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Reviewing the potential of bio-hydrogen production by fermentation

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ABSTRACT

Hydrogen is a common reactant in the petro-chemical industry and moreover recognized as a potential fuel within the next 20 years. The production of hydrogen from biomass and carbohydrate feedstock, though undoubtedly desirable and favored, is still at the level of laboratory or pilot scale. The present work reviews the current researched pathways. Different types of carbohydrates, and waste biomass are identified as feedstock for the fermentative bio-hydrogen production. Although all techniques suffer from drawbacks of a low H_2 yield and the production of a liquid waste stream rich in VFAs that needs further treatment, the technical advances foster the commercial utilization. Bacterial strains capable of high hydrogen yield are assessed, together with advanced techniques of co-culture fermentation and metabolic engineering. Residual VFAs can be converted. The review provides an insight on how fermentation can be conducted for a wide spectrum of feedstock and how fermentation effluent can be valorized by integrating fermentation with other systems, leading to an improved industrial potential of the technique. To boost the fermentation potential, additional research should firstly target its demonstration on pilot or industrial scale to prove the process efficiency, production costs and method reliability. It should secondly focus on optimizing the micro-organism functionality, and should finally develop and demonstrate a viable valorization of the residual VFA-rich waste streams.

1. Hydrogen and traditional production methods

Hydrogen is a common reactant in the petro-chemical industry and recognized as a potential fuel within the next 20 years. IHS Chemical expects an annual worldwide increase of the demand for hydrogen by nearly 4–5% during the next five years. Asia continues to dominate the increased demand due to the progressive growth of its domestic economies [1]. The major applications of hydrogen are at present in processing fossil fuels and in synthesizing ammonia. Hydrogen also serves as a reactant in the production of methanol. Minor applications include the hydrogenation of fats, fuel cells and the demonstration in hydrogen-fuelled vehicles. The worldwide distribution of the consumption of hydrogen is summarized in Fig. 1.

With depleting fossil fuels, bio-hydrogen is increasingly considered as a potential renewable and top energy source for its clean combustion and its high specific energy, i.e. 123 MJ/kg or ~3 times higher than petro-based fuels (~46 MJ/kg). The enhanced demand of hydrogen has been significantly influenced by its use in the desulfurization of transportation fuel, and by the growth of the transportation sector [2]. Co-currently the quality of crudes is diminishing, leading to a decrease of hydrogen generation from crude processing. This has caused refineries to re-evaluate the hydrogen availability. Numerous research is being conducted on appropriate hydrogen production [3–7], storage [7–12] and application [5,6]. Though hydrogen is proposed as an alternative for fossil fuels, this application is however depending on the availability of technologies to enable a sustainable production of hydrogen, most of which are still under development. Bio-hydrogen could soon be entering the fuel market, provided the cost of the pre-cited steps decreases considerably [5,13]. Under this circumstance, worldwide ongoing research on hydrogen generation and storage is intensive.

Hydrogen can be prepared through various methods. Refineries, as mentioned above, are large-volume consumers of hydrogen as well as producers. Till now, fossil fuels are the main feedstock of 96% of all hydrogen: natural gas accounts for ~49%, liquid hydrocarbons are involved for ~29% and coal represents ~18% of the hydrogen production. Electrolysis (3.9%) and other by-product sources of hydrogen

(0.1%) make up the remaining 4% [4–6].

Steam reforming of methane from natural gas or syngas is now economically the most important process of industrial bulk hydrogen production, and also involves removal of hydrogen from hydrocarbons [14]. At high temperature, i.e. 700–1100 °C, steam (water vapor) and methane react to form hydrogen and carbon monoxide:

$$CH_4 + H_2O \rightleftharpoons 3H_2 + CO \tag{1}$$

The CO produced can further react with steam to yields extra H_2 through the water-gas-shift (WGS) reaction:

$$CO + H_2O \rightleftharpoons CO_2 + H_2 \tag{2}$$

Electrolysis of water is responsible for about 3.9% of the worldwide hydrogen production [6], and is normally used onsite:

$$2H_2O \rightarrow 2H_2 \text{ (g)} + O_2 \text{ (g)} \tag{3}$$

Most industrial electrolysis cells apply complex platinum electrodes (plates or honeycombs). Generally, electrolysis as a hydrogen production method is only used for specific applications such as oxy-hydrogen torches or when extremely high purity hydrogen is required.

Innovations such as the application of Polymer Electrolyte Membrane (PEM) electrolysis [15] and microbial reverse-electrodialysis electrolysis cells [16] are currently under investigation. New configurations of a typical water electrolysis system are evaluated. The efficiency of a water electrolysis hydrogen production system under different operating conditions was calculated in detail by Zhang et al. [17], which can be helpful for further understanding and developing water electrolysis systems for H_2 production.

Although bio-hydrogen produced from renewable sources could be a promising production method, its contribution is below 1% so far.

Large industrial-scale hydrogen production from biomass or carbohydrates, i.e. hydrogen by fermentation, is undoubtedly desirable and favored, however still at the level of laboratory or pilot scale, as assessed in section 2 below. Strategies for industrial-scale production need to be developed, and further research is still needed.

Since hydrogen yields are reported to be low, it is important to select feedstock that is cheap, rather than using carbohydrate-rich raw materials such as starch, corn or sugars, of high commercial value and contributing to the human food-requirements. Waste biomass streams, with lower but accessible concentrations of carbohydrates, and offered at a limited or at zero cost (since needing to be treated prior to discharge) are economically the selected feedstock for bio-hydrogen production. It is therefore important to compare the different feedstock towards their real hydrogen production potential.

The hydrogen production by fermentation generally refers to three main categories, i.e. firstly by dark-fermentation, where no light is involved; secondly by photo-fermentation with light as the source of energy; and thirdly by combining photo- and dark-fermentation.

It should also be mentioned that the production of hydrogen by biomass gasification is often considered within the bio-production scope. The gasification technique is however of thermo-chemical nature, and hence not within the category of biochemical production methods. Gasification is well-documented in literature for both laboratory, pilot and industrial scale, with numerous review and research papers available. Gasification is therefore not considered in the present biochemical production review.

2. The bio-production of hydrogen through fermentation

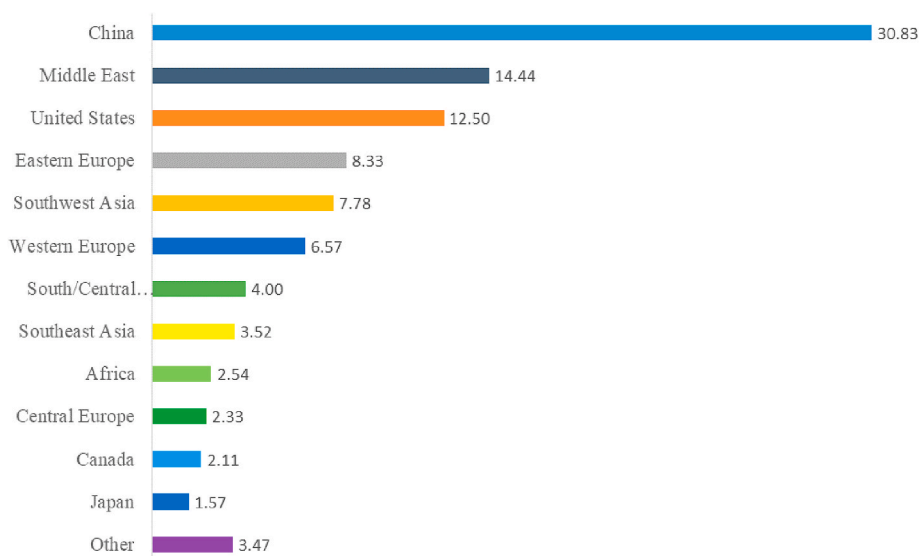In dealing with the fermentation production of H_2, several aspects

Fig. 1. Geographic distribution (%) of the worldwide hydrogen consumption.

should be considered to understand the selected feedstock and the principles of the principal technologies used, i.e. dark and photo fermentation, and their combined 2-step application. These aspects are dealt with below.

2.1. Carbohydrate substrates utilized for bio-hydrogen production

According to the fermentation principles, the use of carbohydrates offers the highest potential. Carbohydrates such as glucose, sucrose and starch were initially investigated, despite their high cost (300–1000 €/ton), hence hampering large-scale production and competing with the food cycle.

There are however numerous waste products that contain a wide range of carbohydrates [18] and are available at very low cost, since otherwise to be treated to meet environmental standards [19,20]. These waste feedstock streams can be of industrial, agricultural, municipal or forestry origin.

For most of these waste feedstock materials, pretreatments are necessary to liberate e.g. glucose by saccharification of the macromolecules. It should however be remembered that the pretreatment processes can generate inhibitors unless the pretreatment method is carefully selected, as illustrated in Table 1, where these substrates and there required pretreatments are summarized.
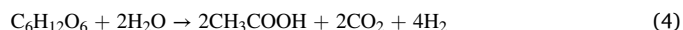
For cellulosic substrates, various chemical, biological and enzymatic pretreatments are possible [21–24]. These pretreatments can disrupt the stable structure of cellulosic materials, resulting in a higher surface area and an increased hydrolysis rate of cellulose materials. They can also decrease the degree of crystallinity and of cellulose/hemicelluloses polymerization [25]. Besides pretreatment, other measures can also be taken to increase the bio-hydrogen yield of cellulose substrates, including manipulation of carbon and electron flows through a metabolic shift caused by end-product inhibition, metabolic engineering at the genetic level, fermentation by co-cultures, and appropriate reactor designs [26,27].

Recently, also algae have been considered as potential feedstock, due to their content of polysaccharides and carbohydrates, e.g. between 8

and 30 wt% in microalgae and a lower wt% in macro-algae [46]. Further research is however required before the H$_2$ yield can be validated and economically assessed.

2.2. Dark fermentation

Dark fermentative avoids the problem of needing light as the necessary energy resource. The production proceeds mostly through the acetate and butyrate pathways [47].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (4)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (5)$$

Most literature sources investigate the microorganism species of the *Clostridium* genus because of their high hydrogen production rates. In addition, these bacteria possess ideal properties for industrial application, including forming endospores and high growth rates [47]. *Clostridium sp.* also allows mixed culture hydrogen production, in which the variety of species present facilitates the efficient decomposition and conversion of organic waste into hydrogen. The conversion process is generally accompanied by organic acid production [47].

The fermentative hydrogen production process is self-inhibiting once the partial pressure of hydrogen exceeds a critical value, which induces a switch to a different metabolic pathway in which acetyl-CoA and CO$_2$ are produced instead of hydrogen and organic acids [48] (Fig. 2).

Different bacterial strains have been used in the dark fermentation of various carbohyfdrate feedstock. Productivities are generally expressed in the molar amount of H$_2$ produced per mol of substrate. Results are depicted in Fig. 3. With molecular weights of 180 g for glucose and galactose, 342 g for sucrose and lactose, and 2 g/mol for H$_2$, the production yields can be converted into g H$_2$/g substrate. Waste water treatment sludge was also used as substrate for DF by *Chlostridium bifermentants* and *Pseudomonas* sp. GZI, however with low H$_2$ yields (0.9 mmol/g dried sludge, or about 0.1 mol/mol dried sludge, if the molecular formula of activated sludge is approximated as C$_5$H$_7$O$_2$N) [50,51].

Other commonly used enteric bacteria, such as *Escherichia coli* and

Table 1
Waste substrates utilized for fermentation bio-hydrogen production.

| Waste sources	Substrates	Pretreatment	pH	Inhibitors	Hydrogen yield
Industrial	Paper solid waste [28]	Crushed to < 0.5 mm	2.5% H$_2$SO$_4$	–	61.1 mmol/h/g
	Waste peach pulp [29]	Boiled for 45 min	–	–	123.27 mL/g TOC
	Wastewater from citrus processing [30]	suspended	–	–	85.4 mmol/L
	Vinasse from citrus processing [30]	solids and settled inorganics removed	–	–	13.4 mmol/L
	Vinegar residue [25]	heat (99 °C for 30 min)	1(10 mL HCl/g)	NH^4–N, acetate, butyrate	53.2 mL/g VS
	Waste activated sludge [31]	γ-irradiation	12.0	Low pH	1.07 mL/L sludge
	Sewage sludge [32]	Heat treatment (150 °C for 30 min) with alkaline condition	–	Ammonia	39.0–220.3 mL/L sludge
	Cassava starch wastewater [33,34]	Without treatment	–	-, VFAs and alcohols	287 mL/g starch, 0.3 mol H$_2$/mol
Agricultural	Dairy cow solid waste [35]	Dried and crushed; hydrolyzed the dilute acid	–	VFAs	97 mL/g
	Cassava starch [36]	Suspended	–	Low pH	1.72 mol/mol glucose
	Corn starch [37]	Heated at 100 °C for 30 min	–	Low pH	1.94 mol/mol glucose
	Wheat straw [38]	120 °C for 90 min	2% H$_2$SO$_2$	–	1.19 mol/mol glucose
	Wheat straw [39]	Enzyme treatment	–	–	19.64 mL/g-VS
	Sugarcane bagasse [40]	Raw bagasse heated at 120 °C for 30 min	sulfuric acid (1%, g/v)	Phenolics	62.1 mL/g non-detoxified sugarcane bagasse
Municipal	Fruits and vegetable wastes [41]	Crushed < 2 mm	–	Acetate, Lactate	3.46 mol/mol
	Waste paper [42]	Crushed <0.15 mm and Heat (120 °C for 90 min)	2.2	Furan	140 mL/g sugar
	Waste pastry [43]	Crushed <0.5 mm	–	–	241.4 mL/g glucose
	Food waste [44]	Crushed <0.5 mm	–	VFAs	57 mL/g-VS (50 °C)
Forestry	Poplar leaves [21]	2% vicozyme	–	Furfural	44.92 mL/g dry poplar leaves
	Waste sorghum leaves [45]	heat at 100 °C for 176 min	5.95% HCl	–	47.3 mL/g sugar
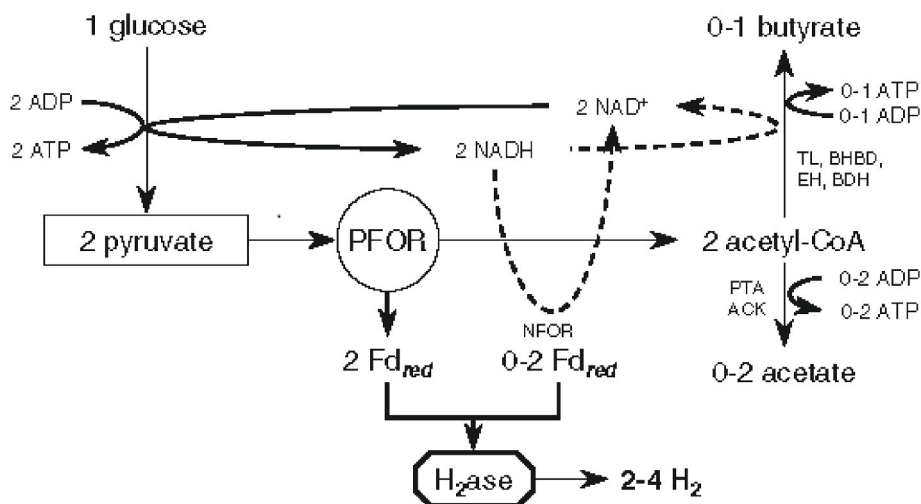
Fig. 2. Fermentation using e.g. *Clostridium butyricum* (Reprinted from Ref. [49]. With permission of Springer Nature).

Enterobacter aerogenes, can also be applied for hydrogen production via fermentation [62]. Unlike *Clostridia*, enteric bacteria primarily yield hydrogen via the decomposition of formic acid and formate, even the exclusive pathway in the case of *E. coli*. Since cleavage is not a redox reaction, it does not influence the redox balance in the fermentation. This conversion route does not suffer from inhibition in case of an increased hydrogen partial pressure since formate cleavage is an irreversible reaction. Furthermore, enteric bacteria are facultative anaerobes, showing high growth rates in the presence of oxygen. After oxygen depletion, an anaerobic metabolism takes over from an aerobic one. Under anaerobic conditions, their growth rate is reduced since less metabolic energy can be extracted from the same substrate. These properties offer the opportunity to develop an industrially interesting scheme of alternating aerobic (fast microbial grown to a high concentration) and anaerobic (high rate hydrogen production) conditions [63].

Controlling the microbial community in the fermenter is necessary to obtaining a sustainable industrial-scale hydrogen production because the used substrate may contain micro-organisms that affect the microbial community within the fermenter, leading to unfavorable conditions and lower hydrogen production rates. Necessary procedures should be taken into consideration, for example, sterilization [64]. Hyperthermophilic archaea (e.g. *Thermotoga neapolitana*) which can be operated at ~70 °C and leave little chance of feedstock contaminants becoming established, can also be used for hydrogen fermentation [65].
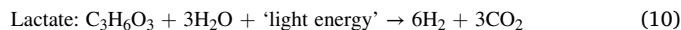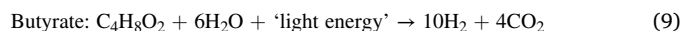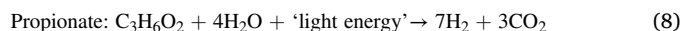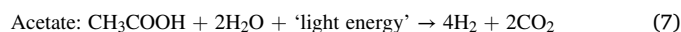
2.3. Photo-fermentation

Fermentation processes which employ light as an energy source for photosynthesis are referred to as photo-fermentation. Comparing to other routes, photo-fermentation has the advantage that light can replace sugars as source of energy, hence reducing the competition for land usage with food crops. The following chemical equation illustrates the hydrogen production via photo-fermentation.

$$16ATP + 16H_2O + N_2 + 10H^+ + 8e^- \xrightarrow{[hv]} 16ADP + 16pi + 2NH_4^+ + H_2 \quad (6)$$
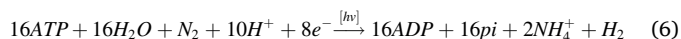
Photosynthetic bacteria such as *Rhodobacter sphaeroides* and *Rhodospirillum* are commonly applied and use small molecule organic acids as carbon sources [66]. Combining dark-fermentation as the first step and photo-fermentation as the second step can enhance the biohydrogen yield due to the enhanced conversion of substrates to H_2 [67]. In photo-fermentation, the purple non sulphur (PNS) bacteria can utilize the light energy for both the production of ATP and high energy electrons by reverse electron flow to reduce ferredoxin. The ATP and reduced ferredoxin thereafter drive the proton reduction to H_2 by

nitrogenase [68]. The accumulated dark-fermentation VFA can be further converted into hydrogen by photo-fermentation. The reactions from VFAs (mainly acetate, propionate, butyrate and lactate) into hydrogen are shown in Eqs. (7)–(10). An obvious advantage for photo-fermentation is the higher theoretical H_2 yield in comparison with dark fermentation. Moreover, the PNS microorganisms are moreover able to absorb and utilize a wide spectrum of light (400–900 nm), and can also use organic substrates derived from various wastes [69,70].

Acetate: $CH_3COOH + 2H_2O + \text{'light energy'} \rightarrow 4H_2 + 2CO_2$ (7)

Propionate: $C_3H_6O_2 + 4H_2O + \text{'light energy'} \rightarrow 7H_2 + 3CO_2$ (8)

Butyrate: $C_4H_8O_2 + 6H_2O + \text{'light energy'} \rightarrow 10H_2 + 4CO_2$ (9)

Lactate: $C_3H_6O_3 + 3H_2O + \text{'light energy'} \rightarrow 6H_2 + 3CO_2$ (10)

Among *algae* and some bacteria, hydrogen production capabilities by oxygenic photosynthesis of *Cyanobacteria* are frequently cited [71]. *Purple non-sulphur* (PNS) bacteria (e.g. *Rhodobacter*) are moreover highly promising to hydrogen production via anoxygenic photosynthesis and photo-fermentation [62]. Light within a wavelength range of 400–1000 nm (visible and near-infrared; 400–700 nm; visible) can be used by *algae* and *cyanobacteria* [72].

The industrialization of natural photo-fermentation is restrained by the availability and the distribution of light, since self-shading needs to be minimized in an industrial scale photo-fermenter. The latter restraint imposes a considerable ratio of surface area to volume for an externally illuminated photo-bioreactor. In turn, this results in a material-intensive and hence expensive construction of the photo-bioreactor.

The optimization of light distribution via optic fibers for light transfer in the fermenter is one method that has been recently employed. An additional advantage of this type of photo-bioreactor is the possibility to filter out wavelengths that are of no use for the organisms, hence also controlling radiant heat-gain by dumping excess light.

A light-emitting diode (LED) fermenter was reported to be a potential industrial-size photo-fermenter with a limited area of land occupied; the LED fermenter is able to prevent self-shading and maintain photosynthesis within the fermenter with limited energy consumption and low installation costs. A maximum light conversion efficiency of about 10% has been reported using PNS bacteria. Combining this finding with a maximum efficiency of about 80% for generating electricity from hydrogen (via a PEM fuel cell) and a subsequent maximum efficiency of about 80% for light generation from electricity (via LED), returns are significantly hampered [62].

Photo fermentation furthermore requires specific substrates, i.e. mostly smaller fatty acids such as acetate, propionate and butyrate.
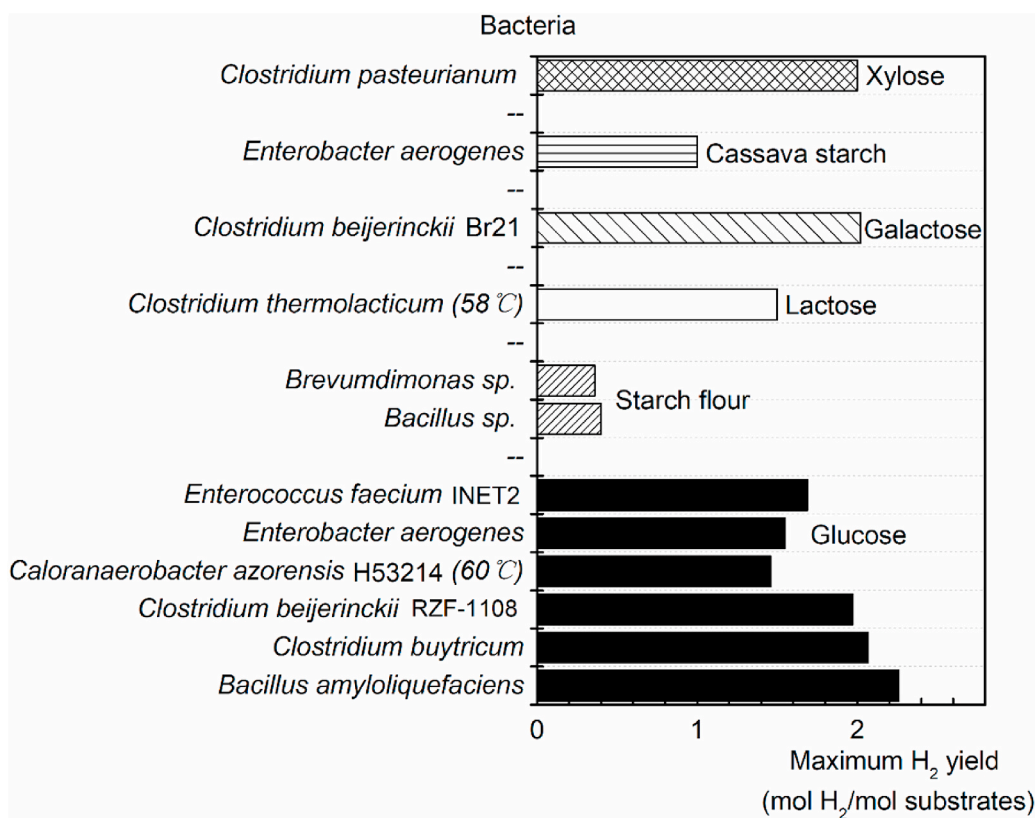
Fig. 3. Single bacteria strains for dark fermentation biohydrogen production, at temperature of 34–37 °C (unless indicated) with substrates xylose [52], cassava starch [53], galactose [54], glucose [50,53,55–59], starch flour [56,60] and lactose [61].

Towards the targets of fuel or energy hydrogen production, artificially lit photo-bioreactors such as the LED-fermenter were proven non-viable.

2.4. Combined fermentation

As briefly mentioned before, there are studies dealing with combining dark- and photo-fermentation and these studies consider the combined fermentation as the most promising technique for industrial fermentation of hydrogen because of its economy and because it reduces the amount of by-products of the fermentation process [62,73,74]. The major advantage of applying a combined fermentation is due to its capability for the photosynthesis step to reuse the otherwise useless organic acids. However, combined fermentation also faces the issues to optimize the design of the reactor and minimize the energy consumption, both major drawbacks of photo-fermentation.

Chen et al. [75] combined dark and photo fermentation for overall hydrogen yield and COD reduction. The dark fermentation effluent mainly comprised acetate and butyrate, and *Rhodopseudomonas palustris* WP3-5 was used as inoculum in photo fermentation. The total hydrogen yield could be enhanced from 3.80 mol H_2/mol sucrose to 10.02 mol H_2/mol sucrose, and a high COD removal of 72.0% could be obtained. They further found that acetate was the most favorable carbon later source for *Rhodopseudomonas palustris* WP3-5 in photo fermentation, whereas inhibition occurred when butyrate was used as the carbon source [76]. Chookaew et al. [77] investigated a two-stage process in which *Klebsiella sp.* TR17 was used in the first stage (dark-fermentation) and *Rhodopseudomonas palustris* TN1 was applied in the second stage (PF) to convert crude glycerol to hydrogen. In the first stage, a hydrogen yield of 5.74 mmol H_2/g COD consumed was obtained. In the second stage, a hydrogen yield of 0.68 mmol H_2/g COD consumed was achieved with addition of yeast extract (2.3 g/L), NaHCO₃ (0.63 g/L), and glutamate (2–8 mmol/L) in 5-fold diluted dark-fermentation effluent,

resulting in a total hydrogen yield of 6.42 mmol H_2/g COD consumed. Other researchers succeeded in converting the dark-fermentation effluent of hydrogen using photo-fermentation [78–80]. These efforts demonstrated that photo-fermentation was an effective in the further valorization of dark-fermentation effluent.

3. Specific problems and bottlenecks involved in the biochemical production methods (like yields, costs, and biomass waste)

This section will firstly demonstrate the pathways of hydrogen fermentation and then discuss the different parameters that affect the process as a result of the fundamental pathway. The overall assessment will highlight the bottlenecks involved in the hydrogen production, and dealt with in detail in section 4.

3.1. The fundamental metabolic pathways of dark fermentation

To investigate the biological effects during dark fermentation, Fig. 4 illustrates the metabolic pathways, where *Clostridium sp.* and *Enterobacter sp.* are the main species that are commonly used in bio-hydrogen production.

The metabolism can be divided into two separate pathways: the facultative anaerobic metabolism, such as through *Escherichia coli* (as indicated by PFL pathway, pyruvate formate-lyase) and strict anaerobic metabolism, through *Clostridium* (as indicated by PFOR pathway, standing for pyruvate: ferredoxin oxidoreductase).

Glucose in both routes breaks into pyruvate through standard glycolysis, in which process the NAD is reduced to NADH and the conversion of ADP to ATP takes place. The key intermediate is pyruvate, since its degradations differ from PFOR and PFL. In principle, formate is formed when facultative anaerobes degrade pyruvate, while acetyl-CoA

is transformed through the action of PFL. Strict anaerobes produce acetyl-CoA, CO_2, and reduced ferredoxin using PFOR (pyruvate: ferredoxin oxidoreductase). Hence, in a facultative anaerobic metabolism pathway, the *fhl* complex which possesses Ni–Fe hydrogenase is responsible for producing hydrogen from formate. Alternatively, reduced ferredoxin promotes the hydrogen production by a FeFe hydrogenase. The by-product is principally ethanol in facultative fermentation, and ethanol, butyric acid, butanol and acetone for the strict anaerobic pathway depending on the microorganism and the physical fermentation conditions.

3.2. The parameter-response of fermentation

The process of fermentative hydrogen production is complex. The H_2 productivity depends mainly on the inoculant, the cultures, the substrates, and the addition of trace elements [13].

3.2.1. Effect of inoculum

Different inoculants have been studied for H_2 production, among which *Enterobacter sp., Bacilus sp.* and *Clostridium* sp. distinguished by its gram-negative, rod-shaped and strict anaerobic nature. *Clostridium sp.* converts glucose to VFAs, H_2 and CO_2. The typical species are *C. buytricum, C. thermolacticum, C. pasteurianum* and *C. bifermentants* [62, 72]. *Enterobacteriaceae* are gram-negative, rod-shaped but facultative anaerobes.

Almost all studies used pure cultures of bacteria in batch mode with six-carbon sugars (e.g. glucose) as substrate in major cases. Studies utilizing five-carbon sugars, such as xylose show low yield though it is a potential solution to release the pressure from the food crisis as five-carbon sugars account for little of food supply. In this condition, renewable feedstock of organic wastes is a more desirable substrate, however demanding a diversity of hydrogen producing microorganisms due to its complexity. Microorganisms which are capable of H_2 production exist wildly in soils, wastewater treatment sludge and compost.

Fig. 5 illustrates the production yield of H_2 with different microbial strains, which show different capabilities as the yield varies from 1 to 2.8mol H_2/mol [50,82–93].

3.2.2. Effect of the cultures

Fermentation with pure cultures has a limited metabolic capacity, therefore mixed cultures were proposed since they extend the range of substrates so that the VFAs produced in dark fermentation can further be used in photosynthesis and converted to hydrogen. There are studies dealing with two combination patterns, i.e. sequencing batch operations of dark- and photo-fermentation, and their combination [94].

Combining *C. butryicum* and *Rhodobacter sp.* produces 4.5 mol H_2/mol glucose, higher than the yield in a sole dark fermentative (1.9 mol H_2/mol glucose) and the obtained value in two consecutive steps (3.7 mol H_2/mol glucose) [95]. The maximum H_2 yield and production rate reported when using the combined and sequencing batch operation is 6.6–7.2 mol H_2/mol glucose and 1.55 mmol H_2/L culture/hr respectively.

Bao et al. [60] however reported a successful fermentation that combines hydrolysis and hydrogen generation in a single bioreactor. The strains include *Bacillus sp.* which is responsible for starch hydrolysis and *Brevumdimonas sp.* being in charge of hydrogen synthesis. The interspecies cooperation allows to produce H_2 in a single reactor with cumulative yield up to 1.04 mol H_2/mol glucose. Table 2 demonstrates the studies on various culture modes which also showed the quantity of hydrogen produced. Higher hydrogen yields are reported for separate hydrolysis and subsequent fermentation.

3.2.3. Effect of substrates

Substrates have a definite influence on the fermentation process due to their different biodegradabilities. Glucose, maltose and xylose are readily used, while other substrates, for example starch, require a preliminary transformation into glucose or maltose, either by acid or by enzymatic hydrolysis. Furthermore cellulose is the most difficult to use as most microorganisms cannot directly or indirectly consume cellulose. On the other hand, the carbohydrate-rich wastes feedstock such as waste water from the sugar mills, from breweries, from cassava processing and others, can to a large extent reduce the cost of bio-H_2. Cassava is a
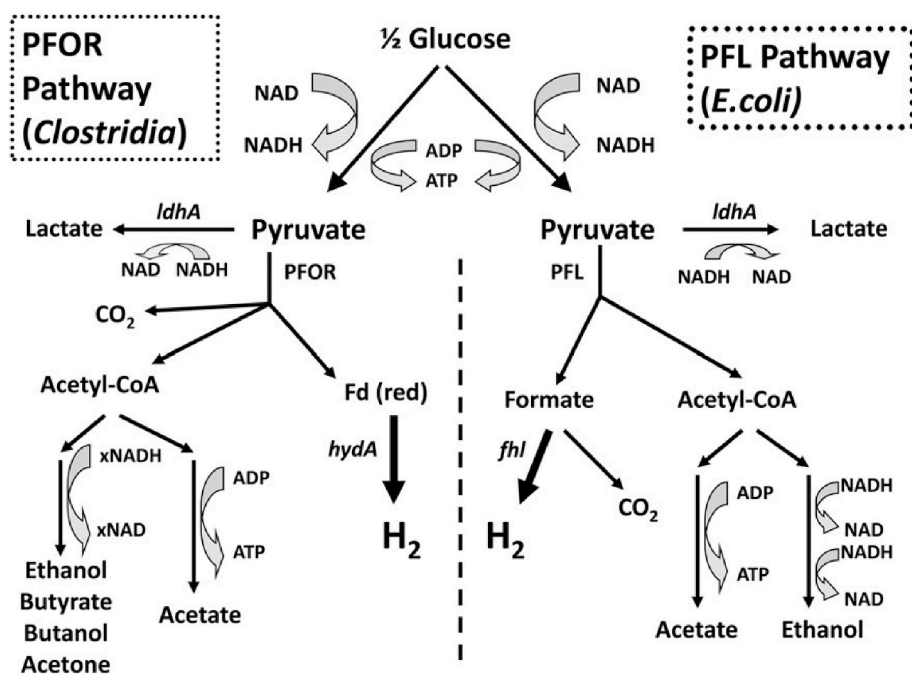

Fig. 4. Metabolic pathways within the hydrogen dark fermentation (Reprinted from Ref. [81]. With permission of Elsevier).
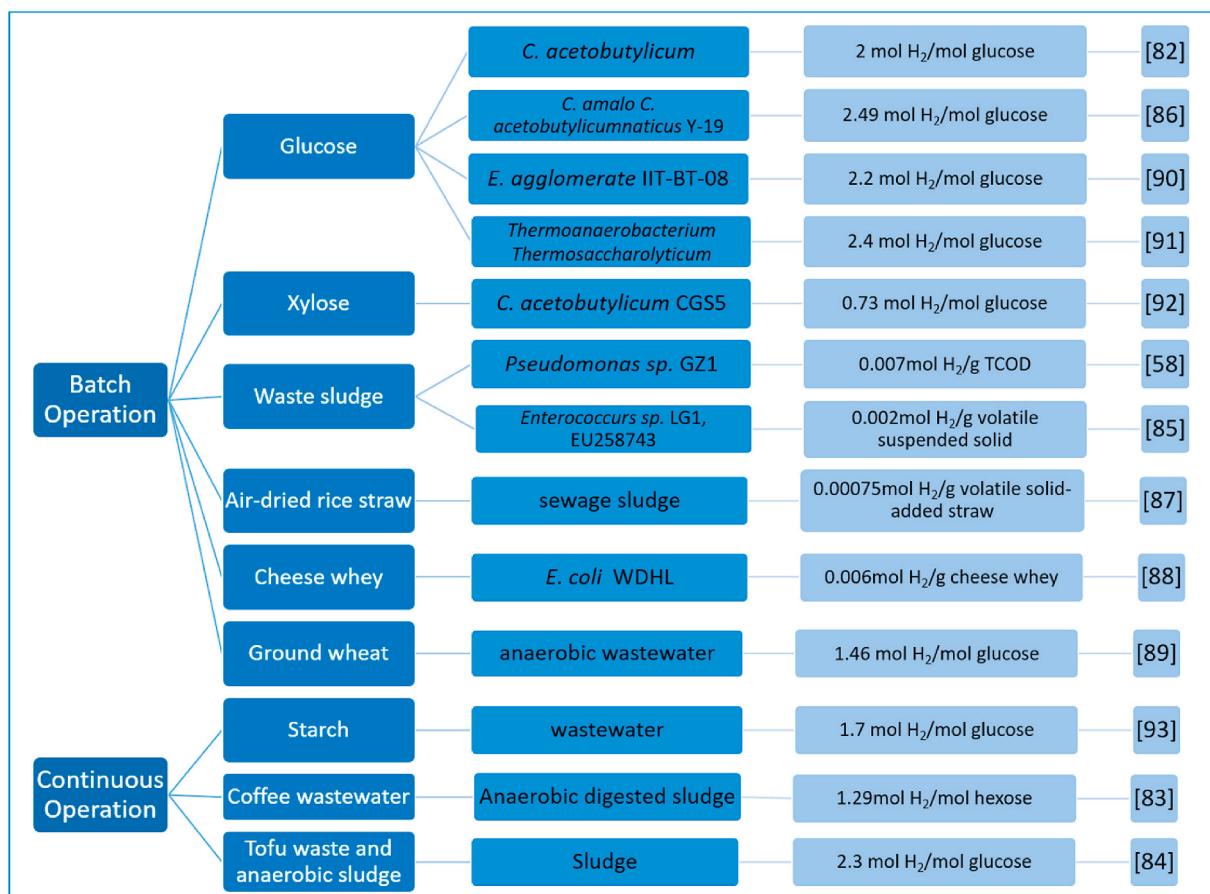
Fig. 5. Different bacterial cultures in batch or continuous fermentative H_2 production modes.

promising hydrogen production feedstock due to the fact that it can be planted on sterile soil [102] and is not used for food at some production locations. The maximum production yield using cassava flour under optimum operating conditions was reported as 1.72mol H_2/mol glucose [103]. Moreover, the waste water from the cassava starch manufacture is also rich in carbohydrates with a high BOD and COD (»10 g/L), which makes it suitable as feedstock. The fermentation process is simultaneously part of the wastewater treatment, which makes it a win-win situation for the cassava starch production.

In addition to substrates summarized in Fig. 3, additional studies with different substrates are illustrated in Table 3.

3.2.4. Effects of trace elements

Enzymes are the specific performers of all the metabolism and bio

reactions including the hydrogen production reactions. Trace elements, for example metal ions (Na^+, Mg^{2+}, Zn^{2+} and Fe^{2+}) are co-factors of enzymes, thus they are essential factors for the hydrogen synthesis: iron and nickel are necessary elements of hydrogenase [37]; magnesium is the activating agent for kinase and synthetase [37]; and some reducing amino acids, for example L-cysteine, are capable of maintaining the oxidation-reduction potential (ORP) [37].

Hydrogenase is involved in both pathways, which are[Fe–Fe] hydrogenase in the PFOR and [Ni–Fe] hydrogenase in the PFL [68,113, 114]. The [Ni–Fe] hydrogenase is significantly present in hydrogen-producing bacteria and it is has a less sensitive inhibitory effect on oxygen in comparison with [Fe–Fe] hydrogenase, therefore Fe^{2+} and Ni^{2+} are the two significant trace elements affecting hydrogen fermentation production.

Table 2
Hydrogen production using different cultures, in batch (B) or continuous (C) operating mode.

Substrate and its concentration	Inoculums	Produced H_2 (mol H_2/mol glucose)	
Glucose, 9 g/L	*C. butyricum* + *R. coprophilus* *Pseudomonas* RLD-53	1.98	B [96]
Acid hydrolysis of wheat starch, 5 g/L	*Rhodobacter sphaeroide*-RV	1.23	B [97]
Hydrolyzed starch, 26 g/L	*C. butyricum* CGS2	1.5	C [98]
Discarded wheat starch, 5 g/L	*C. acetobutylicum* + *Rhodobacter sphaeroide*-RV	0.6	C [99]
Wheat starch, 10 g/L	Anaerobic digestion sludge	1.14	B [100]
Corn starch, 20 g/L	Excess waste activated sludge	0.92	C [101]
Corn starch, 10 g/L	Mixed cultures	1.04	B [60]
Wheat starch, 10 g/L	Dark fermentation +*Rhodobacter sp.*	185 mL H_2 g VFA	C [94]

Table 3
Effect of different substrates on the hydrogen yield.

Inoculum	Substrates	Maximum hydrogen yield	Refs.
C. acetobutylicum CGS2	Starch	9.95 mmol H_2/g COD	[104]
Municipal sewage sludge	Xylose	2.25 mol H_2/mol xylose	[105]
C. Pasteur CH_4	Sucrose	2.07 mol H_2/mol hexose	[92]
C. thermolaticum	Lactose	3.0 mol H_2/mol lactose	[61]
Cow dung compost	Cornstalk wastes	149.69 mL H_2/g TVS	[106]
Class septicumM-21	Chitin wastes	2.2 mol H_2/mol substrate	[107]
Cow dung compost	Beer lees	68.6 mL H_2/g TVS	[108]
C. thermocellum 27405	Cellulose biomass	2.3 mol H_2/mol glucose	[109]
Anaerobic sludge	Food waste	1.8 mol H_2/mol hexose	[110]
E. cloacae E82005	Molasses	3.5 mol H_2/mol sucrose	[111]
Anaerobic Sludge	Dairy wastewater	31.7 mmol H_2/g COD	[112]

Research showed that iron and sulphur influence the function of proteins by acting as electron carrier in the oxidation of pyruvate to acetyl-CoA, CO_2 and H_2. Wang and Wan reported that iron addition boosted the synthesis of hydrogen to the maximum yield of 2.33 moL/mol glucose at 350 mg/L of Fe^{2+} [115]. Moreover, iron could induce metabolic alteration: experiments revealed that the amount of Hydrogen produced increased to 3.06 mol/mol glucose from 2.93 mol/mol glucose after adding 25 mg/L $FeSO_4 \cdot 7H_2O$, and vice versa.

Table 4 summarizes how adding trace elements affects the hydrogen production. Whereas bio-hydrogen production essentially requires the presence of sufficient quantities of essential micro-nutrients for bacterial metabolism during fermentation (such as metal ions and trace elements), the enhancement efficiencies of individual or compound nutrients are different.

3.2.5. Overall assessment

As far as photo-fermentation is concerned, its negative energy balance when using an artificial light source needs further investigations in terms of technical and economic respects.

Biological production of H_2 by dark fermentation of wastes (primary route) and non-food biomass (secondary route) can facilitate the proposed green, sustainable hydrogen economy. It is currently drawing great attention since no additional energy source is needed, and since the technique is capable of using various feedstock, such as waste water and process residuals. The core barrier now is the attainable yield in the typical mesophilic fermentation: glucose has a potential equivalent of 4 mol H_2/mol glucose. Through fermentation, between 25 and 50% of the available hydrogen is released as H_2 [110,118]. One should moreover also consider that the mass balance of the reaction is not in favor of H_2 (despite its 2 mol/mol glucose), but results in a waste water with very high concentrations of VFA's produced (acetate, butyrate, propionate), hence with a high COD, needing further treatment. Given the ideal yield of 4 mol H_2/mol glucose, provided the density of gasoline is 0.72 kg/L, and at a heat capacity of gasoline and hydrogen of 44 MJ/kg and 143 MJ/kg respectively, a vehicle will consume 1.5 kg hydrogen per 100 km (it is equivalent to 7 L gasoline per 100 km). At the production of 4 mol H_2/mol glucose, or 8 g/180 g glucose, the production of 1.5 kg of H_2, needs a theoretical input of 33.8 kg of glucose. At lower conversion efficiencies, the amount of glucose consumed will increase. This low efficiency, together with the required further waste water treatment, will prevent the production of H_2 to compete with fossil fuel for a long period in future. A better conversion and/or an efficient use of the by-products (mainly acetate and butyrate) are the key factor to the future bio-H_2 success.

4. Potential solutions to overcome the bottlenecks and recommended actions towards a better future for biochemical hydrogen production

Although biochemical fermentation is an attractive way of producing bio-hydrogen since a variety of available wastes or biomass can be used, its industrial application is at present hampered by the low yields obtained.

To improve the yield of hydrogen, studies are being conducted in different areas, including mainly: metabolic engineering; third stage fermentation of butyric/acetic acid; separation/upgrading of the acids; and membrane separation of hydrogen.

4.1. Metabolic engineering

Metabolic engineering has been recognized as having a potential to enhance the hydrogen yield. The key obstacles of fermentation are the constraints introduced by the metabolic pathways involved. The fundamental metabolisms leading to H_2 production were outlined in 3.1. Current research investigates increased yields by either modifying the existing pathways, or by metabolic engineering to introduce new enzymes and/or pathways to overcome thermodynamic or metabolic barriers to increase the yields.

Fig. 6 below illustrates different strategies to maximize yields of present hydrogen synthesis pathways by eliminating reactions that compete for the same substrate, or by eliminating the activity of any uptake hydrogenases that could be present.

Inactivating competing reactions and manipulating culture conditions has led to higher H_2 yields, close to values predicted by metabolic schemes, although the final target of H_2 production levels beyond present limits needs further investigation.

H_2 production when using *Clostridium* bacteria could be fostered by disabling the uptake of hydrogenase, or disabling the oxygen system. Such actions will result in a more robust hydrogen production, where the hydrogen yield of the dark-fermentation step could be enhanced [119–121].

Researchers have proposed to introduce new pathways, however all of them end up with low yield due to various factors. One attempt for *E. coli.* intended to express a highly active [Fe–Fe] hydrogenase (hydA) from various sources, but it failed since *E. coli.* does not possess the specific genes (which encode the required maturation factors) required for the expression of hydA, even with the assistance of co-expressed hydEFG [122]. Other modifications have been tried and provided

Table 4
Effects of adding of trace elements on the H_2 production.

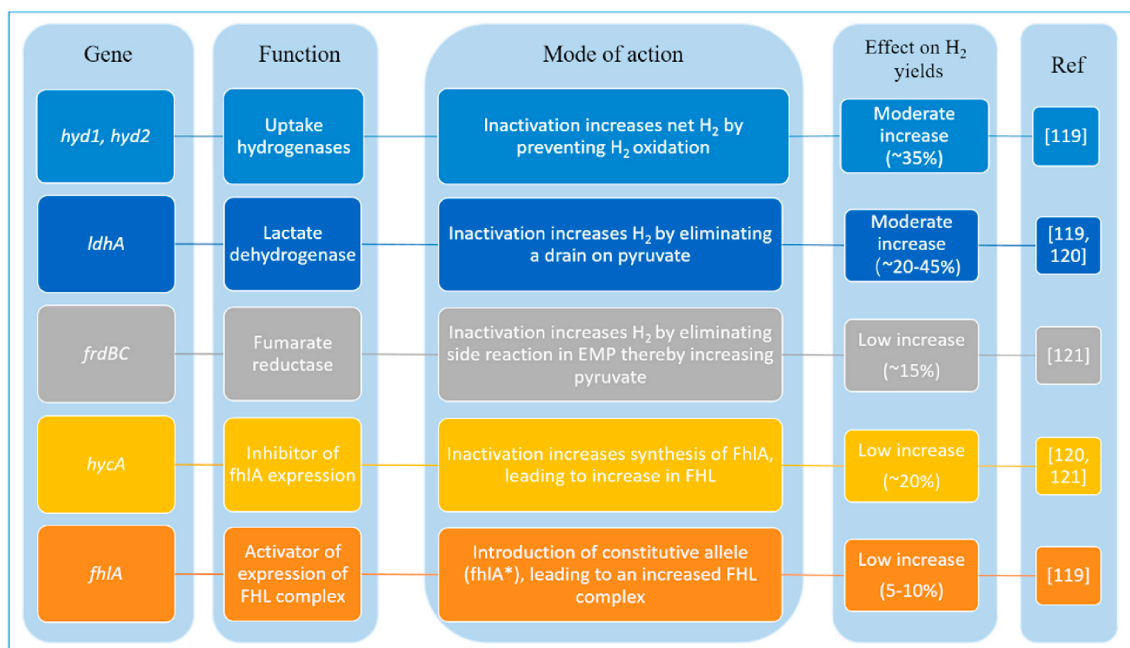| Elements		Remarks	Ref.
Ni^{2+}	Within Ni^{2+} 0–50 mg/L, H_2 production increases as Ni^{2+} increases, and decreases as the Ni^{2+} increases beyond 50 mg/L		[116]
Ni^{2+}	H_2 production was increased by 71% by adding Fe^{2+} and Ni^{2+} comparing to the blank sample and reached the maximum at 50 mg/L of Fe^{2+} and 25 mg/L of Ni^{2+}.	glucose as substrate an anaerobic continuous flow stirred tank	[117]
L-cysteine	Adding L-cysteine (0.1–1.0 mM) improves H_2 yields		
Fe^{2+} and L-cysteine	H_2 yield is increased to maximum 1982 mL and 1.94 mol H_2/mol glucose	Relative enhancement effect: Fe^{2+} > L-cysteine > L-cysteine + Ni^{2+} > L-cysteine + Fe^{2+} > L-cysteine + Mg^{2+}	[37]
Ni^{2+}	H_2 production was reduced at Ni^{2+} concentrations above 20 mg/L		[60]

Fig. 6. Targets of genetic modification of common pathways.

negative conclusions till now. Different metabolic engineering strategies are summarized in Table 5.

Using a reverse electron flow was suggested as alternative solution: the reduction of ferredoxin with NADH could generate enough reducing power to promote hydrogen production by hydrogenase [132]. In this process, it is necessary to overcome the thermodynamic barrier with energy input. A small amount of respiration could be used to overcome the barrier by creating an electrochemical gradient to favor the ferredoxin reduction. This method needs further investigation.

Conclusively, a possible modification of the system could drive it to higher bio-hydrogen yields. However further investigations are needed.

4.2. Third stage fermentation of butyric/acetic acid

As discussed above, the waste water of dark fermentation is rich in butyric/acetic acid and is not environmentally friendly. These volatile fatty acids have to be treated either by VFA recovery or by a following stage of photo fermentation, that can utilize the residual VFA and increase the cumulative yield. The overall yield of hydrogen in such a two-stage operation exceeded the yield of a single stage production process [133]. Bao et al. [37] reported a pH ~4 at the termination of the mixed culture fermentation. Fig. 7 summarizes the pH and the quantities of residual VFA. It appears that the pH is between 4 and 8 due to the generation of VFA. Different substrate were used, i.e. food waste [110], glucose [134], cornstalk waste [106], cattle dung and sludge [73], steam exploded corn straw [135], fruit vegetable waste [136], rice waste

[137], and sewage sludge [138,139]. Reference effluent composition data are converted from mg/L, g/L, % values to total VFA concentrations, given in mM/L.

Experiments revealed that malate and lactate were the most favorable substrates, with the highest H_2 production rates in photo-production [140]. Uyar et al. [141] investigated the growth and hydrogen production of *Rhodobacter sphaeroides O.U. 001* in media containing mixed VFAs (malate, acetate, propionate, butyrate and lactate): their results demonstrate that the highest H_2 production rates during of dark fermentation were obtained in malate (24 mL hydrogen/L reactor h). It was moreover observed that the bacteria sequentially consumed acetate, propionate and then butyrate. For the different VFAs tested, the butyrate H_2-efficiency was lower due to the competitive production of PHB. The substrate conversion efficiencies were globally between 14 and 50% [142].

4.3. Upgrading of the VFAs

VFAs are obtained as key intermediates in dark fermentation processes. As discussed previously, acetate is the most favorable for VFA fermentation, which makes the upgrading of volatile fatty acids a possible pretreatment or biological facilitation to hydrogen production. The upgrading of acids mainly targets on the low or even negative cost of feedstock, such as sludge, organic wastes, meaning the required cost is low, the target end product being VFAs, for example acetate via an anaerobic process. Fig. 8 summarizes literature data for soluble

Table 5
Different metabolic engineering strategies to increase hydrogen production.

| Strains	Metabolic engineering strategy	Improved H_2 production (%)
E. coli K–I2 BW25113 [123]	Protein engineering of large subunit of hydrogenase 3 (hycE)	800 (compared with wild-type strain)
E. coli K–I2 BW25113 [124]	Inactivation of hydrogenase 1 and hydrogenase 2	200
Caldicellulosiruptor bescii [125]	Deletion of lactate dehydrogenase gene (*ldh*)	21–34
E. coli [126]	Heterologous expression of HupSL hydrogenase from *Rhodobacter sphaeroides*	209
Enterobacter aerogenes [127]	Homologous overexpression of NAD synthetize gene *hadE*) and deletion of phosphoenolpyruvate carboxylase gene (*ppc* and *hybO*)	301
Klebsiella oxytoca HP1 [128]	Homologous overexpression of two hydrogenase subunits (*hycE* and *hycG*)	90.5
Clostridium tyrobutyricum [129]	Homologous overexpression of hydrogenase gene (*hydA*)	150
C. pasteurianum [130]	Homologous overexpression of hydrogenase (*hydA*) and glycerol dehydrogenase (*dhaDI* and *dhaK*)	170
C.tyrobutyricum [131]	Inactivation of acetate kinase (*ack*)	150
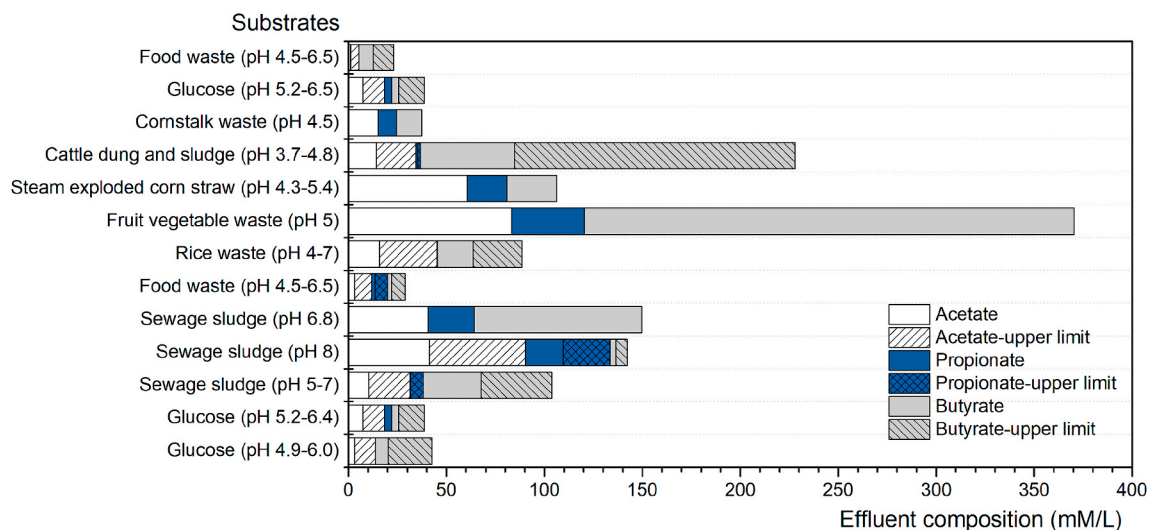
Fig. 7. VFA composition of the effluent.
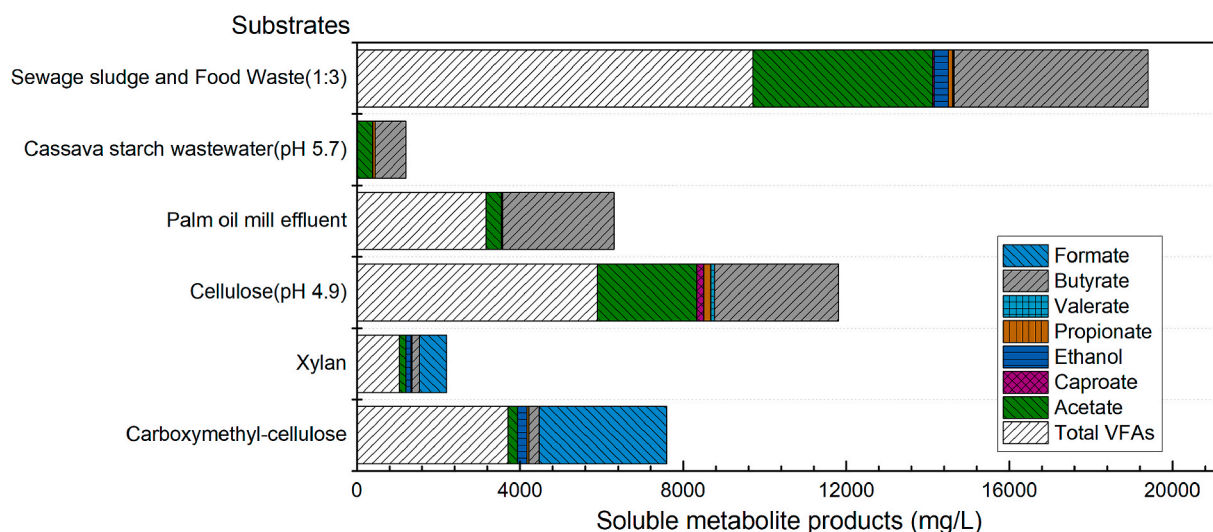
Fig. 8. Soluble metabolite products of effluent from dark hydrogen fermentation. Carboxymethyl-cellulose [143], Xylan [143], Cellulose [144], Palm oil mill effluent [39], Cassava starch Wastewater [33], Mixture of sewage sludge and food waste (1:3) [145].

metabolites, produced during dark fermentation.

The anaerobic process can be described by four steps, being (i) hydrolysis, where larger polymers are decomposed by enzymes; (ii) fermentation, where acidogenic fermentation is dominant and acetate is the main end product (VFAs are also produced along with CO_2 and H_2); (iii) the breakdown of VFAs to acetate and H_2 by acetogenesis; and finally (iv) the conversion of acetate, formaldehyde, hydrogen and carbon dioxide into methane, carbon dioxide and water by methanogenesis.

One of the two most important threats is the suppression of the methanogenesis step. Dictor et al. [146] found that methanogenesis can be inhibited during anaerobic electro-stimulation (application of a weak current to hydrogen producing bacteria) with promoted degradation of organic waste, thus leading to an enhanced hydrogen production.

Dark fermentation is an alternative of the common anaerobic digestion but where anaerobic fermentation is stopped by suppressing methanogenesis, hence not converting VFA to methane or to hydrogen.

A different route that certainly merits further investigation is the recovery and separation of the VFAs, for use in chemical transformations or as raw material. Both acetates and butyrates can be transformed into esters, then separated and purified as chemical feedstock.

4.4. Membrane purification of hydrogen

As the last step of hydrogen production, its purification is very important with Pressure Swing Adsorption, cryogenic distillation, and membrane separation as possible solutions. The first two processes are energy intensive [147], and current research focuses on membrane separation with polymeric and non-polymeric materials [110,148–152].

If proven to be viable in real operating conditions, membrane separation will attractively compete with the more mature technologies of pressure swing adsorption and cryogenic distillation. Apart from the high energy consumption, a cryogenic system can only practically achieve moderate hydrogen purities (~95%) [153].

Hydrogen separation membranes were previously reviewed [149], and the operating conditions and performance of different membranes is illustrated in Fig. 9. Inorganic membranes can cope with more severe operating conditions their organic counterparts.

As illustrated in the figure, the selectivity and working flux both are low for dense polymer membrane. Metallic membranes have a very high selectivity and flux. Highly pure hydrogen can be obtained through using dense metallic membranes. However the metal Pd is expensive, though thin film membranes can to some degree reduce the cost. Thin

Membrane types	T (K)	H_2 selectivity relative to CO_2	H_2 flux (mol/m²s bar)	Stability issues	Poisoning issues	materials	Transport mechanism
Dense polymer	<373	low	low	Swelling, compaction	HCl, SO_x	polymers	Solution diffusion
Microporous ceramic	473–873	5–139	0.06–0.3	Stability in H_2O		Silica, alumina, zirconia, titania, zeolites	Molecular sieving
Dense metallic	573–873	>1000	0.06–0.3	Phase transition	H_2S, HCl, CO	Palladium and its alloys	Solution-diffusion
Porous carbon	773–1173	4–20	0.01–0.1	brittle	Strong absorbing vapors	carbon	Solution-diffusion, molecular sieving
Dense ceramic	873–1173	>1000	0.006–0.08	Stability in CO_2	H_2S	Proton-conducting carbon	Solution-diffusion

Fig. 9. The operating conditions and performance of different membranes.

membranes also increase the hydrogen flux. The drawback of a metallic membrane is its sensitivity to some gases, such as CO, H_2S, thus needing gas pretreatment. Ceramic membranes are inert to hazardous gases, are resistant to high pressure and temperature, thus making it attractive to systems that operate at increased pressures, where permeabilities are considerably increased. However, due to its working mechanism, microporous ceramic membranes can never reach the 100% purity obtained by thin-film-palladium or ion-transport membranes.

4.5. Potential and challenges

Bio-hydrogen production through fermentation has advantages over other approaches, because abundant waste biomass can be used without increasing the amount of greenhouse gases in the atmosphere, and moving closer to a "hydrogen economy". If 88% of the cost of bio-hydrogen through fermentation of glucose is attributed to the feedstock [154,155]. It is important to utilize cost-effective substrates.

The low substrate conversion (usually <30%) and low hydrogen yield which is thermodynamically limited to maximum of 4 mol H_2/mol hexose, are considered to be the main limitations of biohydrogen [156]. The energy conversion efficiency can be improved by integrating fermentation with other techniques such as anaerobic digestion or efficiency can be improved by integrating fermentation with other techniques such as anaerobic digestion or bio-electrochemical systems. To improve the performance of an integrated system in using wasted biomass, the selection of effective bacteria, construction of efficient strains and optimization of the process conditions have been studied, i. e., the utilization of high productive bacteria like *E.coli* or *R.sphaeroides* [157].

Currently, most of studies on dark-fermentation were conducted at lab scale. However, from an economic point of view, a continuous process is required for constant biohydrogen production at large scale. Additional research is also required (1) to explore cost-effective bio-hydrogen using abundant wasted biomass, (2) to develop an energy efficient, environmentally friendly and inhibitor-free pretreatment approaches when cellulosic materials are used as feedstock, (3) to isolate new bacterial species or obtain robust microbes by modifying the pathways of present microorganisms using genetic methods, (4) to assess the economic feasibility of biohydrogen production from waste biomass (i.e., life cycle analysis), (5) to integrate the up- and downstream technologies for multi-outputs from waste biomass. The most possible commercial establishment for biohydrogen production can be at a position near to a wastewater treatment plant, with stable and sufficient supply of feedstock. However, only wastewater supply will not be enough for continuous biohydrogen production since the content of organic substrate of wastewater will be insufficient for dark-fermentation. Thus addition of other organic substrates such as agricultural wastes and municipal organic wastes will be required.

Although the present review focused on the bio-hydrogen production, it is interesting to compare these current lab-scale systems with commercial or significantly developed H_2 production systems that utilize renewable and non-renewable resources. Fig. 10 illustrates the 3 different strategies, using fossil fuels, nuclear energy and bio-energy resources. Within the fossil fuel solutions, hydrocarbon reforming and pyrolysis/gasification are widely applied on a commercial scale. Nuclear energy applications consider high temperature water splitting and electrolysis. The renewable energy routes comprise two alternative pathways for using diverse biomass species in either thermo-chemical processes (mostly pyrolysis, gasification and liquefaction), or in fermentation processes (indirect bio-photolysis, dark fermentation, photo-fermentation, sequential dark and photo-fermentation). While the thermo-chemical processes can be considered of commercial scale, fermentation could see enhanced development on a medium term. Electrolysis, associated with some of the renewable electricity generation is commercially proven.

Although costing data are scarce and widely differing among published data, a tentative comparison is presented in Table 6. Cost components related to H_2 storage, transportation and utilization are not included. It should be remembered that efficiencies for steam reforming are 70–85%, 60–75% for coal gasification, 50–70% for electrolysis and 35–50% for biomass gasification. Efficiencies of fermentation processes are still below 5%.

It is clear that currently the most economic sources of H_2 are fossil fuels and biomass. A kilogram of hydrogen has nearly the energy content of one gallon of gasoline. The cost of hydrogen per kilogram is hence directly comparable to the cost of a gallon of gasoline. From the data, it is clear that advanced nuclear techniques need major improvements to be economically viable. Also renewable energy (wind, solar) driven
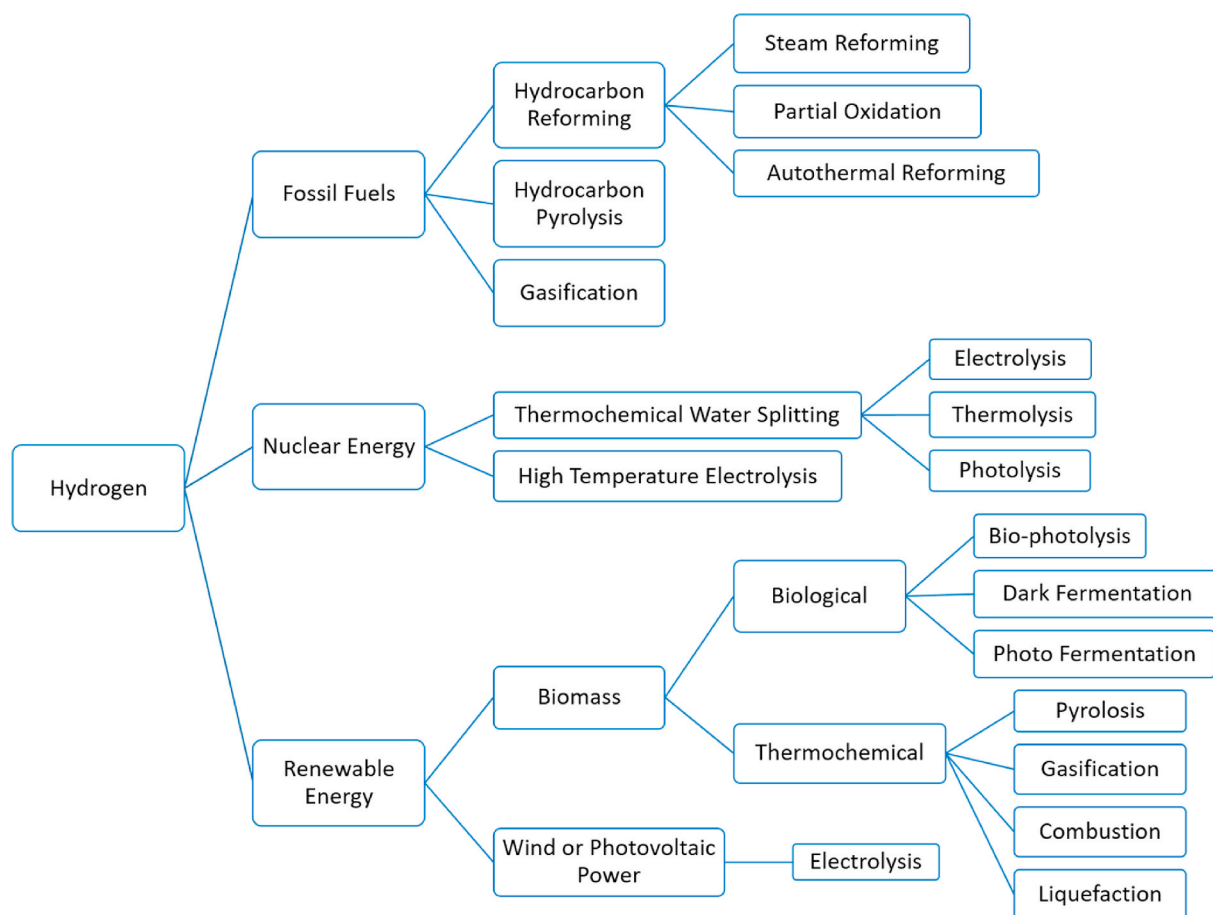
Fig. 10. Technologies for producing H_2 from fossil fuel, nuclear energy or renewable energy resources or feedstock.

Table 6
Operating and cost data for hydrogen production from renewable and non-renewable resources.

Processes	Energy source	References	Feedstock Cost	Plant Output (t H_2/h)	H_2 cost ($/kg)
Steam Methane Reforming (SMR)	Natural gas	Dincer and Acar, 2014 [158]; Acar and Dincer, 2015 [159]; Kalamaras, 2013 [160]; Penner 2006 [161]; Rutkowski, 2005 [162]	9.48 $/GJ	9.8–14.2	1.8–3.0
SMR with CO_2 sequestration	Natural gas	Rutkowski, 2005 [162];	9.48 $/GJ	14.2	2.3
Coal Gasification (CG)	Coal	Kreutz et al., 2002 [163]; Rutkowski, 2005 [162]	1.15–1.26 $/GJ	10.6–32.1	0.8–1.3
CG with CO_2 sequestration	Coal	Kreutz et al., 2002 [163]; Rutkowski, 2005 [162]; Dincer and Acar, 2014 [158]; Acar and Dincer, 2015 [159]	1.15–1.26 $/GJ	10.6–32.1	0.9–1.7
Nuclear Energy and Electrolysis	Nuclear	Petri et al., 2006 [164]; Dincer and Acar, 2014 [158]; Acar and Dincer, 2015 [159]	~0.048 $/kW h	0.04	4.2–7
Sulphur-iodine thermo-chemical water splitting	Nuclear	Richards et al., 2006 [165]; Schultz, 2003 [166]	Not available	24.3–30.1	1.75–2.01
Wind turbines and electrolysis	Wind	Bockris & Veziroglu, 2006 [167] ; Levene et al., 2006 [168]; Dincer and Acar, 2014 [158]; Acar and Dincer, 2015 [159]	0.038 to 0.045 $/kW h	0.04	5.6–7.5
Photovoltaics and electrolysis	Solar	Glatzmaier et al., 2010 [169]; Dincer and Acar, 2014 [158]; Acar and Dincer, 2015 [159]	0.03 to 0.05 $/kWh	0.06–14.8	6.1–9.0
Solar Power Tower and electrolysis	Solar	Glatzmaier et al., 2010 [169] Kolb et al., 2007 [170]	0.08 to 0.10 $/kWh	16–26.3	5.1–6.5
Gasification	Biomass	Mann, 2005 [171]; Dincer and Acar, 2014 [158]; Acar and Dincer, 2015 [159]	16.5–46.3 $/t	0.08–8.1	1.0–2.0
Pyrolysis	Biomass	Padro and Putsche, 1999 [172]	16.5–46.3 $/t	0.11–30.4	1.0–2.0
Dark Fermentation	Biomass	Jeffrey, 2010 [173]	Various resources	Lab-scale only	2.6
Photo Fermentation	Solar	Demirbas MF, 2006 [174]	Various resources	Lab-scale only	2.8

techniques are still too expensive, despite being currently promoted on a large scale to utilize excess electricity produced by wind turbines or photovoltaics in periods of low electricity demand of the grid. Gasification and pyrolysis of biomass in its different forms can be attractive, especially due to the volatility of fossil fuel prices. Bio-hydrogen production by fermentation is still rather expensive, but one should remember that only lab-scale data are currently available.

5. Conclusions and recommendations

Large industrial-scale of hydrogen production from biomass or carbohydrates by fermentation, though undoubtedly desirable and favored, is however still at the level of laboratory or pilot scale. Strategies for industrial-scale production, at an economically viable level, need to be developed, and further research is still needed. A wide spectrum of wasted biomass of industrial, agricultural, municipal and forestry origins can be considered to be used as substrate. Although results are interesting, and confirm the possibility of generating bio-hydrogen, all techniques suffer from 3 main drawbacks, i.e. (i) a low production yield of H_2 (a few mol H_2/per mol of equivalent glucose only in the case of fermentation; (ii) the production of a liquid waste stream rich in VFAs and hence needing further treatment when fermentation is used.

The present work reviewed the literature relative to dark fermentation, photo-fermentation, and combined processes. Dark-fermentation is an environment-friendly and cost effective approach of used for waste biomass. To improve the performance of dark-fermentation, it is essential to use several microorganisms capable of high hydrogen yield such as *Bacillus amyloliquefaciens* and *C. pasteurianum* and to use some advanced techniques of co-culture fermentation. The application of mutant strain obtained from genetic engineering becomes a promising trend in dark-fermentation. The development of integrated systems of dark-fermentation with other technologies such as anaerobic digestion is important for valorization of dark-fermentation effluent, making the production of biohydrogen profitable and sustainable.

Although literature offers some solutions to the problems, as outlined in Section 4, these solutions are either incomplete, economically debatable, or in initial stages of assessment.

To enhance the future of bio-hydrogen, an important step forward would involve the further use and valorization of the residual waste streams produced by fermentation. At current market values of C_2, C_3 and C_4 chemicals, it is certainly worth focusing research efforts on the recovery and additional use of VFAs and minor organic compounds formed by bio-H_2 fermentation.

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Declaration of competing interest

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

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