipids, proteins, carbohydrates, and various secondary metabolites, among which are carotenoids (astaxanthin, β-Carotene), xanthophylls (lutein, zeaxanthin) and phycocyanin [25]. So far, the economic and environmental sustainability of large-scale microalgae farming has been hampered by the high energy requirements, especially in the harvesting and extraction phases, and the need for low-cost nutrient sources, especially nitrogen and phosphorus [26–29].

Coupling DF and microalgae cultivation (Figure 2) can improve the sustainability of both processes in a biorefinery approach, which is envisaged for the transition to bioeconomy [30, 31]. DF effluents as cultivation media for microalgae provide VFAs as an inexpensive source of organic carbon, yielding higher biomass and added-value compounds concentrations and productivities concerning autotrophy [32]. This, in turn, can improve the efficiency of the harvesting and extraction steps. Moreover, DF effluents can contain enough N and P to sustain microalgae growth in ammonium and orthophosphate due to the mineralization occurring during DF [33]. As shown in Figure 2, microalgae could also upgrade the biogas by fixing the carbon dioxide that it contains and providing a higher hydrogen content in the biogas (up to 85 % v/v H2) [34]. This process has been extensively studied with methane-rich biogas produced by AD, obtaining a 54-99% v/v CO_2 removal and 65-97% v/v CH_4 recovery [35], while studies on the hydrogen-rich biogas generated via DF are moving their first steps, with a promising 85% v/v CO_2 removal and fixation rate of 95 mL CO_2/L/h [34].

Unlike the other processes coupled to DF, microalgae cultivation does not convert the remaining COD directly into hydrogen. The result is a biomass rich in valuable compounds, including up to 71.1% lipids, 63% proteins and 80% carbohydrates (DM basis) in a percentage that depends on the strain and culture conditions (Figure 2) [36–39]. Carbohydrate-rich biomass might be recirculated as DF feedstock, thus enhancing the hydrogen yield of the whole process. For instance, an experimental yield of 0.93 mol H_2/mol reduced sugars was

obtained from a recirculated hydrolyzed *C. vulgaris* [34, 40]. Also, the spent biomass after high-added value compounds extraction constitutes a suitable substrate for DF. For instance, the fermentation of *Dunaliella salina* lipid-extracted biomass resulted in a high biohydrogen yield of 192 mLH_2/gVS [41].

Multiple interacting factors affect the coupling of DF with microalgae cultivation, from both the abiotic (effluent composition, pH, C:N:P ratio, illumination conditions, feeding mode, and process configurations) and biotic (bacterial and microalgal strains and their interactions) environment [42]. In particular, for optimal coupling, DF should be directed towards the acetate hydrogen production pathway due to i) the higher theoretical hydrogen yield and ii) the high acetate assimilability by many microalgae species, which seems to boost lipids production [43, 44].

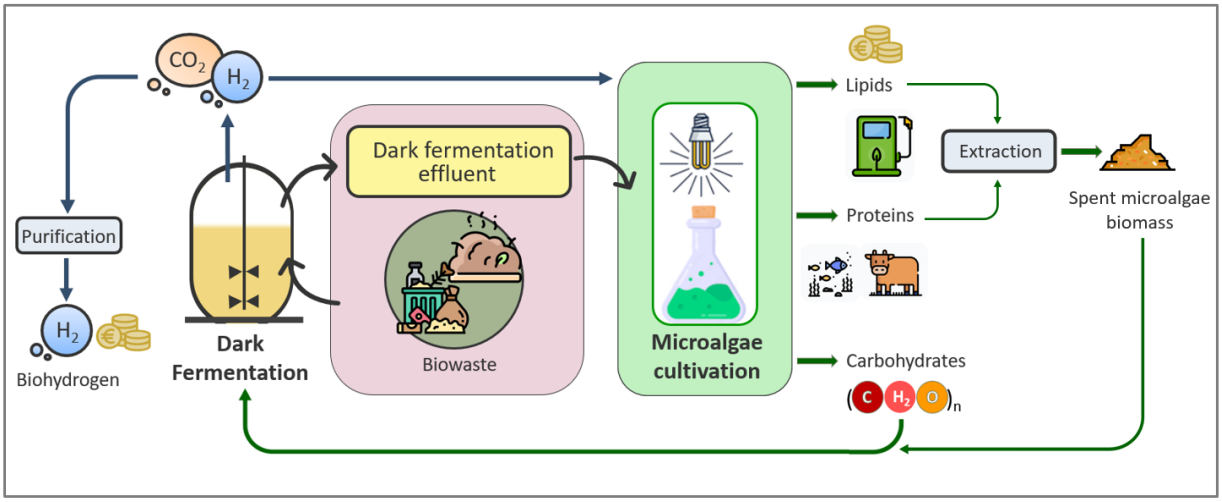

Figure 2. Coupling of DF and microalgae cultivation: conceptual scheme outlining the main processes and outputs. DF generates biogas and an effluent which can be supplied as substrates for microalgae growth and storage of lipids, proteins, and carbohydrates. Carbohydrates can be eventually recirculated as DF feedstock.

Conversely, butyrate uptake is a major bottleneck in coupling the two processes, and the underlying metabolic mechanisms, mainly studied for the model microalga *Chlamydomonas reinhardtii*, are only partially understood [44, 45]. A significant breakthrough has been reported by [46], who proposed a butyrate metabolic network for the non-photosynthetic microalga *Polytomella* sp. through a proteomics approach. After entering the cell through membrane-bound transport proteins, butyrate would be activated to butyryl-CoA before entering the β-oxidation pathway in the peroxisomes [46]. Unlike acetate, butyrate lowered the accumulation of storage products with simultaneous induction of fatty acids synthesis. These fatty acids probably served for peroxisomes reorganization and the production of enzymatic cofactors involved in butyrate assimilation. Analysis of the butyrate-related metabolic network of *Polytomella sp.* identified the issues to be tackled to understand the poor butyrate assimilation in green microalgae, potentially serving as a metabolic reference [46]. DF effluents are principally composed of a mixture of acetate and butyrate. In such cases, diauxic growth was observed, and butyrate consumption started only after acetate depletion [47–49]. Acetate was consumed after 1.5-3 days, sustaining a microalgal growth rate of 3.4-0.81 d^{-1} depending on the strain, while butyrate was consumed after 6-10 days and resulted in a lower growth rate of 1.28-0.28 d^{-1} [36, 47]. *Polytomella* sp. stood out as the most rapidly

growing strain on acetate and butyrate, with a growth rate of 4.1 d^{-1} and 2.5 d^{-1}, respectively [36]. Whereas acetate concentrations as high as 30 g/L were used to support the growth of *C. sorokiniana* and *A. prototechoides* at pH 6.8 [49], butyrate was reported to inhibit their growth at concentrations as low as 0.1 and 0.5 g/L, respectively, at pH 6.5 [47]. The mechanism underlying butyrate inhibition has been recently clarified and is detailed elsewhere [50]. It is important to highlight that microalgae growth is strongly affected by the undissociated form of the organic acids (ROOH), which rises as the pH lowers (pKa of VFAs ~ 4.8-4.9). Therefore, ROOH concentration can be maintained under the inhibitory threshold by controlling the pH at alkaline values. The inhibitory threshold is species-specific, with maximum ROOH concentrations ranging from 71 to 207 mg/L for acetic acid and 13-25 mg/L for butyric acid for the five most commonly cultivated strains [50].

In microalgae cultivation on DF effluents, pH determines the chemical form of VFAs, the VFA's chemical species, and the total ammonium nitrogen content (TAN, *i.e.*, free ammonia and ammonium). Ammonium is the optimal nitrogen source for microalgae growth, while the other forms of nitrogen need to be reduced to ammonium ions [51]. Additionally, despite ammonium being the preferred form for microalgae utilization, high TAN levels can cause inhibition (50-260 mg TAN/L), varying remarkably depending on the microalgae strain and cultivation conditions [52]. Although some studies reported that the toxic effect of TAN is mainly attributable to ammonium [53], other researchers proposed ammonia as the major inhibitor of microalgal growth [54, 55]. Since ammonia concentration rises with the pH, this parameter should be monitored depending on the DF effluent composition concerning VFAs and TAN content.

The C:N:P ratio of the DF effluent depends on the substrates used in DF and strongly influences the microalgae growth and their macromolecular composition. Considering the Redfield ratio 106:16:6 as a reference for average algal biomass, nutrient-replete conditions support biomass growth. At the same time, nitrogen starvation seems to trigger storage product accumulation, namely lipids or carbohydrates, depending on the strain [48, 56, 57]. Therefore, optimizing a two-phase cultivation strategy with a nutrient-replete growth phase followed by a nitrogen starvation storage phase can improve the overall conversion of COD to storage products. Fed-batch cultivation mode can be applied in the first stage, thus achieving a high cell density that facilitates the harvesting and extraction [58, 59].

Illumination is a fundamental factor for microorganisms supporting an autotrophic metabolism. Mixotrophy can increase the titer of microalgae cultures by a yield equal to or higher than the sum of the yields obtained under autotrophy and heterotrophy [60, 61]. Under mixotrophy, autotrophic and heterotrophic metabolisms can boost each other. The organic substrate metabolization releases carbon dioxide, which is directly used in autotrophic metabolism. The oxygen produced during photosynthesis is available in turn for cell respiration. In continuous processes, respiratory oxygen consumption and phototrophic oxygen production can be counterbalanced by adjusting the rate at which the organic carbon source is provided to the microalgae culture [62]. Moreover, mixotrophy can alleviate butyrate inhibition thanks to the autotrophic generation of part of the biomass which can consume it [63]. Another advantage of mixotrophy is the enhancement of some cellular

processes (*e.g.*, lipids and carbohydrates storage) and metabolite production associated with light (*e.g.*, astaxanthin, β-carotene) [43, 64–66].

Regarding biotic factors, microalgae-bacteria interactions play a fundamental role in the coupling, especially with a perspective of full-scale application, where effluent sterilization would be economically unsustainable. The process can be positively affected by synergistic interactions, such as the gas exchange between microalgae (O_2) and aerobic bacteria (CO_2), or negatively impacted by substrate competition, namely for acetate consumption. *C. sorokiniana* was shown to outcompete bacteria for acetate when heterotrophically grown on a real DF effluent containing acetate and butyrate [63]. When the aerobic bacterial strains become dominant in the originally anaerobic DF consortium, they can consume butyrate, but this ability depends on the microbial composition of the consortium [63, 67, 68]. When evaluating a microalgae-bacteria consortium, the main obstacle is to differentiate the VFAs uptake by microalgae and bacteria, respectively. Microalgae growth on labeled carbon ($^{13}C/^{14}C$) followed by flux cytometry and cell sorting could be a feasible approach to measure carbon incorporation [69]. Moreover, the carbon dioxide generated by VFAs degradation and not fixed by the biomass should be quantified. Finally, the selection of microalgae strains should focus on tipping the scales in microalgae's favor considering the consortium. This can be done by selecting or adapting strains able to consume butyrate or using microalgae strains that prey on bacteria such as *Ochromonas danica* [70]. A significant step forward in this direction has been made with the fast-butyrate consuming strain *Polytomella* sp., which yielded 0.65 g carbohydrates/g biomass [36]. However, lipid-accumulating microalgae with the same ability to consume butyrate remain to be found. This can be obtained by further exploring the biodiversity or by improving the already known strains (*i.e.*, through genetic modification or adaptive laboratory evolution (ALE)) [71].

To sum up, acetate and hydrogen should be properly targeted in the first step of DF. In contrast, a mixotrophic two-phase microalgae cultivation at controlled pH can favor a high biomass productivity and product storage in the second step. The main bottleneck is the metabolization of longer chain VFAs, which several strategies can overcome, eventually combined: i) exploring the microalgal biodiversity to find butyrate-consuming strains producing the desired storage product, ii) performing ALE on the strains already known and iii) exploiting the synergistic interactions of microalgae-bacteria consortia.

DARK FERMENTATION - ANAEROBIC DIGESTION BASED PROCESSES

Anaerobic Digestion (AD) is a mature and well-established process that has been applied at an industrial scale for decades [72]. Nonetheless, the hydrolysis step still hinders the total exploitation of the substrate, especially when dealing with complex chemical structures. For this reason, hydrolysis enhancement, together with hydrogen and metabolite productions achieved in DF, has recently gained a lot of interest in the so-called two-stage AD concept [73, 74]. This configuration allows the energetic potential optimization of the organic matter employed as feedstock to increase the overall methane yield while producing hydrogen simultaneously in the first step. More recently, biomethanation (Figure 3) has been considered as another strategy to benefit from hydrogen produced during DF by increasing the methane content in the biogas produced during AD thanks to hydrogen injections [75]. Coupling

biomethanation with DF might thus allow the development of the next generation of two-stage AD processes, as depicted in Figure 3 [76].

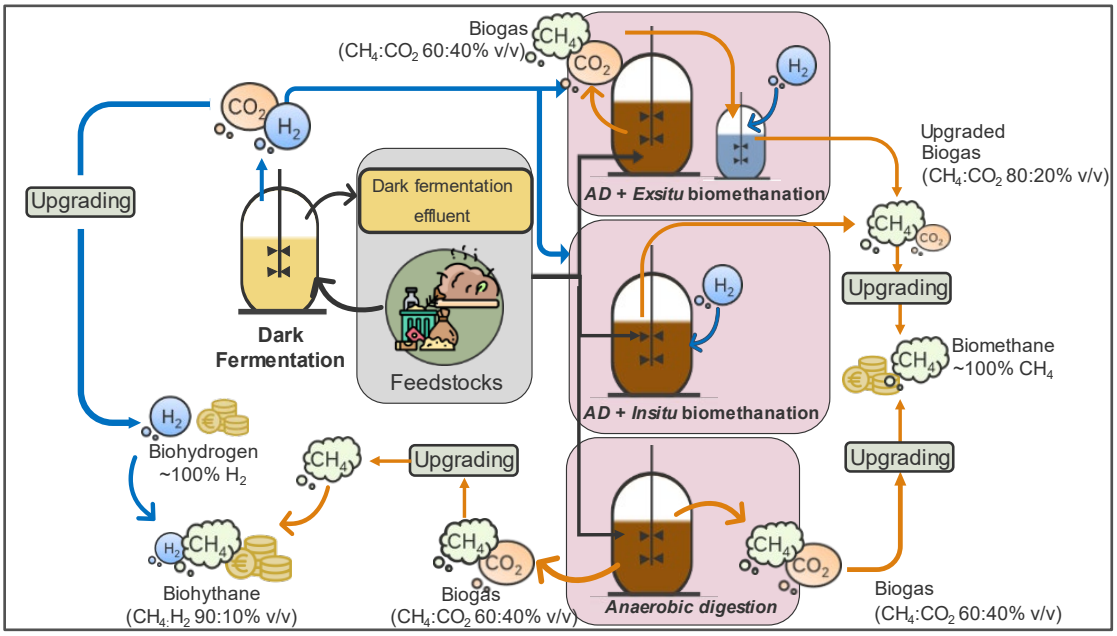

Figure 3. Coupling of DF and AD based processes (two stage-AD and biomethanation); black arrows stand for organic matter flow whereas blue and orange arrows represent hydrogen and methane flow, respectively.

Two-stage DF-AD coupling

A wide range of organic residual streams (manure, straw, food waste, sewage sludge, among others) can be used as substrates in AD or DF [77–79]. However, due to their hydrogen or methane production potential (relying on factors such as their carbohydrate content), there might be an interest in deploying AD, DF, or both [80]. Coupling DF and AD can be an interesting way to improve waste management strategies. The main strength that justifies the coupling is that the effluent obtained from DF is the result of simultaneous partial degradation of organic matter and hydrogen production. As a result, high VFA (such as acetate, propionate, and butyrate), ethanol, and lactate concentrations in the effluent can be further valorized in the subsequent AD unit [74]. Some investigations have compared the Energy Recovery (ER) between single-stage AD and two-stage AD to determine the economic interest of the coupling [81]. Indeed, it was reported that the ER obtained from the coupling was 20-60% higher than in single-stage AD [79, 82–84]. Nonetheless, the ER was not equally distributed among the two stages. According to [79] and confirmed by [81], the highest ER was achieved when the hydrogen produced was a small fraction (*i.e.*, 5-10%vol). This result suggests that the optimal coupling configuration uses DF to improve substrate accessibility through the acidogenic environment in DF, further promoting the methanogenic step in AD. To achieve such improvement, accurate optimization of both stages is required. For this reason, operational parameters such as temperature (mesophilic or thermophilic) [85], pH (acidic in the first step vs. alkaline in the second) [79], ammonium and free ammonia concentrations (methane inhibition at NH_3 concentrations higher than 700 mg/L) [86, 87], initial substrate pretreatment (*e.g.,* thermal or chemical) [82, 84] and digestate recirculation [87, 88] have been shown to impact the yields, stability and efficiency of the coupling. In addition, applied

Hydraulic Retention Time (HRT) and Organic Loading Rate (OLR) should be adjusted considering each process performance but also to ensure the coupling synergy [89–91]. As an illustration, Luo *et al*. (2011) showed a 6.7% improvement in energy generation by applying an HRT ratio of 1:14 instead of 3:12 (days:days), respectively, to DF and AD.

The energy recovery of the two-stage AD can also be enhanced by improving the degree of degradation of organic matter. For that purpose, different reactor configurations for DF and AD can be used. Whereas conventional Continuous Stirred-Tank Reactors (CSTR) are mainly chosen to perform DF, several reactor configurations, such as Up-Flow Anaerobic Sludge blanket (UASB) or fixed bed reactors, studied at bench scale, were in favor of higher ER in AD. As shown by De Souza Almeida et al., (2022) obtained an improvement of 47% of the ER when using an anaerobic fluidized bed reactor for co-digestion of cheese when and glycerol [92]. However, those configurations are not suitable for all feedstocks, which might limit their use at a larger scale [90].

Another key aspect that should be considered is the two types of microorganisms that should be promoted for each process: fermentative bacteria in DF and methanogenic archaea in AD [93]. It is well known that the growth rate of fermentative bacteria is much higher than the one observed for methanogenic archaea (*e.g.,* 0.125 vs. 1.5-7 days for fermentative bacteria and methanogenic archaea, respectively) [94]. As a result, fermentation kinetics are more rapid than methanogenesis, resulting in lower DF reactor volumes due to lower HRTs applied (hours or a few days in DF vs several weeks in AD) [5].

Finally, the coupling of DF and AD has also been applied to produce biohythane (*i.e.*, a mixture of hydrogen and methane containing 5 to 20% v/v of H_2) [95]. The added volume of H_2 in the gas grid will gradually increase in the coming years. Some recent estimations within the European Union suggest that this value can rise from 5-10% to 15-20% by 2030 [96]. Nevertheless, this theoretical value is never reached in natural gas and is subject to controversy regarding synthetic methane (CH_4 produced through methanation processes, either biologicals or chemicals). Biohythane production has several advantages over methane production, such as lower ignition temperature, a wide flammability range, and reduced NOx emissions [97, 98]. Moreover, the mass-specific heating value of biohythane (119.930 kJ/kg) is 2.5 times higher than the one of biomethane (50.020 kJ/kg) [97]. Furthermore, methane and hydrogen production from two-stage AD production through the coupling mentioned above allows anaerobic digestion to operate at higher OLR and solid removal efficiency, both in lower HRT [99, 100]. Subsequently, a technically relevant way to increase biohythane production will rely on addressing two-stage AD optimization.

Biomethanation

Biomethanation is a bioprocess in which hydrogen and carbon dioxide are converted into methane. From an operational point of view, biomethanation can be done either *in situ* [101] or *ex situ* [102]. During *in situ* biomethanation, hydrogen is injected in the same anaerobic digester where biogas is produced from organic substrates (Figure 3). As for *ex situ* biomethanation, biogas is transferred to another bioreactor, and hydrogen is mixed with only the biogas allowing either pure culture or mixed culture of archaea to convert hydrogen and

carbon dioxide into methane. Here, an external source of hydrogen is needed to perform biomethanation. A way to obtain this compound is from water electrolysis, where the excess electricity obtained from renewable resources is used to produce hydrogen, a concept referred to as Power-to-Gas (PtG) [35, 103]. PtG is the main coupling concept when referring to biomethanation [89]. Nonetheless, PtG projects associated with biomethanation still own the fewest installed power (in MW_{el} terms) compared to hydrogen and chemical methane formation [103]. Despite a rapid fall in the capital expenditure for electrolysis technology (*i.e.*, from 1300 €/kW_{el} in 2017 to 500 €/kW_{el} predicted by 2050 [103]), the electricity price and consumption, as well as the maintenance of those devices, still represent a major part of methane annual production cost with PtG [104, 105]. In addition, the combination of drastically different technologies (*i.e.*, water electrolysis and biomethanation) is a technical barrier at operational and societal levels [106, 107]. As a possible solution, DF could be used instead of water electrolysis as a bio-based technology to produce hydrogen. DF would contribute to better waste management and improve methane production (Table 1) [73]. Using DF, some associated costs derived from the upgrade and storage of the gas mixture generated in DF (H_2:CO_2, 50:50% v/v) should be considered [108, 109]. However, carbon dioxide presence might be useful to stabilize the H_2:CO_2 ratio during the biomethanation process, preventing carbon dioxide depletion from the gas phase and associated pH drop and acetate accumulation [110]. The compatibility between DF and biomethanation for feeding, maintenance, and gas production control is also crucial to envision the future use of this technology as it allows the industrial development of existing AD facilities [76]. The main challenges that should be faced in the coming years are related to hydrogen production and consumption optimization. In particular, specific objectives such as i) which feedstocks should be employed considering their hydrogen and methane production potentials, ii) to pursue the development of adapted equipment (responding to legislation about the use of hydrogen), and iii) to overcome limitations resulting from the hydrogen low gas-liquid mass transfer, have to be faced to allow the development of this coupling at industrial scale. Indeed, the gas-liquid mass transfer rate remains the process bottleneck when hydrogen is converted to methane either by *in situ* or *ex situ* biomethanation [111]. The main reason lies in the physicochemical properties of hydrogen gas (solubility $1.6x10^{-4}$ g/100 g water, Henry constant $7.8x10^{-4}$ mol/kg/bar), which limit its methanogen consumption [112]. To overcome those boundaries, several strategies have been developed, such as different bioreactor configurations (membrane bioreactors [113, 114] and trickling bed bioreactors [115]) and optimization of operational parameters (mesophilic and thermophilic temperatures [116] and partial pressure of hydrogen [117]). Likewise, changes in the microbial community of mixed cultures are also influenced by hydrogen partial pressure in the system. Acetogenesis is carried out by syntrophic microorganisms, which are thermodynamically constrained by the H_2 partial pressure, which should remain under 10^{-4} atm to allow VFAs degradation and methanogenesis [117]. According to different authors, archaeal community adaptation to hydrogen inputs is required to avoid acetate accumulation and optimize methane production [110, 117, 118]. In the same way, during *in situ* biomethanation, continuous hydrogen injection into the anaerobic digester was reported to inhibit VFA degradation resulting in a pH decrease, which finally caused process failure. Therefore, coupling biomethanation with mixed cultures and DF might lead to biomethanation failure due to high VFA concentration in DF effluent without an adapted community. To avoid this accumulation, accurate choice of initial inoculum [117, 118], as well as pulsed hydrogen injection [119] and use of additives [120, 121], are strategies that are promising to promote community activity and adaptation during biomethanation

processes. In addition, the feeding strategy of DF effluent to the biomethanation reactor could be adapted to avoid the increase of VFA concentration in the biomethanation reactor (*e.g.*, co-digestion with other substrates or slow stepwise feeding). Considering the Technology Readiness Level (TRL), the *ex situ* biomethanation is more advanced than *in situ* biomethanation. Whereas several industrial *ex situ* biomethanation units are currently operational (*e.g.*, DEMETHA project (mixed culture) [122] or Electrochaea company (pure culture) [123]), *in situ* processes are mainly performed at lab scale, with few trials at pilot scale [124, 125]. This delay in developing *in situ* biomethanation is due to the impact mentioned above of hydrogen on the AD process. On the contrary, with *ex situ* biomethanation, hydrogen injection does not inhibit the microbial community but at the expense of building a new reactor.

Table 1. Opportunities and limits of coupling DF with AD and DF with biomethanation [35, 81, 97, 126–129]

Coupling	Opportunities	Limits
DF – AD	• Producing hydrogen and methane in separate processes but on the same plant. • Improvement of the ER from residues • Avoiding methanogens inhibition • Producing biohythane with higher OLR and shorter HRT for AD	• Upgrading cost of gas produced with both processes • New constraints associated to hydrogen production and selling (storage, transports) or biohythane introduction in the gas network (restrictions from legislations) • Additional capital and operational expenditures (or CAPEX and OPEX) due to DF implementation and coupling control • Development is required to optimize DF (TRL 7) at industrial scale
DF - Biomethanation	• Increasing methane content in biogas • Decrease in carbon dioxide emissions • Improvement of the ER from residues • Avoiding methanogens inhibition (for *ex situ* biomethanation) • No investment for H_2 storage and distribution • Reduced upgrading cost for methane production	• Additional CAPEX and OPEX associated to the creation of DF reactor and biomethanation sub-reactor (for *ex situ* biomethanation) • Development is required to perform *in situ* biomethanation without process failure and optimize DF at industrial scale

DARK FERMENTATION - PHOTOFERMENTATION

Purple phototrophic bacteria (PPB) are diverse bacteria that can grow using various metabolic pathways. This versatility allows PPB to survive in various environments [130]. Figure 4 shows their most relevant metabolic features, highlighting those related to hydrogen production/consumption.

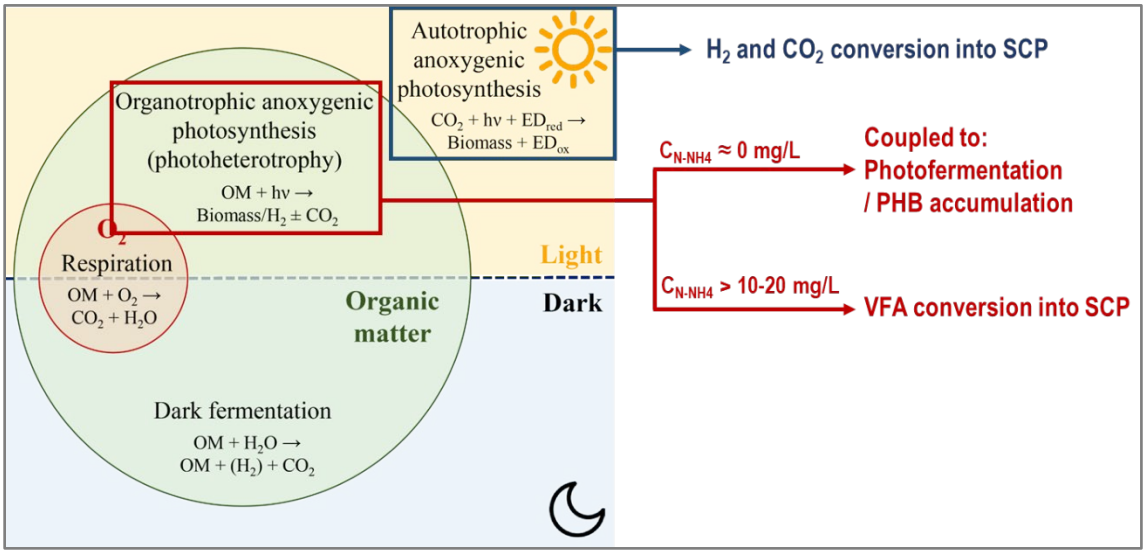

Figure 4. Simplified representation of the main metabolic modes of PPB structured according to energy and carbon sources and electron acceptors. Relevant metabolic modes for hydrogen production/consumption are highlighted. Adapted from [131]. SCP stands for single-cell proteins, OM for organic matter, hv for light energy, ED for electron donor (reduced or oxidized), and C_{N-NH4} for ammonium-N concentration.

Their most unique characteristic feature is their capability to grow via anoxic photosynthesis. Under anaerobic conditions and in the presence of light (mostly infrared, with absorption peaks at 750-1,100 nm), PPB can grow using light as an energy source and a wide range of electron donors. They can fix carbon dioxide when growing on inorganic electron donors (*i.e.,* photoautotrophy) or use organic carbon as a C source instead (*i.e.*, photoheterotrophy) [131]. In addition, in the absence of light and under anaerobic conditions, PPB can grow via fermentation in the presence of organic matter. If oxygen and organic matter are present, chemotrophic growth via respiration is the prevalent growth mode [132]. PPB can be classified into purple sulfur bacteria (PSB) and purple non-sulfur bacteria (PNSB). PSB grow mainly photoautotrophycally using reduced sulfur compounds as electron donors. At the same time, PNSB have a more versatile metabolism, using a wide range of organic and inorganic electron donors (*e.g.*, organic matter, hydrogen, hydrogen sulfide, reduced metals, etc.) [133]. PPB also have a diverse metabolism to regenerate reduced cofactors (*e.g.*, NADH or NADPH) [134]. If carbon dioxide is available, its fixation is the main mechanism for cofactor recycling [135]. In addition, in the presence of an excess of organic carbon, PPB can accumulate polyhydroxyalkanoates (PHAs) or produce hydrogen, both mechanisms serving as electron sinks [136].

Regarding resource recovery, the outstanding ability of PPB to grow at high biomass yields (up to 1 g $COD_{biomass}$/g $COD_{removed}$) and to accumulate added-value products has attracted increased attention in recent years, particularly when growing PPB photoheterotrophically [137, 138]. Nevertheless, the most widely researched application of PPB involves hydrogen production. Hydrogen is synthetized by PPB, such as *Rhodobacter sp.*, *Rhodopseudomonas sp.,* or *Rhodospirillum sp.*, under anaerobic, illuminated, and ammonia-limited conditions (Figure 4) [139]. During the so-called photofermentation, the nitrogenase enzyme can uptake electrons generated from the anaerobic oxidation of organic substrates, use protons as electron acceptors and light as an energy source, and generate molecular hydrogen [136]. The light energy collected by light-harvesting complexes is used to generate ATP via photophosphorylation, and high-energy electrons reduce ferredoxin through reverse electron flow. The reduced ferredoxin (electron carrier) and ATP are then used to produce hydrogen via proton reduction catalyzed by a nitrogenase [140]. This enzyme is also responsible for ammonia production from the reduction of molecular nitrogen. Therefore, molecular nitrogen decreases hydrogen production due to competition at the enzymatic reaction centres [141]. More importantly, hydrogen production via photofermentation must be performed at low ammonia concentrations (above 10-20 mg N-NH_4^+/L), as nitrogenase activity is inhibited due to product inhibition [131]. Therefore, efficient photofermentation is limited to low-N streams.

ATP generation from light makes photofermentation interesting compared to other processes because hydrogen production is not linked to catabolic processes. Therefore, simple organic compounds, including VFAs such as acetic acid and butyric acid, can be used as substrates for hydrogen production. Other organic substrates can also be consumed via photofermentation, including simple sugars (*e.g.*, glucose, sucrose) and alcohols. Despite the advantages, the low hydrogen production rates (maximum volumetric productivities of 3.6 L/Ld and average values of 2.2 L/Ld) hamper the cost-effective hydrogen production via photofermentation due to low biomass concentrations [131]. Direct use of complex substrates like food or agro-industrial waste requires a pretreatment, mainly hydrolysis, to enhance their biodegradability [142, 143] (see Figure 5, process 1). The light requirement is another limitation of photofermentation, as it entails high operational and capital costs. All the challenges mentioned above limit the potential application of single-stage photofermentation.

Coupling DF with photofermentation might be a niche application of photofermentation. Thanks to the possibility of further consuming short-chain VFAs for hydrogen production, photofermentation can be used to overcome the main bottleneck of DF, which is characterised by lower hydrogen yields (0.11 g COD_{H2}/g CODfed on average). [74]. PPB can theoretically convert 1 mol of acetate into 4 mol of hydrogen, increasing the yield to 12 mol hydrogen/mol glucose [144]. However, this theoretical yield is hardly achieved in reality since the growth and maintenance of PPB require part of the electrons and carbon (and competition with PHA production always occurs to some extent) [145]. Thus, average hydrogen yields around 0.25 g COD_{H2}/g COD_{fed} are often reached in DF, followed by photofermentation [131].

Therefore, photofermentation can be used for the bioconversion of the VFAs produced during DF into hydrogen, enhancing the overall yields without jeopardizing the overall rates (Figure 5, process 2) [146]. As an additional benefit, the biomass obtained during photofermentation could be further valorized as an animal feed substitute due to the high protein content of PPB and its adequate amino acid profile [147].

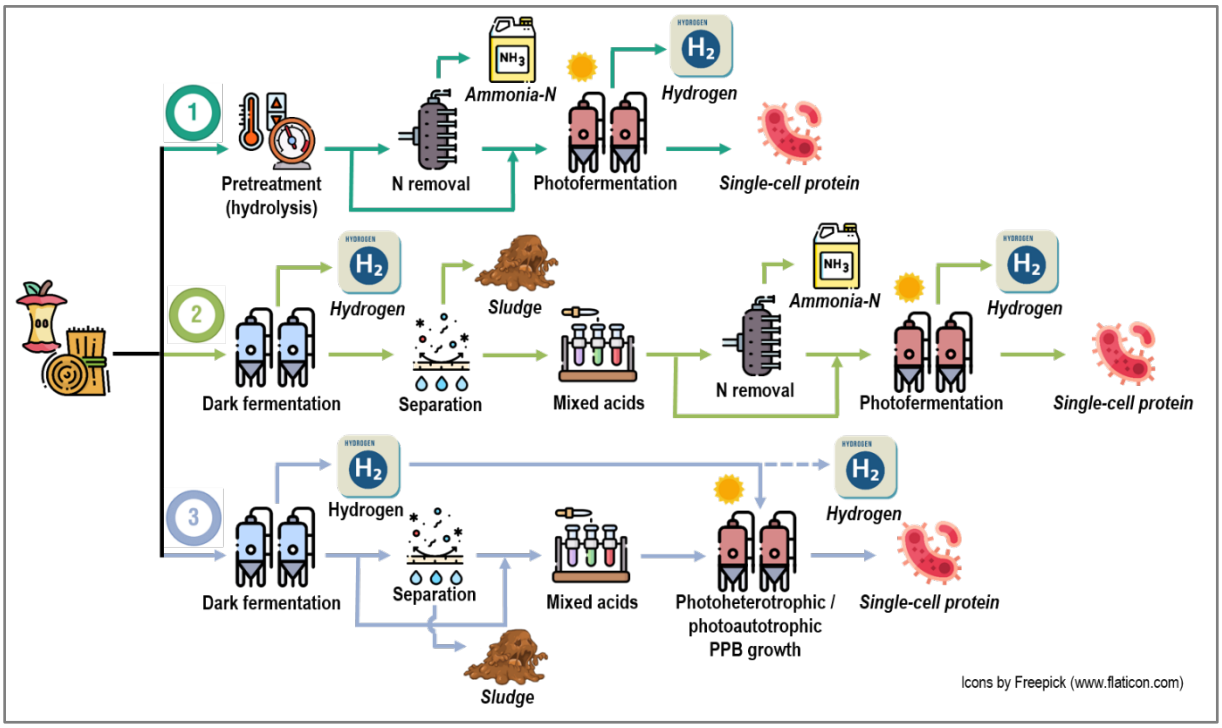

Figure 5. Potential operational configurations for the bioconversion of organic waste into value-added products via (1) photofermentation, (2) sequential DF and photofermentation for hydrogen production, and (3) sequential DF and photofermentation for single-cell protein production.

Some technical barriers need to be overcome when considering the coupling DF-photofermentation. Before photofermentation, the separation of the sludge by filtration or centrifugation is required for effective light distribution. Moreover, dilution or a previous N removal step (*e.g.*, via membranes, adsorption, or stripping) is also necessary when using substrates with high N contents to avoid hydrogen production inhibition by ammonia [136, 145]. In addition, an important factor to consider in photofermentation is the energy consumption due to light supply. Artificial light for hydrogen production exhibits prohibitive costs [131]. Therefore, the economic feasibility of photofermentation after DF must be considered, and efforts should be carried out using natural light and optimal operational conditions to maximize production rates.

Optimal conditions for hydrogen production via photofermentation (Figure 5, process 1) have intensively been studied (Figure 6). pH values above 5.5 promote hydrogen production with an optimal range between 6.5 and 7.4 (Figure 6A). Since low pH values lead to hydrogen production inhibition, the applicable OLRs must be limited due to the risk of reactor acidification. OLRs higher than ~2-6 g COD/Ld significantly decrease the hydrogen yields, although this drop depends on the photobioreactor configuration [131]. No pH control is

required at appropriate loads since organic acid consumption increases the pH. Increasing in the light intensity up to 3,500 lux favors hydrogen production by photofermentation (Figure 6B). It must be considered that light attenuation is particularly relevant in PPB-based processes, as near-infrared light (the main energy source for PPB) is more attenuated by water than light within the visible spectrum [148]. The increase in light intensity above 4,000-4,500 lux causes a decrease in the hydrogen yields due to photoinhibition [139].

Regarding temperature, high hydrogen yields are obtained even at low temperatures (<25 °C), whereas values over 40 °C result in decreasing hydrogen yields due to microbial inhibition (Figure 6C). The operation at low temperatures is essential since no energy requirements for reactor heating might be needed for photofermentation. Organic matter concentrations in the substrates above 4-8 g COD/L have a negative impact on hydrogen yields (Figure 6D). This factor, along with the inhibition due to ammonia-N, considerably limits the direct use of photofermentation to valorize DF effluents, restricting its application to streams with low organic and nitrogen contents. Dilution strategies could be applied, but they would increase the operational costs of the process, thus compromising the economic feasibility. The reduction state of the C source impacts the carbon dioxide production/fixation by PPB, which might directly affect the hydrogen yields. This implies that the optimal conditions for individual DF and photofermentation processes might be different from those for the coupled process.

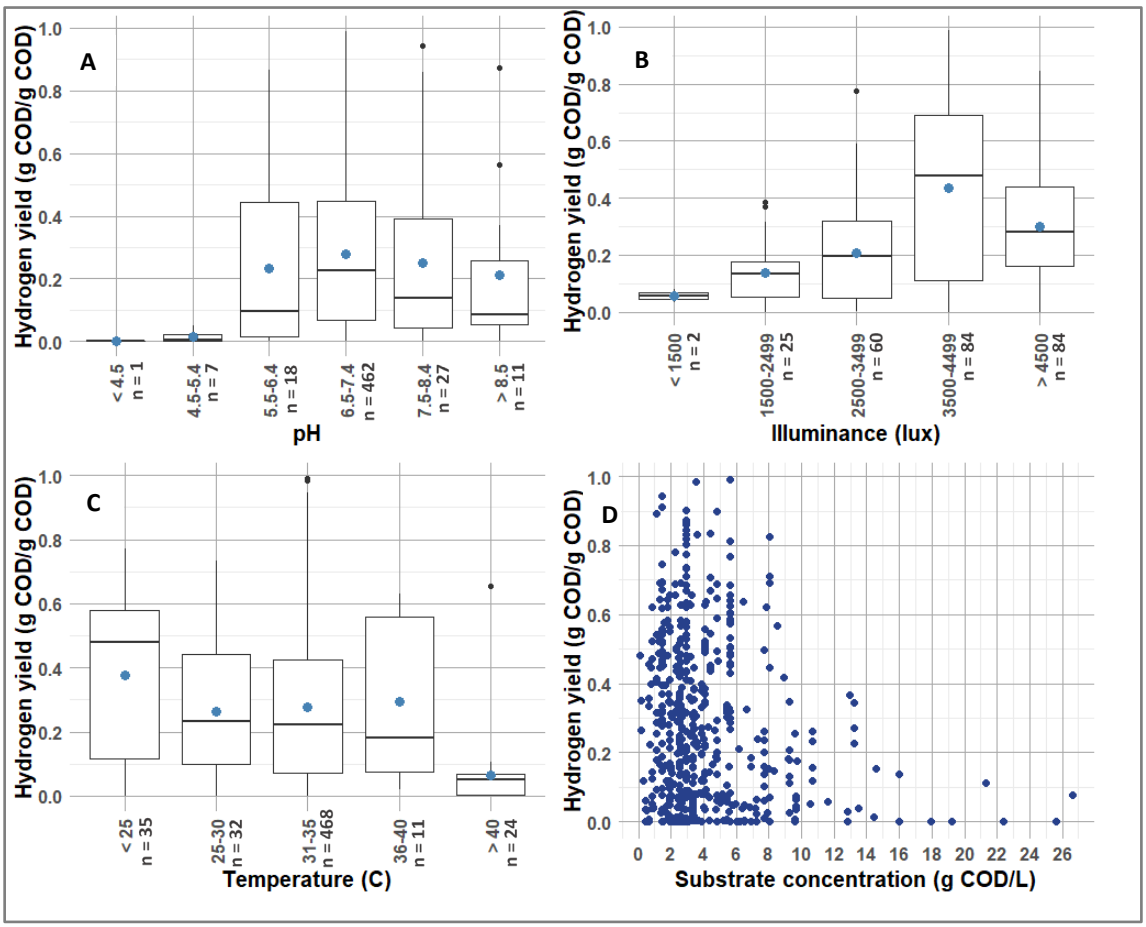

Figure 6. Hydrogen yields produced by PPB in photofermentation processes at different (A) pH values, (B) illuminances, (C) temperatures, and (D) substrate concentrations. Light blue dots in boxplots represent mean values. COD stands for chemical oxygen demand, and "n" for the number of data points. Adapted from [131].

Another way to couple DF with PPB processes is the bioconversion of the DF gaseous effluents into single-cell protein. This approach has recently emerged as a promising solution for feed and food scarcity (Figure 5, process 3). PPB can effectively use hydrogen as an electron donor and carbon dioxide as a carbon source for their growth (Figure 4) [149]. Thanks to photophosphorylation, high yields of 1 g $COD_{biomass}/COD_{H2}$ have been achieved in mixed PPB cultures (own unpublished results). In addition, biomass productivities of 0.3-0.5 g VSS/Ld (own results), along with high protein and amino acid contents in the PPB biomass (50-60 and 40-50 % on VS basis, respectively) have been reported, confirming the potential of this approach [147, 150]. However, autotrophic PPB growth entails lower biomass production rates (up to 0.5 g $COD_{biomass}$ /Ld) than heterotrophic PPB growth (up to 6 g $COD_{biomass}$ /Ld) [137]. As a result, more research is required to improve biological growth along with the gas-liquid mass transfer of hydrogen and carbon dioxide.

LACTIC ACID FERMENTATION - DARK FERMENTATION

The motivation for coupling lactic acid fermentation (LAF) and DF is to overcome the negative effect of the accumulation of lactic acid bacteria (LAB) in DF reactors. LAB are gram-positive, non-spore forming bacteria that ferment carbohydrates producing mainly lactic acid [151]. LAB taxonomic classification has had many adjustments over time but generally agrees that LAB belongs to the family *Lactobacillaceae* and order *Lactobacillales* [151]. The presence of LAB in DF was widely considered detrimental to the process, but recently it was deemed inconclusive or poorly understood [152]. Three negative impacts of LAB in DF reactors are i) substrate competition, ii) bacteriocins release, and iii) reactor over-acidification [152]. Substrate competition occurs when carbohydrates are converted to lactate via homolactic and heterolactic fermentations, steering the process from hydrogen to lactate production (Eq. 1 and 2).

Glucose → 2 Lactate Eq [1]

Glucose → Lactate + Ethanol + CO_2 Eq [2]

Furthermore, the LAB community outcompetes other microbial groups by releasing bacteriocins, specifically inhibiting hydrogen-producing bacteria (HPB), particularly *Clostridium* sp. [153]. Additionally, lactate production mediated by LAB can reduce the pH in DF reactors below the optimum range of 5.5 – 6.0 for hydrogen production. LAB might thrive at pH values as low as 3.5 [154]. Nonetheless, LAB were also reported to impact the HPB positively. A few of the positive relationships between LAB and HPB are i) higher substrate hydrolysis, ii) a contribution of LAB to oxygen depletion, iii) a cross-feeding between LAB and

HPB, and iv) a direct contribution of lactic acid to hydrogen production [152]. Illustratively, a study evaluating starch as a substrate in DF concluded that *Bifidobacterium* assisted in breaking down starch into less complex molecules before being consumed by *Clostridium* for hydrogen production [155]. Facultative LAB *Lactobacillus* was suggested to consume oxygen producing lactate, thus providing an anaerobic environment for anaerobic HPB to produce hydrogen [156]. Cross-feeding of LAB and HPB was shown in multiple studies, where the lactate and acetate produced by LAB were subsequently consumed by HPB [157, 158]. The inability to convert lactate to hydrogen during DF was also associated with the common practice of heat-pretreatment of inoculum to deactivate methanogens and enrich HPB, which was found to also inhibit Lactate-Utilizing Hydrogen-Producing Bacteria (LU-HPB) such as *Megasphaera elsdenii* [159]. Circumventing this blind spot, lactate has successfully been converted to hydrogen by excluding heat pretreatment of inoculum for DF, with the suppression of hydrogenotrophic methanogens by incubation time [159]. Considering these positive findings, studies have been carried out to utilize lactate as one of the carbon sources for hydrogen production. There are multiple pathways reported for the conversion of lactate to hydrogen, summarized by [152], a few of which are as follows (Eq. [3-5]).

$$\text{Lactate} \rightarrow 0.5\ \text{Butyrate} + CO_2 + H_2 + 0.5\ H_2O \qquad \text{Eq [3]}$$

$$\text{Lactate} \rightarrow 0.5\ \text{Acetate} + 0.5\ \text{Ethanol} + CO_2 + H_2 \qquad \text{Eq [4]}$$

$$\text{Lactate} + H_2O \rightarrow \text{Acetate} + CO_2 + 2H_2 \qquad \text{Eq [5]}$$

A better understanding of the interrelated factors such as inoculum source, pretreatment or enrichment method, reactor configurations, and operational conditions is essential in achieving efficient lactate-driven DF (LD-DF) [152]. LAF-DF coupling was shown in different configurations according to (Figure 7).

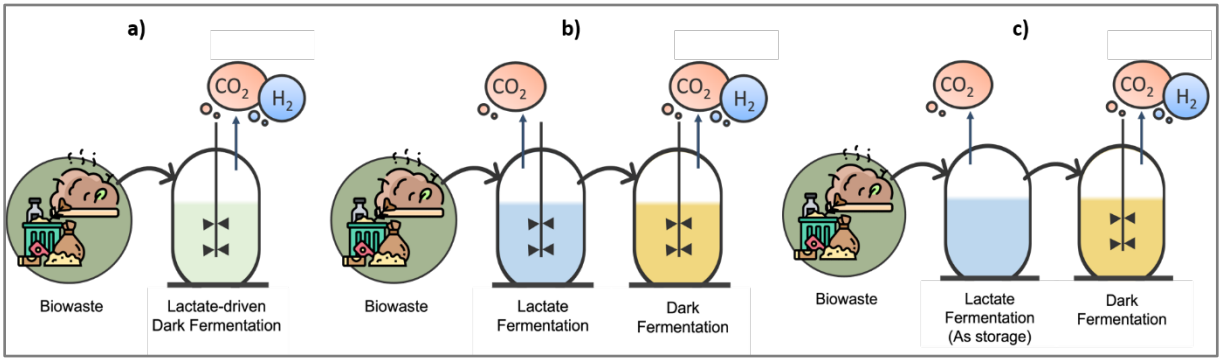

Figure 7: Different process configurations assessed for the coupling of LAF-DF; a) Lactate-driven DF, b) two-step LAF + DF, and c) LAF as storage method + DF.

LD-DF in a single reactor (Figure 7, a) relies on a positive and balanced relationship between LAF and DF, at which lactate production and consumption rate do not cause instability. For instance, the importance of process pH to maintain a correct balance in a single reactor was highlighted [160]. The highest hydrogen yield was achieved at pH 7 (61.9 ± 0.2 NmL H_2/g VS) from fruit and vegetable waste, where simultaneous lactate production (below 10 g /L) and

consumption were observed. The process was unbalanced at low pH values (uncontrolled, pH 5.5, pH 6.0, and pH 6.5), resulting in higher lactate accumulation (up to 17 g/L) and lower hydrogen production (41-59 NmL H_2/g VS).

In a two-stage LAF-DF (Figure 7, b), substrates are pre-fermented in a separate reactor to favor lactate production, and effluents are subsequently converted into hydrogen in a second DF reactor. Optimal operating conditions are essential in differentiating the two reactors, where lactate production is favored in the first reactor, and the second reactor is driven towards hydrogen production. In a recent study, tequila vinasse was pre-fermented at an HRT of 13.3 h and pH 5.5 to produce lactate-rich effluent (13.2 ± 1.7 g/L) [161]. This effluent was then fed to a CSTR (HRT 12 h and pH 5.8), which produced a maximum hydrogen yield of 109.8 ± 7.2 NmL H_2/g VS_{added}. Likewise, inoculum is important in providing suitable microbial communities for both carbohydrate and lactate conversions. Suitable inoculum can be obtained from the enrichment of various sources of wastewater or a specific mix of strains such as *Megasphaera elsdenii* with *Clostridium butyricum* as HPB together with *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, and *Enterococcus mundtii* as LAB [162, 163]. A key takeaway in two-stage LAF-DF is that the LAB in the first stage reactor did not negatively impact the performance of the second reactor for hydrogen production. It was inferred that the inhibiting effect of LAB on HPB was species-specific, but further research is required [161].

Finally, LAF has been used to preserve food and as part of the ensiling process to preserve crops for animal feed [164]. Recently LAF has been considered as a storage strategy to preserve the biomethane potential of organic substrate (Figure 7, c) [164]. However, information on utilizing LAF as a storage method before DF for hydrogen production is scarce. Storage is essential in allowing biorefineries to run continuously despite varying feedstock availability and is critical for easily biodegradable substrates such as food waste, where premature fermentation and organic carbon losses can occur during transportation [165–167]. With regard to the coupling of LAF and DF, there are many opportunities for further investigations of biohydrogen production, such as looking at the effects of LAF storage parameters (*e.g.,* storage temperature, concentration, duration) on the biohydrogen potential of substrates or stabilising a continuous reactor by eliminating substrate competition between LAB and HPB. Such studies would be helpful for further understanding of the underlying causes in positive and negative interactions between LAB and HPB.

CONCLUSIONS

The production and use of renewable hydrogen via coupling processes with DF technology is now regarded as an attractive biotechnological approach for the utilisation of residual streams. The metabolic profile and anaerobic microbiome obtained after DF are essential variables to optimize the coupling regardless of the second process. The change in operational conditions, separation step, and presence of unwanted microbial activity are some of the key

challenges that deserve further specific investigation depending on the type of coupled process. Additionally, the grade of DF effluent purity needed is crucial to select a suitable separation technology to balance the economic cost. Overall, the potential benefits of coupling different biological processes with the DF studied in this chapter have been demonstrated, which may become a key biotechnological process in the future.

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