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Abstract

This review provides the alternative routes towards the valorization of dark H_2 fermentation effluents that are rich in volatile fatty acids mainly acetate and butyrate. Various enhancement and alternative routes such as photo fermentation, anaerobic digestion, utilization of microbial electrochemical systems and algal system for the generation of bioenergy and electricity are highlighted. What's more, various integration schemes and two-stage fermentation for the possible scale up are reviewed. Moreover recent progress in process efficiency for the performance achieved in wastes stabilization, overall recovery of useful and higher COD removal into value-added products are discussed extensively.

Keywords: Biohydrogen; Volatile fatty acids; Dark and Photo-fermentation; Bioelectrochemical Systems (BESs); Biomethane; Bioplastics

1. Introduction

Over the past decades, Hydrogen (H_2) has gained a great interest for its potential to be used as new, clean and sustainable energy vector. Indeed, H_2 has the intrinsic advantages of a very high energy content per mass unit (120 $kJ.g^{-1}$) that is much superior to other usual energy vectors, as well as combustion properties that only produces water vapour [1, 2] The energetic value of H_2 is more than twice higher than natural gas or propane and gasoline, but also seven times higher than wood [3] All these factors make the H_2-based technologies as serious candidates to

replace the polluting fossil fuel–based transportation systems in a near future. This is particularly true since many concerns are worldwide rising about global warming and fossil fuel depletion. In a near future, an emerging economy market based on H_2 energy is foreseen to become an earnest alternative to substitute fossil-based fuels for transportation.

Presently, H_2 is mainly produced for non-energetic uses in chemical and petrochemical industries. The production of ammonia represents the major part of H_2 consumption (54%) with more than 80 Mt of ammonia was used as fertilizer in agriculture in 2013 [4]. Other industrial applications of H_2 mainly include concerning oil refining and methanol production (35%) and, at a lower extent, in metallurgical, electronical, glass and agro-industries [5]. Beyond actual industrial uses of H_2, new routes for local and diffuse sources of H_2 production will be required in case of the emergence of H_2-based transportation system. To date, the production of H_2 at large scale is principally based on natural gas reforming which having the negative environmental impacts by releasing extensive amounts of carbon dioxide (CO_2). The steam reforming technology generates about 96% of total H_2 (Lin et al., 2012). The development of environment-friendly technologies is therefore crucial for sustaining H_2 in the transportation sector. The remaining 4% of H_2 are produced through water electrolysis that can be considered as clean and sustainable when renewable resources are considered (wind, sun, water, etc.). Although renewable energy based water electrolysis will be certainly widely implemented to answer to the growing demand in H_2 energy. In this context, technologies using raw biomass or any type of organic waste through thermochemical and biological processes could be considered as a very promising for the production of fully green hydrogen with the lowest environmental impact [6, 7]. For now, these bio-technologies remain at pilot scale demonstration however there are many chances for the upscaling in the next coming decades.

When considering waste materials, dark fermentation (DF) process has to be seriously considered for its proficiency to convert and adapt to an ample array of wastes to produce H_2 [5]. Among all biological processes, DF process has the main advantage do not require light to occur, with regards to photo-fermentation or green algae photobiolysis. The generation of biohydrogen through DF processes is based on the activity of anaerobic microorganisms that are able to degrade and convert many types of organic matter into H_2 and other by-products [8, 9]. These microorganisms are found in natural and anthropogenic environments such as marshes, sediments, manure, sewage sludge or digestive animal tracts, and are thus easily available for sampling. Indeed, this microbial process is derived from anaerobic digestion and is widespread in natural ecosystems [10]. In nature, firstly hydrolytic bacteria hydrolyze organic biomass into simple molecules which are further converted into H_2 and CO_2 mainly through the acetate and butyrate pathways [11]. Both pathways generate 2 moles of H_2 per mole of co-product:

$$C_6H_{12}O_6\ (Glucose) + 2H_2O \rightarrow 2\ CH_3COOH\ (Acetate) + 2\ CO_2 + 4\ H_2 \quad (\Delta G_0' = -215\ kJ.mol^{-1})$$

$$C_6H_{12}O_6(Glucose) + 2H_2O \rightarrow CH_3CH_2CH_2COOH\ (Butyrate) + 2CO_2 + 2H_2\ (\Delta G_0' = -264\ kJ.mol^{-1})$$

Among all biomass constituents, mainly carbohydrates participate to produce H_2 through these pathways [12]. However, carbohydrates can also be converted into other metabolic products such as ethanol, butanol, lactic acid or other volatile fatty acids. The metabolic shift of the process depends on the environmental parameters, the microbial inoculum and the type of used biomass [5, 13]. Additionally, H_2 could be directly consumed by homoacetogens or propionic acid-producing bacteria and the presence of such H_2-consuming bacteria might also considerably reduce the amounts of cumulated H_2, even when methanogenesis is unfavoured due

to low pH, and high organic loads. Nonetheless, in mixed culture, Hawkes et al. [14] proposed an equation of an average of 2.5 moles of H_2 when considering mixed culture fermentation.

$$4\ C_6H_{12}O_6\ (Glucose) + 2\ H_2O \rightarrow$$
$$3\ CH_3CH_2CH_2COOH\ (Butyrate) + \ 2\ CH_3COOH\ (Acetate) + 8\ CO_2 + 10\ H_2$$

That implies a butyric/acidic acids ratio of 1.5 which has been controversially discussed in several studies. In particular, Guo et al [12] demonstrated a statistical independence between acetate accumulation as generated by both the acetate pathway and homoacetogenesis, and the H_2 yield, invalidating the relevance of the butyric/acidic acid ratio. Moreover, in the case of more complex biomass such as lignocellulosic biomass, the degree of polymerisation of the carbohydrates can strongly affect the H_2 yield since a slow hydrolytic step is required which could subsequently favour H_2 consumers. Nonetheless, the heterogeneous composition of lignocellulosic materials requires the use of different types of hydrolytic enzymes to be fully degraded. Such complex enzymatic potential is produced by microbial consortia increasing biomass degradation thanks to their synergistic enzymatic functions [15]. Thus, the use of complex microbial consortia represents having many advantages including not require sterile conditions and can easily adapt to culture condition changes.

However, considering the flexibility and the variability of metabolic pathways in mixed cultures, the H_2 produced during the conversion of organic matter in mixed cultures represents an average maximum COD ranging between 20% and 25% of the initial COD from the substrate, which is equivalent to 2 or 3 moles H_2/mole Glucose. In general, the remaining 80% are retrieved in the forms of fermentative metabolites co-produced. To work out an integrated process which embodies most of the metrics that could bring economic viability, such amounts

of COD has to be further considered in downstream processes within an integrated scheme. However, fermentative metabolic routes may influence the downstream process, especially when considering biological processes that can be more or less sensitive to by-product patterns. So far, various methods have been proposed to utilize DF effluents efficiently and adding further revenue to the process.

Numerous reports demonstrated the application of integrated schemes for the valorization of DF effluents to generate value added end-products. However, the least amount of review papers with a limited discussion on the integrated schemes were found in the literature [16, 17]. For example, a review by Guwy et al. [16] doesn't elaborate the key challenges and overcoming opportunities associated with the integrated systems, in particular on the aspect of BESs. Recently, Turon et al. [17] reviewed the potential application of dark fermentative effluents for microalgae cultivation and assessed controlling both biotic and abiotic factors is essential for enhanced microalgal biomass production from dark fermentation effluents. To fulfill the existing research gaps in the area of integrated schemes for the valorization of dark fermentative effluents to generate value added end-products, this review article overviewed the challenges associated with various integration schemes and their upcoming opportunities. Also, the enhancement of H_2 production routes are discussed in the frame of photofermentation and BES systems, the effectiveness of two-stage methanogenic systems are discussed. The potential of heterotrophic algae cultivation process for lipids production and anoxygenic nutrient-limiting process for bioplastics production also reviewed. Finally, the scientific and technical challenges for integrated schemes are discussed to provide the insights of the integrated biorefinery scheme for value-added chemicals and energy production.

2. Enhancement routes for H_2 production

The greatest challenge of the DF process is its low hydrogen yield with a maximum of 2.5–3 mol H_2/mol glucose ($C_6H_{12}O_6$) as proposed theoretically [14] and which is also observed in practice [5]. However, this represents only 20–25% of the 12 mol of hydrogen available according to the stoichiometric conversion of $C_6H_{12}O_6$ to hydrogen [18] and leads to the formation of fractions of organic matter which consist of weak organic and ethanolic molecules not further convertible by fermentative metabolisms to H_2 [19]. In DF, enhancement of overall hydrogen yield over 4 mol H_2/mol glucose can be made DF economically viable [4].

In the course of bio-hydrogen production, intermediate metabolites/by-products produced by the biocatalyst will compete with the metabolic pathways responsible for H_2 production, and this rerouting of metabolic pathways leads to a drastic reduction in the overall H_2 yields [20]. However, several researchers made an effort to redirect the H_2-evolving metabolic bioreactions to reduce the generation of low-end molecules [21]. To overwhelm the stoichiometric limitation of DF and in order to produce closely theoretical hydrogen yield that is 4 mol H_2/mol glucose, new strategies need to be examined.

2.1. Strategies to enhance the process efficacy of H_2 fermentations

In the course of conventional bio-hydrogen production, low substrate utilization, its conversion efficiency and accumulation of low-end metabolites were considered as great challenges [22]. In specific, the DF process has major problems for practical applications due to the low hydrogen yield (4 mol H_2/mole glucose), with a maximum conversion efficiency of 33% [23]. Moreover, significant amounts of residual organic acids/low-end metabolites were still present in the DF process [24]. As a result, further treatments are necessary prior to reactor

effluent disposal. In view of environmental and economic factors, it is preferable and wise to reuse the residual organic fraction of bioreactor effluents at the end of the DF process for additional energy or value-added products production together with waste remediation (Figure 1).

2.2 . Integration schemes

Over the past years, in the direction of enhancing DF H_2 production process efficiency, several process integration schemes have been suggested to overcome the existing limitations and challenges. Utilization of the residual low-end metabolites (such as volatile fatty acids) issued from hydrogen bioreactor effluents as potential organic feed stock for additional energy recovery has been proposed in the form of integrated schemes (Table 1). Numerous secondary bioprocesses together with DF process including, photofermentation process for biohydrogen production, electro-fermentation/bioelectrochemical systems for H_2 and bioelectricity production, methanogenesis process for methane production, nutrient-limiting process for bioplastics production, and heterotrophic algae cultivation process for lipids production have been investigated [18, 25-28]. Secondary bioprocesses which can be integrated with DF hydrogen production process are recent emergence. This review describes the possibilities in integrated schemes for the utilization/reuse of effluents from the primary DF process as potential carbon-rich substrate in a secondary process for additional energy generation, as well as value-added bio-fuel and chemicals production through which the entire process could become economically viable and practically applicable (Figure 1). The integrated schemes for the utilization of DF effluents are depicted in Figure 1 and discussed in details in further sections.

2.2.1. Integrating DF with photobiological process

In this scheme, the residual low-end metabolites issued from the DF reactions will be considered as carbon-rich organic substrate in a photobiological H_2 production process for additional energy generation (Figure 2). In this scheme, residual organic acids such as volatile fatty acids (VFAs) can be readily consumed by photosynthetic bacteria for their metabolic requirements [29]. While low-end metabolites of the DF process are efficiently consumed by few purple non sulphur (PNS) phototrophic bacteria, the 2-stage integration scheme of DF bioprocesses with anoxygenic photo-fermentation process has the dual benefits of a higher H_2 yield and simultaneous treatment of the effluents. Photosynthetic microbial colonies which convert solar energy to chemical energy can be further engineered to transform the weak organic acids to essentially biohydrogen through photo-fermentation processes [30]. The extensively studied bacteria for photo-fermentation of volatile fatty acids are *Rhodobacter* species. However, green microalgae can also consume low-end metabolic intermediates produced from the DF process, specifically when acetic acid is used as a potential substrate [31, 32]. The maximum theoretical biohydrogen yield of the photo-fermentation process of acetic acid is 4 moles of biohydrogen from one mole of acetate as recorded [5]. If we assume that acetic acid is only the low-end metabolite and used by the dark and photo-fermentation, biohydrogen yields of 12 moles of hydrogen per mole glucose could be achieved. Here, 4 moles of hydrogen were retrieved from the DF process and the remaining 8 moles of hydrogen were produced by photo-fermentation. So far, the maximum average biohydrogen yields was reported to be in the middle of 6 and 7.2 moles of biohydrogen for every one mole of glucose by integrated DF with photo-fermentation process [18]. Su et al. [33] conducted an extensive investigation in a 2-stage combined dark and photo-fermentation process to enhance the H_2 production from glucose

(20 g L^{-1}), where 1.32 mol H_2 mol^{-1} glucose and 4.16 mol H_2 mol^{-1} glucose, respectively by dark and photo fermentative hydrogen production was observed. The cumulative hydrogen yield of the integrated system was of 5.48 mol H_2 mol^{-1}glucose. Recently, Lo et al. [34] reported the utilization of starch feedstock (35 g L^{-1}) for combined dark and photo-fermentation strategy where the overall yield was 16.1 mmol H_2 g^{-1} COD equivalent to 3.1 mol H_2 mol^{-1} glucose, with a COD elimination efficiency of 54.3%. The literature survey suggests that an efficient and effective photo-fermentation of DF effluent has to meet certain conditions such as total volatile fatty acid and NH_4^+ concentrations should be lower than 2500 mg L^{-1} and 40 mg L^{-1}, respectively [35-39]. In addition to this, the DF effluents should be glucose deficient since it mainly affects the efficiency of photo-fermentative hydrogen production [33, 38] The quantities of CO_2 generated were very high. Microalgae have better efficiency in CO_2 fixation and thus may be considered as yet one more resource for the generation of biofuels [9, 40, 41]. Lee et al. [42] have studied biohydrogen evolution from effluents in CSTR, ASBR, and UASB using a dark and photo fermentation system to achieve better H_2 production and waste remediation. They reported that the stoichiometric evolution of biohydrogen seemingly occurred because of the reactions from the anaerobic citric-acid cycle accompanied by the photo-mediated reactions which lead to the prompt oxidation of the "reduced NAD^+" produced by the former species [43]. On the other hand, photo-fermentation of these residual low-end organic metabolites from the hydrogen bioreactor is considered to be more challenging than DF process with respect to process efficiency, sensitivity to fixed nitrogen, due to poor light diffusion, inadequacy in harnessing the energy from high photo-intensities, maintaining microenvironment, substrate inhibition, nutritional requirements for microbial growth, risk of contamination and the quest to reduce costs in the design of process-effective photo-bioreactors [30].

2.2.2. Integrating DF with bioelectrochemical systems (BESs)

Compared to conventional treatment technologies, bioelectrochemical systems (BESs) offer a novel and transformative solutions for integrated waste treatment and energy generation [44]. These BESs offers an appropriate platform for both reduction and oxidation reaction-oriented processes [45, 46] . However, all the BESs share one similar principle in the anode compartment, in which organic substrates like glucose, fructose and starch etc., or carbon rich biodegradable waste materials get oxidized and generate electrical current. However, a wide variety of applications have been realized by employing this self-induced *in situ* current, hydrogen/chemical production in microbial electrolysis cells (MEC) and microbial electrosynthesis (MES) hydrolysis of complex organic substrate in bio-electro hydrolysis system (BEH), or water desalination in microbial desalination cells (MDC) [2, 28].

2.2.2.1. DF and MEC coupled system can achieve high H_2 rate and yield

MECs can be applied to biohydrogen production [2] , CH_4, H_2O_2 and formic acid production [2, 47, 48] , wastewater treatment [49], environmental sensors [50], and bioremediation [51] . In recent years, a remarkable interest on MEC technology, majorly due to the possibility of integration with other bioprocess technologies such as DF biohydrogen process [18, 52]. Electrochemically active bacteria (EAB) can completely convert biodegradable organic matter into H_2 and CO_2. EAB are very ineffective in metabolizing directly complex organics as electron donors do in MFCs or MECs [53-55]. DF effluents consist of a high proportion of volatile fatty acids (VFAs) and MECs that may be viewed as '*electrochemical factories'* where electricity is generated under microbial action. The possible organic matter for MEC are confined to certain compounds, such as acetate, cellulose, starch and wastewater [56, 57]. Such

two-stage process integration scheme has been investigated to exploit carbon rich effluents of DF biohydrogen reactor as potential substrate for harnessing more biohydrogen. It was supposed that this two-stage process integration schemes have the high substrate conversion efficiency (90%) of MEC system. Moreover, integration of MEC technology with DF process reactions accounting for more H_2 generation could be potentially a solution to achieve higher overall hydrogen yield and to improve total substrate conversion efficiency [18]. Several research groups have reported a significant enhancement in the generation of H_2 and the corresponding yields in a joint DF and MECs system using acid-rich DF effluents from various substrates [52, 58, 59] (Figure 3). Lalaurette et al. [60] assessed a two-stage bioprocess for hydrogen generation which used both DF with cellulose and a MEC, and found that there had been an enhancement in the net biohydrogen yield to 9.95 mol H_2/ mole glucose from the fermentative hydrogen yield of 1.64 mol H_2/ mol glucose using cellulose. Babu et al. [61] reported a peak H_2 generation rate of 0.53 mmol/h and a cumulative biohydrogen generation of 3.6 mmol, for a 49.8% of the VFAs metabolized at 0.6 V [61]. Similarly, Liu et al. [58] reported that the biohydrogen yield and generation rate had peaked at 1.2 mL H_2/mg COD and 120 mL H_2/g VSS/d, respectively, whilst in another work, Li et al. [54] indicated a 5 mmol H_2/g-corn stalk yield coupled with a 81–91% removal of acetate. Moreover, Moreno et al. (2015) recorded 94.2 L H_2 /kg VS from a two-stage DF-MEC system with cheese whey wastewater, whilst Dhar et al. [62] calculated net H_2 yield of 25% of the total COD from the metabolic conversion of sugar beet juice in an integrated biohydrogen generation uint.

2.2.2.2. DF and MFC coupled system for efficient treatment of DF effluents

One of the main shortcomings with DF H_2 generation relates to the much-observed low bioconversion extents of the organic matter in the substrates. MFC technique has fortunately addressed this limitation reasonably very well by further harnessing the residual untapped bioenergy from the available substrates [46]. MFC is a bio-electrochemical device which converts the chemical energy in the naturally available substrates into electric energy with the use of exoelectrogenic microorganisms [63]. MFCs consist of anode where the bio-catalytic action takes place between substrates and the biofilm formed on the electrode surface [63]. The generated electrons reach anode electrode and passes through the external circuit were current is produced. The protons migrate through the proton exchange membrane via anolyte and gets collected at the cathode were the electron, proton and oxygen combined to form water [64]. MFCs are simultaneously used for wastewater treatment and the production of clean energy from biomass. Thus, use of this MFCs has a great potential in broad applications, such as bio-sensing applications, emergency locator transmitters (ELTs), recently researchers designed origami MFC which is able to transmit radio signals in parallel configuration [65].

MFCs can be operated with a wide range of complex organics [63]. Besides, the effluents from the bioreactor are rich in volatile fatty acids such as acetate, propionate, butyrate etc., which serve as a potential source of MFC fuel. The acid rich effluents of bioreactor are rich in readily biodegradable organic matter and could be effectively consumed by the electrochemically active anodic biocatalyst for bio-electricity generation with concurrent waste remediation [66]. Such two-stage process integration strategy has been investigated to exploit carbon rich effluents of dark fermentative biohydrogen reactor as potential substrate for additional energy generation in the form of bio-electricity (Figure 4) [18, 63].

Sharma and Li [67] reported coupled biohydrogen and electricity generation from glucose fractions from wastewaters fed at variable organic loading rates. Furthermore, Mohanakrishna et al. [68] demonstrated the bioconversion of VFAs in batch-mode hydrogen-producing DF bioreactor equipped with an MFC and successfully came to observe the generation of electrical energy and organic matter solubilisation of up to 80%. Moreover,Pandit et al. [20] reported the following in their work based on MFC: 8.23 mol H_2/kg COD; reductions in the amounts of COD and total carbohydrates to the tune of 85% and 88%, respectively; and an electrical power production of 3.02 W/m^3.

2.2.3. Integrating DF with biomethane production process

Biomethane production process involves oxidation of carbon-rich substrate by a group of microorganisms that works syntrophically under anaerobic conditions. However, hydrolysis/breakdown of the complex organic substrate into simple sugars/compounds and methanogenesis are considered as major limiting steps in this process. Substrate hydrolysis is limited/influenced by fermentative biocatalysts whose optimum pH is around 5.5, despite the fact the optimum pH for methanogenic biocatalysts is around 7.0. Therefore, by separating this substrate hydrolysis process from methanogenesis, both processes can be improved independently. The benefits of acidogenesis-methnaogenesis two-stage integration schemes include: (i) utilizing complex organic substrate for biohydrogen production in the first stage which is cleaner than biogas alone; (ii) utilization of reactor effluents from H_2 bioreactor for reaching better recoveries in the second stage [18]. This type of two-stage system found to be suitable for solid organic wastes those are rich in carbohydrates [69] whereby significant biogas and COD removal efficiency was observed. Thus, a two-stage integration scheme of bio-H_2 and bio-CH_4 generation needs a dissimilar process handling to single stage bio-H_2/CH_4 generation.

Sequential anaerobic digestion (methanogenesis) of the residual low-end carbon rich metabolites issued from the H_2 bioreactor for additional energy generation has indeed to be considered to make the entire process viable [18].

2.2.4. Integrating DF with biopolymers/bioplastics production process

The utilization of DF effluents waste streams for biopolymers production makes the process more sustainable, economically feasible. These carbon-rich effluents are promising feedstocks for efficient production and accumulation of biopolymers (namely polyhydroxyalkanoates, PHA) and polyhydroxybutyrate (PHB) in bacterial cells at second stage integrated bioprocess. The PHAs are a biopolyesters of PHA which accumulates as cellular reserve storage materials and are formed under additional nutrient and carbon deprived circumstances [66, 70, 71]. The production of PHA by single-strains cultures by feeding synthetic substrates as carbon source (e.g., acetate, butyrate, etc), which is economically not viable for its production at large scale. These volatile fatty acids are simple low-end acid rich metabolites with a lower number of carbons, which enables PHA production by the involvement of a less number of enzymes when compared to glycolysis and β-oxidation [66]. During application of mixed culture for bioplastics production using DF effluents enrichment step to select microorganisms it is important to enhance PHA storing capacity and PHA yield [72]. However, enrichment step mainly depends on the presence or absence of carbon source, an electron acceptor as well as cycle length and aeration. Moreover, organic loading rate, pH and nitrogen limitation are also need to be optimized to enhance the PHB accumulation in the microbial system [73, 74]. Recently, the production of PHB from diverse fatty acids and carbon rich organic effluents from a dark fermentative biohydrogen process were investigated under an

anoxic condition by employing a mixed culture/microbial population [18, 66, 75]. Ntaikou et al. [76] explored the combined production of biohydrogen from DF of olive mill wastewater and polyhydroxyalkanoates (PHAs) production using the resulted DF effluents. Recently, Patel et al. [26] utilized pea shell slurry for integrated H_2 and PHB production using defined mixed culture (MMC4) of *Enterobacter*, *Proteus*, *Bacillus* spp.) in batch as well as continuous mode operation. They have also immobilized the microbial culture on coconut coir which results in increase in both H_2 yield and PHB content. Moreover, some investigators studied the feasibility of coupled biohydrogen and polyhydroxyalkanoate production using *Calophyllum inophyllum* oil cake by *Enterobacter aerogenes* and *Rhodobacter sphaeroides* under dark and photo fermentation conditions [77] .The biopolymers of PHAs could be used in food processing industries for packaging purposes [73, 78]. Thus, biopolymers production integrated with biohydrogen production process enabled the whole process to be more economically viable [79].

2.3. Microalgae cultivation on DF effluents

In this scheme, DF effluents that contain 67 to 80% of the initial COD in forms of variable liquid metabolites can be used to support heterotrophic or mixotrophic microalgae growth [17] . Such process combination is in favour of a full valorization of DF end-products, where microalgae biomass could return to the fermentation process as additional biomass. Although the organic matter that can be used as a substrate in DF can be from various origins, it is well accepted that H_2 generation from first generation biomass, ie. crops, enters in competition with food usages of lands and subsequent ethics conflicts, and should be firmly precluded. Therefore, second and third generation biomass, respectively the remaining biomass after crop harvesting and waste/algal biomass should be seriously considered for bioenergy production. Amongst the possibilities of 2^{nd} generation biomass to generate hydrogen by DF, the substrates

containing high amount of soluble sugars have only to be considered, in regard to methanogenic reactors where more complex biomass could also be degraded by hydrolytic bacteria ([80]. Recently, microalgae biomass as 3rd generation feedstock was considered as potential biomass feedstock with significant H_2 production [81, 82]. In this case, mixed bacterial cultures are strictly required to increase the potential of biomass conversion and the origin of seed microbial inoculum plays a crucial role, suggesting a prerequisite of time adaptation to the biomass.

Overall, the fermentation process can be coupled to a microalgae-based bioreactor where microalgae are grown under heterotrophic (no light) or mixotrophic (low light) conditions to produce more biomass feedstock that can be further recycled in the first step process [17, 81]. Heterophic/mixotrophic growth of microalgae could also be used to accumulate carbohydrates or biolipids that are naturally produced when microalgae are grown on carboxylic acids under starvation conditions, eg. nitrogen deficient conditions [17, 81]. As heterotrophic microalgae model, *Chlorella sorokiniana* was reported as an efficient consumer of several types of carbon sources, such as acetate, in absence of light to produce lipid-rich microalgal biomass [17]. Many other genera of microalgae have been reported to be able to accumulate carbohydrates or biolipids (from 20 to 77% of biomass weight) under starvation conditions (nitrogen, phosphorus, sulfur, and silicon) either under autotrophic or heterotrophic growth [83]. Most efficient strains are related to *Botryococcus braunii* (high lipid accumulator), *Chlorella* sp. - *Chlamydomonas reinhardtii* - *Scenedesmus* sp. - *Dunaliella* sp. (as models of study), new strains from the *Selenastraceae* family such as *Monoraphidium* sp., as well as more particular strains of *Monodus subterraneus*, *Monallanthus salina*, *Nannochloropsis Sp*, *Neochloris oleoabundans*, *Schizochytrium sp*, [83-86]. Some of the genera are able to grow under strict heterotrophic (no light) or mixotrophic (low light) conditions making the bioprocesses more compact with higher

cell concentrations (from 4 g/L with raw effluents up to 109 g/L with synthetic organic carbon) [17]. By using organic compounds as external carbon source, lipid oil content can be increased up to four times when compared to autotrophic conditions [86]. However, most of the study focusing on microalgal heterotrophy have dealt with simple sugars, ie. glucose or fructose, as main carbon sources, making the process costly and unsustainable. The use of DF effluents is an interesting alternative to make the process more economically feasible while similarly supporting the microalgal growth in well-known fermenter technology based reactors [17].

In this context, *Chlorella* sp. has been widely investigated as a model of heterotrophic growth. Miao and Wu [87] reported a lipid content of 55% when grown heterotrophically on glucose at 10g/L, versus less than 15 % under phototrophic conditions. Chen et al. [81] compared the mixotrophic growth of *Chlorella vulgaris* FSP-E, *Scenedesmus subspicatus* GY-16 and *Anistrodesmus gracilis* GY-09 under N depletion for their ability to store carbohydrates with acetate as sole carbon source. Interestingly, when compared to phototrophic growth, the authors reported an increase of 25% and 30% of biomass and carbohydrate productivities, respectively. When considering the growth on fermentation end-products, only few studies have dealt with lipid production [17]. Especially, the metabolic pattern of the fermentation effluents constitutes the key bottleneck when coupling DF and microalgal growth [17]. Indeed, while acetate was found favorable to microalgal hetrotrophic growth, butyrate may affect the growth of *Chlorella* sp. even at a low concentration of 0.25 g/L [88]. Interestingly, when considering a mixture of acetate and butyrate, as found in DF effluents, a diauxic growth of *Auxenochlorella protothecoides* and *Chlorella sorokiniana* was observed with first consumption of acetate prior to butyrate uptake [88]. Turon et al. [17]. suggested considering the total VFAs concentration as well as the acetate:butyrate ratio that has to be respectively lower than 10g/L and higher than 2.5

to be favourable to heterotrophic microalgal growth. Venkata Mohan and Devi [89] showed that a mixture of fermented fatty acids issued from a DF reactor supported the biomass growth up to 1.42 g/L with a lipid yield of 26.4%. Although the acetate:butyrate ratio was not optimal with a value of approximately 1.4, the growth was mainly supported by acetate with only low impact of butyrate and propionate. Consistently, Fei et al. [90] reported that VFAs supported the heterotrophic growth of *Chlorella protothecoides*. With an acetate:butyrate ratio of 2 and a total concentration of 2 g/L and with urea (500 mg/L) as sole nitrogen source, the authors reported a high lipid content of 48.7% and a maximal microalgae cell concentration of around 0.55 g/L with only low degradation of butyrate and propionate. The use of these compounds by heterotrophic microalgae constitutes the main current challenge remains therefore to full valorization of DF effluents and the selection of microalgae potentially able to grow on butyrate and others DF metabolites, such as propionate, lactate for further carbohydrates or lipid production are crucial to make sustainable such process coupling [17].

Apart from the composition in metabolites, another factor is the possible contamination of the microalgal reactor by bacteria issued from the DF fermenter. When considering *Chlorella* sp. on raw or synthetic DF effluents, the microalgae outcompeted successfully the bacterial populations, with an extra carbon yield reaching 55% on DF effluents likely due to nutrients issued from the effluents [27].

3. Scientific and technological challenges for integrated schemes

Successfully developing and implementing such integrated schemes for the utilization of DF effluents to energy and value-added bio-products would demand to approach a number of interrelated scientific, engineering, and to some extent, the technological challenges before these

techniques and bioenergy-producing equipment may be brought into large scale usage. Elucidating the interdependencies and sensitivity of the key operational process parameters is more than needed in view to optimize the process performances and energy output.

- During photofermentation, main scientific challenges are the high cost of photo-bioreactors, low light conversion efficiency as well as excess energy demand of nitrogenase which make the process expensive and less efficient. These bottlenecks can be overcome by maintaining proper media composition, to increase light conversion efficiencies can be possible by reducing antenna size, developing H_2 impermeable plastics bioreactor, and using metabolic engineering approaches to replace N_2ase with H_2ase.
- Similarly, during methanogenesis process main challenges are requirement of different media as well as microbial growth is not constant. This can be solved by using large size reactors, applying higher HRT and using low-cost alkalinization method.
- Moreover, microbial electrolysis processes have some limitation for commercial applications including the requirement of expensive precious metal cathodes, the higher voltage required for significant yield and resulted current densities need to be increased. These challenges can be overwhelmed by developing inexpensive cathodes (Ni, stainless steel), by designing better anode geometry, by eliminating H_2 cycling metabolic reactions as well as by developing engineered cells with lower internal resistance.
- Reproducible and reliable factorial experimental design approaches have to be formulated to better probe the influence of the different biological, physical and chemical parameters, their optimization and interactions on the desired process dynamics.

- Moreover, the essential relevant techniques of metabolic and biological engineering should be tapped to produce microbial strains which may champion the metabolic process reactions in view to overexpress the biohydrogen and/or electrical power production in integrated systems.
- Although tremendous and extensive R&D is required before the integrated hybrid system can be deployed on a practical level and economically feasible.

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Table 1: List of few two-stage integration schemes investigated with DF hydrogen production.

	First stage DF (H_2)	**Second stage Photo Fermentation**	**References**
Substrate	**HY mol H_2/ mol substrate**	**HY mol H_2/ mol substrate**	
Cassava starch	2.53	3.54	[36]
Cassava starch	2.00	0.86	[39]

Hydrolyzed			
Cheese Whey Wastewater	2.04	2.69	[29]
Corn stalk	163[a]	339[a]	[91]
Glycerol	0.57	0.68	[92]
Starch hydrolysate	0.54	1.07	[34]
Sucrose	0.98	4.48	[41]
Sucrose	1.90	3.22	[93]
Sugar beet molasses	2.10	4.75	[35]
Sweet potato starch	2.4	4.6	[94]
Sweet potato starch	2.7	4.5	[95]
	First stage DF (H_2)	**Second stage MFC (Electricity)**	
Fruit juice industry WW	1.4	550 [c]	[96]
Cereal WW	0.79	371 [c]	[97]
Cellulose	2.92	85 [c]	[98]
Glycerol	0.55	92 [c]	[99]
Glucose	2.72	4.2 [d]	[67]
Molasses	1.58	3.02 [c]	[20]
Vegetable waste	0.56	111.76 [c]	[68]
	First stage DF (H_2)	**Second stage MEC (H_2)**	
Cassava starch WW	260 [e]	205 [e]	[100]
Cellobiose	1.64	0.96 [f]	[61]
Corn stover	1.6f	1.00 [f]	[60]
Corn stalk	129 [a]	257 [a]	[101]
Fruit juice wastewater	95 [f]	1478[f]	[102]
Sugar production wastewater	18.8 [f]	344 [f]	[102]
Vinnase residues	35.4 [f]	1399 [f]	[102]
	First stage DF (H_2)	**Second stage AD (CH_4)**	
Cassava WW	54 [a]	164 [g]	[103]
Cornstalk	58 [a]	200 [g]	[104]
Food waste	85 [a]	63 [g]	[70]
Food waste	290 [a]	240 [g]	[105]
Food waste	205 [a]	464 [g]	[106]
Food waste (pilot scale)	66.7 [a] and 1 [f]	490 [g] and 1.9 [f]	[107]
Laminaria japonica)	115.2 [a]	329 [g]	[108]
Microalgal biomass	135 [a]	414 [g]	[109]
OFMSW	43 [a]	500 [g]	[110]
OFMSW:WAS (Waste activated sludge) 1:5	29 [a]	287 [g]	[111]
Pulverized garbage	5.4 [f]	6.1 [f]	[112]

and shredded paper wastes			
Rice straw	20 [a]	260 [g]	[23]
Vegetable waste	17 [a]	61.7 [g]	[70]
Water hyacinth	0.38 [f]	0.29 [f]	[113]
Water hyacinth	51.7 [a]	43.4 [g]	[114]
	First stage DF (H_2)	**Second stage PHA**	
Distillery spent wash	142 [e]	40% [h]	[66]
Biological waste	54 [a]	41.7% [h]	[115]
Food waste	118 [e]	36% [h]	[116]
Food waste	9.3 [e]	39.6% [h]	[117]
Glucose	1.92	11.3% [h]	[118]
Glucose	0.58	18.6% [h]	[119]
Glucose	1.92	8.8% [h]	[120]
Olive oil mill WW	196.2 [a]	8.9% [h]	[76]
Pea-shells	54 [e]	64.7% [h]	[26]
Sweet sorghum	0.68	71.4% [h]	[121]
Taihu blue algae	105 [e]	43.3% [h]	[122]
	First stage DF (H_2)	**Microalgae**	
Food waste	69 [a]	26.4% [h]	[89]
glucose	1.37	55% C-based biomass yield	[88]
Glucose:xylose (9:1)	2.78	1.12 [i]	[123]
Glucose	110 [a]	1.57 [i]	[124]
Glucose	272 [a]	1.98 [i]	[125]
Starch	198 [a]	1.27 [i]	[126]

a- mL H2/g- substrate; b- mmol H_2/day ; c- mW/m^2; d- mW/m3; e- mL H_2/g COD; f - L/L-d; g- ml CH_4/g biomass, h- dry cell weight; i- algal biomass g/L; WW- wastewater

Table 2 Pros and Cons for the Integrated schemes with dark fermentation process

Process	Pros	Cons	Strategies to overcome/improve process performances

PF	+Maximum substrate utilization +Additonal energy production (H_2) + Catabolize DF effluents	- ammonia inhbition - biomass from DF effluents affects growth and hydrogen yield - high cost photobioreactors	Co culture of DF and PF process to reduce the toxicity of PF bacteria
BESs	+ Harnessing more H_2 production + Electricity generation + Effective COD removal	- Energy input needed to stimualate the electrochemical reaction - Expensive bioreactor setup	Modification of the BESs bioreactor to improve the process efficiency
AD	+ Effective COD removal + Maximum bioenergy potential + Combined with hydrogen to produce a upgraded hythane biofuel	- Slow digestion process - Microbial community senstive to high organic loading rates	Large size bioreactors with different support media to colonize the methanogens and application of long HRT to enhance methane production.
PHA	+ Alternative source for renewable PHA production + Biotransformation of DF effluent to useful product	- Sterilization needed for maximum PHA production	Combination of defined mixed culture is an useful strategy to improve the PHA production from unsterile DF effluent
Microalgae	+ Effective	- Microbes present in	Selective enrichment of algal

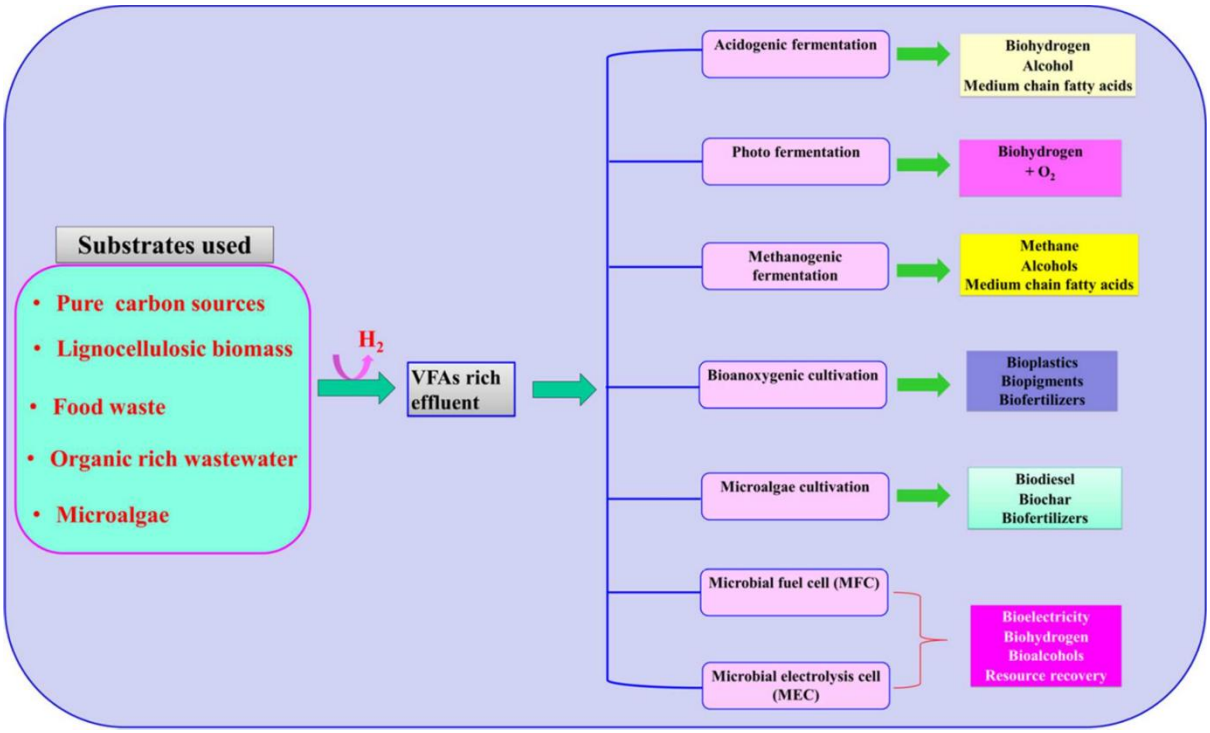
	utilization of nitrogen and phosphorus source + Utilization of VFAS for biodiesel production	DF effluent affects the algal growth - Distrubution ratio of VFAs affected the biomass yield	species to utilize the DF effluents towards carbohydrates or lipid production

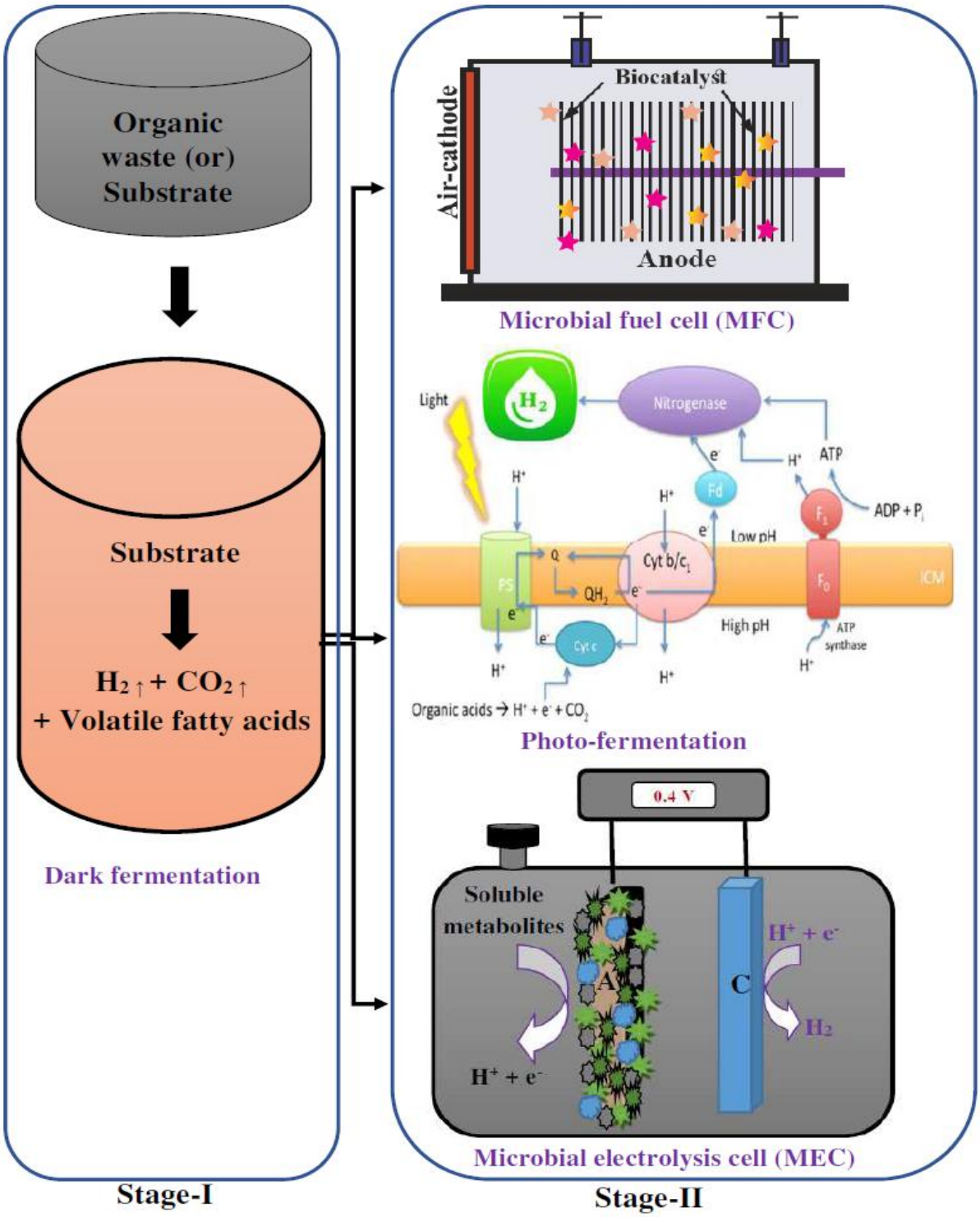
PF- photofermentation; BESs-bioelectrochemical systems; AD- anerobic digestion; PHA-polyhydroxyalkonates

Figure captions

Figure 1: Integrative scheme of valorization of VFAs rich effluent to various value-added products.

Figure 2: Schematic presentation of integration scheme, Stage-I: Dark fermentation (DF) and Stage-II (Microbial fuel cell (MFC), Photo-fermentative hydrogen production process and Microbial electrolysis cell (MEC).


Substrates used
Pure carbon sources
Lignocellulosic biomass
Food waste
Organic rich wastewater
Microalgae
H2
VFAs rich effluent
Acidogenic fermentation
Biohydrogen
Alcohol
Medium chain fatty acids
Photo fermentation
Biohydrogen
+ O2
Methanogenic fermentation
Methane
Alcohols
Medium chain fatty acids
Bioanoxygenic cultivation
Bioplastics
Biopigments
Biofertilizers
Microalgae cultivation
Biodiesel
Biochar
Biofertilizers
Microbial fuel cell (MFC)
Microbial electrolysis cell (MEC)
Bioelectricity
Biohydrogen
Bioalcohols
Resource recovery


Organic waste (or) Substrate
Substrate
$H_2 \uparrow + CO_2 \uparrow$ + Volatile fatty acids
Dark fermentation
Stage-I
Air-cathode
Biocatalyst
Anode
Microbial fuel cell (MFC)
Light
H_2
Nitrogenase
ATP
ADP + P_i
Fd
Low pH
Cyt b/c_1
PS
Q
QH_2
ICM
High pH
Cyt c
ATP synthase
Organic acids → $H^+ + e^- + CO_2$
Photo-fermentation
0.4 V
Soluble metabolites
A
C
$H^+ + e^-$
H_2
Microbial electrolysis cell (MEC)
Stage-II

1

2

3

4