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Review of Continuous Fermentative Hydrogen-Producing Bioreactors from Complex Wastewater

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<http://dx.doi.org/10.5772/110416>

Abstract

In recent years, the production of hydrogen through dark fermentation has become increasingly popular because it is a sustainable approach to produce clean energy. Thus, an evaluation of studies reported on hydrogen production from different complex wastewaters will be of immense importance in economizing production technologies. This work presents a review of the advances in the bioreactor and bioprocess design for biohydrogen production from different complex wastewaters. The biohydrogen production is discussed emphasizing the production metabolic pathways, bioreactor configuration and operation, organic loading rate (OLR), pretreatment of wastewater, as well as microbial diversity. Also, in this review, various bioreactor configurations and performance parameters including H₂ yield (HY) and hydrogen production rate (HPR) are evaluated and presented. The work concludes with challenges and prospects of biohydrogen production and claims for more systematic and comprehensive studies on the subject.

Keywords: biogas, global warming, dark fermentation, bioreactor, process parameters

1. Introduction

According to the IPCC [1] (Intergovernmental Panel on Climate Change), global warming of more than 2°C would have serious consequences, such as an increase in the number of extreme climate events. In Copenhagen in 2009, the countries stated their determination to limit global warming to 2°C between 2015 and 2100. To reach this target, climate experts estimate that global greenhouse gas (GHG) emissions need to be reduced by 40–70% by 2050 and that carbon neutrality (zero emissions) needs to be reached by the end of the century at the latest. To reduce global warming, substantial effort is being made at a global scale to explore renewable energy sources that could replace fossil fuels.

Hydrogen gas can be an ideal sustainable energy carrier, which can reduce the over-reliance on fossil fuels. Some of the advantages of hydrogen can be listed as follows: (i) high energy conversion efficiencies, (ii) production from water with no emissions and (iii) abundance [2].

Dark fermentation is a biological approach commonly used to produce H₂ in the absence of light. It is driven by anaerobic bacteria that can produce hydrogen from wastewaters [3]. This technology has attracted attention because it can use a versatile range of substrates, particularly renewable resources that are organically rich such as stillage, sludge, leachate, pomace, stalks and bagasse [4]. Wastewaters generated from various industrial processes are considered to be the ideal substrates because they contain high levels of easily degradable organic material, which results in a net positive energy or economic balance [5]. From the anaerobic digestion process, complex wastewater can be converted into hydrogen, while promoting the treatment of these wastewaters, providing environmental sustainability.

Studies on batch, semi-continuous and continuous hydrogen-producing bioreactors have been conducted. Batch hydrogen fermentation normally brings about lower hydrogen production rates (HPRs) in comparison with its semi-continuous or continuous counterpart. Besides the extensively studied continuous stirred tank reactor (CSTR), numerous biohydrogen bioreactor processes such as anaerobic sequencing batch reactor (ASBR), fixed-bed bioreactor, fluidized-bed bioreactor and upflow anaerobic sludge blanket (UASB) bioreactor have been developed with high production yields and output [6].

Inevitably, performance of hydrogen-producing bioreactor systems and operation are determined by various factors that are associated with environmental conditions, process operating conditions and chemical conditions, such as inoculum, nutrients, hydrogen partial pressure, temperature, hydraulic retention time (HRT) and substrate concentration [6]. Variations in these factors result in different microbial communities, resulting in different hydrogen yields. In this context, this review summarizes the above factors that influence hydrogen production by dark fermentation from different complex wastewaters.

2. Microbiology of hydrogen production: metabolic pathways

Hydrogen can be produced through different metabolic pathways that can be broadly grouped into two distinct categories—light-dependent and light-independent processes. Light-dependent processes include direct or indirect photolysis and photo-fermentation, whereas dark fermentation is a major light-independent process [7]. According to Sinha and Pandey [8], compared to the photosynthetic processes of hydrogen production, fermentation processes have the advantage of a rapid rate of hydrogen production and simplicity of operation.

The anaerobic digestion process generally consists of the four stages, i.e. hydrolysis, fermentation, acetogenesis and methanogenesis (**Figure 1**). In the first two stages, dark fermentation is involved in the production of hydrogen. Various microorganisms are involved in each step and cooperated with each other to achieve carbohydrates that are converted into hydrogen gas, VFAs and alcohols, which are organic pollutants and energy carriers.

Acidogenesis reactions	Eq. no.	Bacteria
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	(1)	<i>Bacteriodes, Clostridium, Butyrivibrie, Eubacterium, Bifidobacterium, Lactobacillus, Acetobacterium, Butyribacterium, Eubacterium, Peptostreptococcus</i>
$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	(2)	
$C_6H_{12}O_6 + 2H_2 \rightarrow CH_3CH_2COOH + 2H_2O$	(3)	
$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2$	(4)	
$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + CH_3CH_2OH + CO_2$	(5)	
$C_6H_{12}O_6 \rightarrow 3CH_3COOH + 2CH_3CHOHCOOH$	(6)	
$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	(7)	
$C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2H_2 + 2CO_2$	(8)	
$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + (1/2)CH_3COOH + (3/4)CH_3(CH_2)2COOH$	(9)	
$C_6H_{12}O_6 \rightarrow (4/3)CH_3CH_2COOH + (2/3)CH_3COOH + (2/3)CO_2 + (2/3)H_2O$	(10)	
$CH_3CH_2COOH + 2CO_2 + 6H_2 \rightarrow CH_3(CH_2)_3COOH + 4H_2O$	(11)	
$CH_3CH_2COOH + CH_3(CH_2)2COOH \rightarrow CH_3(CH_2)_3COOH + CH_3COOH$	(12)	
$CH_3CH_2COOH + CH_3COOH + H_2 \rightarrow CH_3(CH_2)_3COOH + 2H_2O$	(13)	
$CH_3(CH_2)_2COOH + CH_3COOH + 2H_2 \rightarrow CH_3(CH_2)_4COOH + 2H_2O$	(14)	
$CH_3(CH_2)_2COOH + CH_3COOH + 2H_2 \rightarrow CH_3(CH_2)_4COOH + 2H_2O$	(15)	
Acetogenic reactions		
$CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$	(16)	<i>Desulfovibrio, Syntrophobacter wolinii, Syntrophomonas</i>
$CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H_2$	(17)	
$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	(18)	
$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2$	(19)	
$CH_3(CH_2)_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$	(20)	
Methanogenic reactions		
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	(21)	<i>Methanobacterium formicicum, M. bryantii, Methanobrevibacter ruminantium, M. arboriphilus, Methanospirillum hungatei, Methanosarcina barkeri</i>
$CH_3COOH \rightarrow CH_4 + CO_2$	(22)	
$2CH_3(CH_2)_2COOH + 2H_2O + CO_2 \rightarrow 4CH_3COOH + CH_4$	(23)	
$4HCOOH \rightarrow 3CH_4 + CO_2 + 2H_2O$	(24)	
$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	(25)	
$2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COOH$	(26)	

Adapted from Abbasi et al. [11] and Saady et al. [12].

Table 1. Reactions during acetogenic hydrogen fermentative.

In the step of acetogenesis, the hydrogen could be formatted from lactic acid, ethanol, propionic acid and butyric acid (Eqs. (17)–(20)).

Hydrogen could be consumed for archaea hydrogenotrophic (Eq. (21)). Approximately 70% of all the methane produced in anaerobic digestion process stems from Eq. (22). Furthermore, methane is formed from acetate, butyrate, formate, ethanol and methanol (Eqs. (22)–(26)).

Among the fermentative anaerobes, *Clostridia* have been well known and studied extensively, not for their hydrogen production capability but for their role in the industrial solvent production from various carbohydrates [7]. Hydrogen production by these bacteria is highly dependent on the process conditions such as pH, hydraulic retention time (HRT), and gas partial pressure, which affect metabolic balance. Thus, fermentation end-products produced by microorganism depend on the environmental conditions in which it grows [10].

There are also some bacteria that consumed hydrogen, such as *Lactobacillus* spp. and *Bifidobacterium* spp. [4]. Moreover, the major H₂-consuming microorganisms other than hydrogenotrophic methanogens are homoacetogens, such as *Methanobacterium*.

Depending on the pathway, the theoretical biogas composition is around 67% of H₂ (acetate pathway) or 50% of H₂ (butyrate pathway). The various metabolic pathways that may establish can either be promoted or inhibited, depending on the adopted operating conditions, which govern the production of specific volatile fatty acids (VFAs) and alcohols including acetate, propionate, butyrate, lactate and ethanol [16].

3. Dark fermentation from complex wastewaters

Dark fermentation is a biological approach commonly used to produce H₂ in the absence of light and hence the configuration of the bioreactor is simpler and cheaper. Hydrogen production by dark fermentation has several other advantages such as the ability to produce hydrogen from organic waste and therefore control and stabilize biological waste which has a potential danger of contamination. For instance, dark fermentation can be integrated into wastewater treatment systems to produce H₂ from wastewater. Producing hydrogen from organic waste has a potential to reduce hydrogen production costs since organic waste (including wastewater) is cheap and easily available [2]. Moreover, the regulatory need of treatment of wastewater prior to disposal is making them an ideal commodity to produce biohydrogen from the anaerobic treatment [17].

The main source for the fermentative H₂ production is complex wastewater containing carbohydrate substances. Crucial points for improving the efficiency of hydrogen production that are frequently emphasized throughout literature are associated with facilitated access to cheap wastewater, such as vinasse, cassava wastewater, cheese whey, glycerol, sago wastewater and textile wastewater. There are several articles in the literature that demonstrate the hydrogen production from these wastewaters, indicated in **Table 2**, including the process parameters such as substrate concentration, pH, temperature, HRT, reactor type and seed sludge.

Numerous works have been focused on vinasse. This wastewater, one of the major by-products of the ethanol production process with nearly 14 L of vinasse produced per litre of ethanol, can cause extensive pollution due to its high organic load (up to 40 g COD/L) [18]. Cassava wastewater and cheese whey, main components of agro industrial processes, are considered as highly polluting due to their high organic load and the volume generated, representing a significant environmental impact for the agro-industry [19, 20]. They were also used for the successful H₂ production [21–25].

Substrate	Reactor	Inoculum	Range of pH	T	Substrate conc. (mg COD/L)	HRT (h)	OLR (kg COD/m ³ d)	Maximum HY (mmol/g COD)	HPR (L/dL)	Methane production	Reference
Mixture of glucose and cheese whey	AFBR	Sludge from poultry slaughterhouse	4.0–4.5	30°C	5000	6 h	20	1.6	2.4		Ferreira Rosa et al. [21]
Cheese whey powder	UASB	Full-scale methanogenic UASB reactor treating wastewater from a confectionery factory	4.5–5.63	–		13–3 h	20–48	–	1.62	0.2–0.6 L/dL	Carrillo-Reyes et al. [22]
Cassava wastewater	UASB	Sludge collected from the bottom of a first anaerobic pond treating a cassava wastewater	5.5	37°C	–		10–30	39.83 l H ₂ /kg COD removed	0.39		Intanoo et al. [23]
Cassava flour wastewater	Continuous multiple tube reactor	Natural fermentation	6.5	25°C	4	4	24	2.07 mol/mol substrate	1.94		Gomes et al. [24]
Cassava processing wastewater	AFBR	Anaerobic sludge from a UASB reactor was used for the treatment of swine wastewater	4.5–5.0	30°C	2000–15,000	12–10	4–30	2.0 (12 h)	1.66 (12 h)	14–27% 0.9 L CH ₄ d ₁ L ₁ - 1.5 L CH ₄ -d ₁ L ₁	Rosa e al. [25]
Molasses wastewater	Continuous mixed immobilized sludge reactor	Anaerobic sludge obtained from a local municipal wastewater treatment plant	4–5	35°C	2–6	6 h	8–32	130.57 mmol/mol	12.51 mmol/h L		Han et al. [26]
Tapioca wastewater	ABR	Anaerobic mixed cultures	pH initial—9 effluent = 5.2–5.8	32.3°C	–	24, 18, 12, and 3 h	6 16–130 kg m ⁻³ d ⁻¹	0.745 mmol/g DQO (OLR = 31)	0.883 (6 h HRT)	0.63–3.7%	Thanwiset et al. [27]

Substrate	Reactor	Inoculum	Range of pH	T	Substrate conc. (mg COD/L)	HRT (h)	OLR (kg COD/m ³ d)	Maximum HY (mmol/g COD)	HPR (L/dL)	Methane production	Reference
Crude glycerol	UASB	Sludge from UASB reactor of a seafood wastewater treatment system	Initial = 8	40°C	10–30	12–2	–	44.27 mmol H ₂ /g glycerol	242.15 mmol H ₂ /L/d		Chookaew et al. [28]
Beverage industry wastewater	CSTR	Anaerobic sludge	5.6–6.3 without any pH control	37°C	–	8–1.5	60–320	1.7 mol/mol hexose utilized	55 L/L-d		Sivagurunathan et al. [29]
Sugarcane vinasse	AFBR	Sludge from an upflow anaerobic sludge blanket (UASB) reactor used for the treatment of swine	4–5	30°C	5000–10,000	6–1 h	20–240	3.07	13.68	0–40%	Reis et al. [30]
Washing wastewater of beverage production process	Continuously stirred anaerobic bioreactor (CSABR)	Seed sludge from municipal wastewater treatment plant	5.5	37°C	5	1 h		0.36 mol/mol	11.39		Liu et al. [31]
Alcohol wastewater	UASB	Sludge from the UASB reactor treating an alcohol wastewater	5.5	37°C	–	1.93–0.72	23–62	125.1 ml /g COD removed	18 L/d		Poontaweegeratigarn et al. [32]
Tequila vinasses	ASBR	Sludge from an UASB treating the wastewater from a brewery plant	5.5	25 and 35°C	0.5–5	24–12 h		–	1.2	35–44%	Buitrón and Carvajal [33]
Tofu-processing wastewater	CSTR	Sludge from wastewater treatment plants	5.5	35°C	20 g COD/L	24–6 h	–	–	1.73		Lay et al. [34]

Substrate	Reactor	Inoculum	Range of pH	T	Substrate conc. (mg COD/L)	HRT (h)	OLR (kg COD/m ³ d)	Maximum HY (mmol/g COD)	HPR (L/dL)	Methane production	Reference
Brewery wastewater	Batch	Sludge from a full-scale upflow anaerobic sludge blanket reactor treating citrate-producing wastewater	4–8	25–45°C	2–12	–	–	158 mL/g COD	59 mL/h		Shi et al. [35]
Alcohol distillery wastewater	ASBR	Sludge from the anaerobic tank of Red Bull Distillery	5.5	37°C	20–60	32–13	15–112.5	172 ml H ₂ /g COD removed,	3.3	6.5–35%	Searmsirimongkolet al. [36]
Palm oil mill effluent	UASB	Seed sludge	5.5	37°C	10–40	32–8 h	–	0.38 L H ₂ /g COD added	8.76		Singh et al. [37]
Glycerol	UASB	UASB granules obtained from a UASB reactor	5.5	37°C	–	–	25–75	410 (mmol H ₂ /mol glycerol)	9 mmol H ₂ /L h		Reungsang et al. [38]
Sugarcane vinasse	Up flow anaerobic packed bed reactors (APBR)	Natural wastewater fermentation process	5.4–5.7	25°C	–	24 h	36.3	Maximum 1.8 average 0.3	0.509		Ferraz Júnior et al. [39]
Textile wastewater	Batch	Sludge anaerobic from the treatment plant	Initial 7.0	37°C	20			1.37 mol H ₂ /molreducing sugar	0.312 L d/l		Li et al. [40]
Cheese whey	Batch	Anaerobic sludge	Initial 8	36°C				3.3 mol/mol lactose	16.2 mL/h	–	Seo et al. [41]
Rice mill wastewater	Batch	<i>E. aerogenes</i> RM 08	Initial 7.0 final 5.1	33°C				0.97 mol H ₂ /mol of suga	134.6 mL/h	–	Ramprakash and Muthukumar [42]
Mixture of sugar cane stillage and glucose	AFBR	Sludge from a granular sludge of a thermophilic upflow anaerobic sludge blanket reactor	4.1–4.3	55°C	5000–5300 mg COD/L	8, 6, 4, 2 and 1 h	26–216	5.73 mmol g COD added (HRT 4 h)	18.72		Santos et al. [43]

Substrate	Reactor	Inoculum	Range of pH	T	Substrate conc. (mg COD/L)	HRT (h)	OLR (kg COD/m ³ d)	Maximum HY (mmol/g COD)	HPR (L/dL)	Methane production	Reference
Corn starch wastewater	Batch	<i>Bacillus cereus</i> and <i>Brevundimonas naejangsanensis</i> isolated from sludge anaerobic	Initial 6.5	35°C	10–20	–	–	1.88 mol/mol glucose	3.96	–	Wang et al. [44]
Coffee drink manufacturing wastewater	CSTR	Anaerobic sludge	5.5	35°C	20	12–6	–	0.2 mol/mol	0.34	–	Jung et al. [45]
Sago wastewater	Batch	Fresh cattle dung	7.0	30°C	0.5–5 (% w/v)	–	–	323.4 mL g ⁻¹ starch	3.48	–	Sen et al. [46]
Soft-drink wastewater	Upflow anaerobic packed bed reactor	Natural fermentation	Initial 6.5	25°C	2.3	0.5	–	3.5 mol H ₂ mol of sucrose	9.6	–	Peixoto et al. [47]
Condensed molasses	Continuously stirred anaerobic bioreactor (CSABR)	Municipal sewage treatment plant	5.5	37°C	40–60	8–0.5 h	–	5.3	14.04 (HRT 0.5 h)	–	Chu et al. [48]
Glycerol	Batch	Anaerobic sludge	6.5	–	–	–	–	2.2 mmol/L	–	–	Trchounian et al. [49]

Table 2. Studies of anaerobic biohydrogen production processes using complex wastewater.

The performance parameters were hydrogen production yield (HY) and hydrogen production rate (HPR). The process parameters including pH [22, 26], hydraulic retention time [22, 27–32], temperature [33–35], substrate concentration [30, 31, 36–38], different sludge [21], support materials [39], pretreatment of wastewater [24, 25, 40–42], use of co-substrate [21, 25, 30, 43, 44], inoculum pretreatment [22, 45, 46], addition of nutrients [44, 47], reactor configuration [45, 48] and effects of some heavy metal ions [49] have already been evaluated. Several types of wastewaters listed in this review could produce hydrogen with a HY range of 0.74–5.3 mmol-H₂/g-COD and a HPR range of 0.03–14.04 L/L/d. Among the wastewaters studied, one of highest HY of 5.3 mmol/g COD was obtained using continuously stirred anaerobic bioreactor (CSABR) from condensed molasses; it was successfully operated for 300 days [48].

4. Simultaneous hydrogen and methane production in a single-stage biosystem

The key point in the fermentative production of hydrogen is the inhibition of the methanogenic step so that the formed hydrogen is not consumed for the formation of methane. Two routes can lead to the formation of methane: the route acetoclastic from acetic acid and methanol (methanogenic acetoclastic or acetotrophic microorganism (**Table 1**, Eqs. (22) and (25)) and the hydrogenotrophic route from H₂ (hydrogen consumers microorganisms or hydrogenotrophic (**Table 1**, Eq. (21))).

Among the forms of control, methanogenic activity can be used to maintain the acidic pH medium for cultivation and processing of the inoculum in order to inactivate methanogens [50]. Furthermore, for continuous reactors, the reduction of HRT and consequently higher organic loading rate (OLR), they can avoid the use of H₂ as a substrate for methanogenesis.

Recently produced hythane (H₂+ methane) in a single-stage biosystem, using complex wastewaters with low pH, short HRT and high concentration of organic matter suggested that some archaea can survive at conditions that do not favour methanogens [22, 25, 27, 30, 33, 36]. The hythane production has also received much commercial attention in the transportation sector [51], and a production in a single stage has the advantage of being economically more viable due to economic financial, energy and manpower, than the hythane production by two-stage fermentation [52].

Kim et al. [53] conducted a study on the influence of pH on the activity of users consuming hydrogen methanogens. According to the authors, the formed methane left in consumers of hydrogen archaeas, which are commonly inhibited at pH below 5.0, proved to be more tolerant of acidic conditions than other methane-producing microorganisms.

Carrillo-Reyes et al. [22] evaluated the reduction of pH (5.63–4.5) in UASB reactors fed cheese whey, with a HRT of 6 h and OLR of 20 kg COD/m³.d (**Table 2**). The authors reported that the strategy of reducing the pH to 4.5 to avoid methane production was not efficient. This fact did not favour the hydrogen production and even caused a sharp drop in the total gas production. Similar results were found by Taconi et al. [54], who found a 30% increase in methanogenic activity when the pH was decreased from 7 to 4.5.

As maintaining the pH in acidic conditions does not guarantee the inactivation of methanogens, the heat treatment of the inoculum is not conclusive. For instance, the acetoclásticas microorganisms can survive thermal shock, leading to the consumption of hydrogen to acetic acid formation [50]. The formed acetic acid is then converted into methane (**Table 1**, Eq. (23)).

This fact is reported by Luo et al. [55] who used cassava stillage as the substrate for hydrogen production and found that thermal pretreatment of inoculum does not improve the yield of hydrogen in continuous reactors under mesophilic temperatures. The study analysed the effect of different pretreatments of the inoculum such as acid treatment, heat treatment and shock load in repeated batch tests, demonstrated that inoculum pretreatment could not permanently inhibit methanogenesis either. According to the authors, the methane inhibition only occurs by proper control of fermentation, pH and temperature.

Given the resistance of methanogenic archaea of pretreatment of inoculum, Carrillo-Reyes et al. [22] showed that repeated heat treatment of the granular sludge was the only strategy that completely inhibited methane production, leading to high volumetric hydrogen production rates (1.67 L H₂/L-d). In the same study, the authors use a strategy to decrease methane production: the shock loads (from 20 to 30 g COD/L-d) was a more effective strategy to decrease the methane production rate (75%) and to increase the hydrogen production rate (172%), without stopping reactor operation.

Methanogens were detected in different hydrogen-producing reactors operated at low pH (values between 4.0 and 5.63) and with high organic loading rate (**Table 2**) revealing that they can survive under these extreme conditions.

In ASBRs, Buitrón and Carvajal [33] reported the production of methane (35–44%) concomitant with the production of H₂ when employed with HRT of 24 h. They found that the higher the concentration of vinasse, the greater the percentage of methane achieved. According to the authors, methanogens could be already present in vinasse and before the source of organic acids, and H₂ produced by the reactor found an environment conducive to development on the other hand, using similar wastewater, and even reactor. Searmsirimongkol et al. [36] evaluated the effect of concentration (20, 30, 40 g/L) on the hydrogen production. The highest methane yields (approximately 40% methane content of the biogas) were found at lower concentration (20 g/L); however, concentration higher than 60 g/L did not verify the presence of methane. Serious methanogeneses were reported in high rate reactors, such as UASB [22] and AFBR [25, 30].

The hydraulic retention time (HRT) is also an important parameter in the fermentation processes. Higher rates of volumetric hydrogen production and increased percentages of hydrogen in biogas can be obtained by decreasing the HRT and thus increasing the organic loading rate (OLR) [56]. In addition, low HRT could suppress methane producers and inhibit methanogenesis. However, in many complex wastewaters, this behaviour is not checked. Exemplifying, Rosa et al. [57] evaluated the effects of different hydraulic retention times (HRTs) of 4, 2 and 1 h and varying sources of inoculum (sludge from swine and sludge from poultry) on the hydrogen production in two AFBRs from cheese whey. When the HRT was

reduced, methane was produced concurrently with hydrogen in both reactors, with maximum methane production of 0.68 L CH₄/h/L with an applied HRT of 1 h. Carrillo-Reyes et al. [56] found that the application of a OLR of 20 g COD/L/d and a gradual decrease of HRT from 24 to 6 h led to a decrease in H₂ production from 0.03 to 0.015 LH₂/L/h, due to the presence of methane. According to the authors, the delay in the production of methane from this reactor, when compared to other reactors in their study, was due to the application of high substrate concentrations. The maximum methane yields of 0.02 L/h/L were obtained in reactors with the application of HRT of 6 h, and OLR from 5 to 20 g COD/L. Other studies have also found the simultaneous hydrogen and methane production in short HRTs, from different wastewaters, such as stillage [30, 33], rich in starch wastewater [25, 27].

These results indicated that the low HRT in different configurations of reactors might reduce microbial richness through the washout of microbes and increase microbial diversity through accelerating the proliferation of non-hydrogen-producing microorganism. So, methanogens could adapt to the conditions imposed in hydrogen-producing reactors (low pH, high OLR and low HRT). In spite of the negative effect of these organisms in hydrogen production, they may have an important application in the production of hythane (H₂ and CH₄) using wastewaters with low pH and high concentration of organic matter.

5. Bioreactor configuration

The reactor configuration and the improvement of operating parameters is essential to obtain best hydrogen production rates, indicating that the system performance is largely influenced by the retention of biomass reactor [58]. The batch modes of operation and continuity have been reported in the literature for producing hydrogen. Most batch studies have the advantage of being easily operated, flexible, generating a series of studies with different wastes to produce hydrogen [9]. However, these reactors provide lower H₂ production rates as compared to continuous systems.

Continuous stirred tank reactors (CSTR) are the most common continuous system used for hydrogen production by dark fermentation from olive milk wastewater, cheese whey and condensed molasses (**Table 2**). Reactors upflow anaerobic sludge blanket (UASB), anaerobic fluidized bed (AFBR) and anaerobic packed bed reactor (APBR) also are used for the production of hydrogen in different complex wastewater. The advantages and disadvantages of different types of bioreactors for H₂ production are listed in **Table 3** [59, 60].

Glycerol [49], sago wastewater [46] and brewery wastewater [35] were proved to be feasible substrates by batch tests showing the maximum HY of 2.2 mmol/L, 323.4 mL/g starch and 158 mL/g COD, respectively. In continuous H₂ production, the main reactor used was AFBR [25, 30, 43], UASB [22, 23, 28, 32, 37, 38] and CSTR [29, 34, 45].

In fermentative hydrogen production, the HRT, and in turn the OLR, affect the substrate conversion efficiency, the type of active microbial population as well as the metabolic pathways established in the system [16]. In the following sections, a discussion of literature findings about the influence of these parameters is presented.

Reactor type	Advantages	Drawbacks
CSTR	Simple construction, easy to operate and control	Low biomass retention
UASB	Good retention of biomass in all reactor areas (bed and sludge blanket)	Slow development of granules
AFBR	Good retention of biomass Good mass transfer due to efficient mixing	Excessive shear stress can detach biomass Energy required for fluidization bed
APBR	Good retention of biomass	Clogging Lower mass transfer than FBR

Table 3. Bioreactors for H₂ production: advantages and drawbacks.

5.1. Influence of OLR

The parameters that constitute the OLR are the concentration of organic matter and HRT. For it is a design variable which determines the capacity and the reactor operating conditions. Changes in OLR have a considerable influence on the diversity of the microbial population and on the metabolism pathways of bacteria that may favour hydrogen production [61].

According to De Gioannis et al. [16], there is a discrepancy in the literature regarding the effect of OLR and HY. According to these authors, the OLR is affected by the accumulation of acid, pH changes and variations in the composition that subsequently change the metabolic pathways.

5.1.1. Substrate concentration

The substrate concentration should be selected in order to meet the needs of microbial growth and hydrogen production and its increase can ensure a stable production of hydrogen in high yield [43]. However, concentrations of organic matter in excess decrease substrate conversion and the yield of hydrogen due to the accumulation of inhibitory compounds in the medium, reducing the competitiveness of hydrogen producers for other microorganisms [3, 43].

In the batch tests, optimal substrate concentration varied and was deeply influenced by other operational parameters such as the pH. When the pH was not controlled, HY usually decreased with increasing substrate concentration due to low pH condition. In contrast, finding the optimal substrate concentration in continuous operation mode is more meaningful and practical, since the batch mode does not take into consideration the hydrodynamic effect, steady state of the substrate concentration and pH condition for bacterial growth [62].

Higher feeding concentrations of the substrate could increase H₂ production [22, 26]; however, excessive substrate concentrations may decrease this capacity [25, 28, 31, 34, 36, 37]. Chu et al. [48] in a suspended sludge bioreactor producing H₂ fed with condensed molasses fermentation soluble, increased the H₂ production rate by 2.3 times by elevating the substrate concentration from 40 to 60 g COD/L at a HRT of 2 h. Already, in continuous mixed immobilized sludge reactor from molasses wastewater, Han et al. [26] increased the HPR 3.36 times by elevating the substrate concentration from 2 to 6 g COD/L at a HRT of 6 h. In contrast, when varying the

tofu-processing wastewater concentration from 10 to 40 g COD/L in a batch reactor, Lay et al. [34] found that 20 g COD/L was the optimum concentration for H₂ production.

Most studies reported that hydrogen production from complex wastewaters had substrate concentrations lower than 40 g COD/L (**Table 2**). Often it is noted that higher concentration of any substrate leads to a drop in HY [34]. Moreover, it has been reported that in some cases hydrogen production can be inhibited by the toxicity of the complex wastewaters. This fact is noted by Searmsirimongkol et al. [36] who then diluted alcohol distillery wastewater to obtain various feed COD values of 20,000, 40,000 and 60,000 mg/L. The highest concentrations of hydrogen production resulted in inhibition due to the presence of high potassium concentration. Already Liu et al. [31] showed that SO₃²⁻ affected the hydrogen production at the substrate concentration of 10 g total sugar/L process, when the performance of hydrogen production decreases, HPR was reduced from 34.59 to 6.50 L/L/d, yield was reduced from 0.92 hexose to 0.08 mol H₂/mol hexose, when SO₃²⁻ increased from 0 to 80 mg/L. Sulphate-reducing bacteria (SRBs) causes hydrogen gas converting hydrogen sulphide become less efficient in hydrogen production.

A maximum hydrogen production of 11.39 L/d/L was obtained at HRT 1.0 h (a concentration of 10 g) from washing wastewater of beverage production process with continuously stirred anaerobic bioreactor [31]. The authors suggested that the hydrogen-producing bacteria (HPBs) were adaptive to the system.

High substrate concentration allows more energy-efficient operation but product inhibition is likely to set the upper limit. Certain level of metabolic products in the dark fermentation may inhibit H₂ producing pathway as well as microbial activity [58].

5.1.2. HRT

HRT indicates the time that the organic matter remains in the reactor. This time depends on the metabolism rate of organic matter by microbial community and may vary according to the process. HRT can be used to select a producer of hydrogen community depending on the substrate used.

HRT is also an important parameter in the fermentation process. Higher rates of volumetric hydrogen production and increased percentages of hydrogen in biogas can be obtained by decreasing the HRT and thus increasing the organic loading rate (OLR) [56]. Shortening hydraulic retention times (HRTs) is a well-used and effective operation strategy to enhance hydrogen production from organic wastewater and solid wastes because of its ability to exclude methanogens which have longer generation time.

In most studies on continuously dark fermentative hydrogen production, continuous systems are expected to operate at a low HRT 36–12 h [27, 33, 34, 37, 39] and very low of HRT 12–2 h [21, 22, 25, 26, 28–30, 43, 45, 48] for obtaining a high biohydrogen production that can be operated at extremely low HRT 2–0.5 h [30, 47, 48] with immobilized cell in the biohydrogen.

As shown in **Table 2**, the range of organic loading rate (OLR) was 16–320 kg/m³/d equivalent by a gradual decrease in HRT from 32 to 0.5 h. When considering the variation in hydrogen

production with respect to the HRT, it can be seen that the HRT greatly affected microbial activity and metabolic products, leading to variations in gas production rate, gas composition and hydrogen production rate [36].

With regard to the microbial community, short HRT is also preferred from beverage wastewater [29, 31], sugarcane stillage and glucose [43] and crude glycerol [28]. In contrast, most studies had a drop in hydrogen production because: of too low mixing and poor contact of glycerol with the microorganisms [28]; of the occurrence of OLR shock from tapioca wastewater [27]; of longer reaction time, which allowed for more time to metabolize the Tequila vinasses [33]; microbial cells were washed out from the system as a result from the toxicity of VFA accumulation from alcohol wastewater [32] and of lactate accumulation from tofu-processing wastewater [34].

A maximum hydrogen production of 55 L/d/L was obtained at HRT 1.5 h (an OLR of 320 g/L-d hexose equivalent) from beverage industry wastewater (20 g/L hexose equivalent) with CSTR [29]. This HPR value is much higher than those of other complex wastewaters employed in fermentative hydrogen production.

Therefore, it is essential to define a range of OLR, which will enable to achieve constant efficiency in the biological reactor, or an optimum OLR value for maximum H₂ yield. As a result, the fermentative routes and final metabolites products may be modified due to the OLR applied, as well as the conversion efficiency of the substrate and the microbial community established in the system [16].

6. Strategies for improved hydrogen production

Fermentative hydrogen production is a very complex process and is influenced by many factors such as inoculum, substrate, reactor type, temperature and pH. The effects of these factors on hydrogen production have been reported by a great number of studies throughout the world in the last few years.

Wang and Wan [63] showed that there usually existed some disagreements on the optimal condition of a given factor for fermentative hydrogen production, thus more researches in this respect are recommended.

6.1. Pretreatment of complex wastewater

To enhance the fermentations of some complex wastewaters, such as cassava wastewater, tofu-processing wastewater, corn starch wastewater and textile wastewater, pretreatment must be done to make the process feasible and sustainable. These processes include various combinations of biological, physical and chemical treatment processes [24, 25, 34, 40–42]. Each of these pretreatment methods has a unique purpose and will depend on the wastewater used.

Starch can be hydrolyzed into glucose and maltose by acid or enzymatic hydrolysis followed by biological conversion of the carbohydrates into organic acids and then into hydrogen gas [64]. Moreover, mixed culture could produce more various hydrolases which could utilize complex substrates present in wastewater than pure culture [65]. Rosa et al. [25] used the technique of

acid hydrolysis with sulphuric acid and heated the cassava wastewater at 120°C for 30 min before being used as a substrate. A maximum hydrogen yield of 2.0 mmol/g COD was achieved with OLRs of 10 kg COD/m³/d.

The heat treatment of complex wastewater rich in starch (corn starch wastewaters, rice mill wastewater, cassava wastewater) is common, with the purpose to remove the mixed population of microorganism in the wastewater, which could either compete with biohydrogen producers or inhibit their growth [24, 42, 44]. In these studies, the heat treatment was made in 120°C with times between 15 and 25 min. This pretreatment was also used for tofu-processing wastewater, but at temperature 70°C for 30 min to inhibit the hydrogen-consuming bacteria [34].

Lactic acid bacteria (LABs) are members of the autochthonous microbiota of cassava and are responsible for the fermentation of the root; furthermore, LAB reduces cassava toxicity and prevents post-harvest deterioration [66]. However, in hydrogen-producing reactors, a few LAB strains may have an inhibitory effect due to their bacteriocins, which are antimicrobial peptides that have a deleterious effect on H₂-producing bacteria [67]. Gomes et al. [24] conducted pretreatment heat (121°C; 15 min) in order to eliminate probable negative effects of the presence of LAB in the cassava wastewater used. The bacteriocins as well as their degradation products were detected in both the raw and heat-treated cassava wastewater samples. Their presence suggests that the poor results of hydrogen production observed in all assays could be attributed to these compounds, and demonstrated that the heat treatment of wastewaters may not completely deactivate bacteriocins. In contrast, Seo et al. [41] evaluated the effect of different pretreatment of cheese whey for hydrogen production (heat pretreatment; sonication pretreatment; and hydrodynamic cavitation). All the treated samples exhibited H₂ production activity, suggesting the fact that LABs, which exist predominantly in the raw cheese whey and produce lactic acid, were effectively suppressed. The maximum H₂ yield of 1.89 mol H₂/mol lactose was obtained from the cheese whey pretreated with hydrodynamic cavitation for 15 min.

The production of bio-H₂, particularly from more complex wastewater, such as textile wastewater, has been treated with activated carbon. This technique is available for wastewater industries, solvent recovery, chemical catalyst, gold extraction, gas separation and liquid adsorption. Li et al. [40] used the textile wastewater, hydrolyzed by α -amylase with a concentration of 0.2 mL/L for 20 min. After α -amylase hydrolysis, the hydrolysate was pretreated with activated carbon and cation exchange resin with a concentration of 1% w/w for 30 min. The removal efficiency of ion concentration was 95.85%. After that, the hydrolysate was fed into the batch reactor and the best hydrogen yield was 1.37 mol H₂/mol, reducing sugar.

The application of pretreatment to the complex wastewater was tested in an attempt to overcome eventual limitations these wastewater. The selection of suitable hydrolysis method and/or control of inhibitors production will improve the fermentation, resulting in a positive effect and improving the degradability of the complex wastewater during the biological process [24, 25, 42].

6.2. Nutrients

Excluding the main substrate, carbohydrate materials, dark fermentative hydrogen production requires nutrients for bacterial activity like all biological treatment processes. The nutrients

include nitrogen (N), phosphorous (P), ferrous (Fe) and some trace metals. Among the many kinds of nutrients, N is the most essential one for bacterial growth. Optimal C/N ratio is 47 according to Lin and Lay [68]. P and Fe concentrations affect the metabolic pathway of *Clostridium* sp., and hydrogen production potential decreases when their concentrations are limited.

Appropriate ratios of carbon and nitrogen, carbon and phosphorus, and between carbon and sulphate increase bioproduction of hydrogen by modifying metabolic pathways associated with the nutritional requirement of microorganisms [69]. Argun et al. [70] observed that an adequate nitrogen concentration depends on the phosphorus concentration in the medium. That is, systems with a low phosphorus concentration require a low nitrogen concentration and vice versa. However, in their research, the best hydrogen yield of 281 mL H₂/g starch was obtained at a C/N ratio of 200 and a C/P ratio of 1000, namely, for lower concentrations of nutrients. High nitrogen and phosphorous concentrations could inhibit hydrogen formation by dark fermentation, which likely alters the metabolic pathway [47]. In contrast, low at C/N and the pH values below 3.5 suggests that surplus carbon source could cause rapid acidification and influence the metabolism and growth of microorganism [44].

Some complex wastewater, such as cheese whey [21, 22], Tequila vinasses [33], cassava wastewater [24, 25], soft-drink wastewater [47] and corn starch wastewaters [44], added nutrients to ensure that all the required components were present.

Peixoto et al. [47] showed a similar example when added urea (COD:N of 100:0.7) was used as the nitrogen source in one of their upflow fixed-bed reactors. Under that condition, the hydrogen production ceased completely after 8 days of operation. In contrast, the reactor with a COD:N ratio of 100:0.3 produced hydrogen continuously for 70 days with an average hydrogen yield of 3.5 mol H₂/mol substrate. These authors suggested that the excessive cell growth caused by the addition of nutrients affected the reactor's hydrodynamic pattern, hindering the liquid-gas transfer mass of hydrogen. In addition, the decrease of the HRT increased the production of non-reduced compounds.

Searmsirimongkol et al. [36] evaluated hydrogen production using as source substrate wastewater from ethanol processing produced from sugarcane in anaerobic sequencing batch reactor (ASBR). Through concentration of 40 g/L, OLR of 60 kg COD/m³/d, HRY of 16 h and pH 5.5, at 37°, reached 3320 mL H₂/L/d and 172 mL H₂/g COD removed. The high concentrations of potassium and sulphate observed in raw stillage (with COD of 150 g/L), 8.8 and 7.0 g/L, respectively, show the need to dilute the affluent to avoid toxic effect to the hydrogen-producing bacteria. At concentrations above 40 g COD/L, system performance decreased in terms of hydrogen production due to higher concentrations of PO₄-3 and SO₄-2. Gomes et al. [24] showed that hydrogen production from cassava wastewater quickly decreased and terminated even in the presence of the heat-treated wastewater with or without nutrient supplementation. The authors suggested that the problems were not due to lack of nutrients, but due to the presence of lactic bacteria.

6.3. Temperature

Temperature affects the growth rate and the metabolic pathways of microorganisms, and is considered one of the most important operating parameters which affect fermentative pro-

duction of hydrogen. Microorganisms are capable of producing hydrogen at a temperature ranging from 15 to 85°C. Fermentative reactions for hydrogen production are mainly conducted at mesophilic (25–40°C) and thermophilic (40–65°C) temperatures, while few studies have been conducted in hyperthermophilic temperatures (above 80°C) [8].

As shown in **Table 1**, most of the studies were conducted under mesophilic conditions (25–40°C). Only a few studies [35, 43] were conducted under thermophilic conditions (45–55°C). High temperature can promote hydrolysis and simplify microbial diversity in a manner favourable to H₂ production, but it can also bring about monotonous microbial diversity, resulting in incomplete substrate degradation, especially in the treatment of actual waste. Also, operation at high temperature places an economic burden, as it requires a tight and closed structure and immense energy to heat and maintain the temperature of the reactor. Therefore, the temperature effect must be thoroughly investigated considering not only the H₂ fermentation performance but also substrate degradation and economic factors [62].

Few studies have evaluated the effect of temperature, and the substrates during the investigation of the effect of temperature on fermentative hydrogen production were tofu-processing wastewater [34], brewery wastewater [35] and Tequila vinasses [33].

It should be noted that fermentative processes operating under thermophilic conditions have some advantages over mesophilic processes. This is due to: (i) higher temperature has lower solubility gas (Henry's law); (ii) the hydrogen synthesis pathways are less affected by the partial pressure of hydrogen (pH₂) [10] and (iii) the rates of chemical and enzymatic reactions are higher [71]. However, according to Mohan et al. [72], the optimal temperature for the production of hydrogen depends on the nature of the biocatalyst and the type of wastewater to be used as a substrate.

The effect of the temperature (25 and 35°C) on hydrogen production from Tequila vinasse was studied using a sequencing batch reactor, with HRT of 24 h [33]. A maximum HPR of 50.5 mL H₂/h/L and an average hydrogen content in the biogas of 29.2 ± 8.8% were obtained when the reactor was fed with 3 g COD/L, at 35 °C and 12-h HRT. It is 6.2 times greater than the temperature of 25°C under the same conditions.

Lay et al. [34] used two different temperatures (35 and 55°C) and two different seed sludges to evaluate the hydrogen production performance and obtain the best criteria for maximum production from tofu-processing wastewater. The temperature variation did not affect the HY significantly. The maximum HY of 61.2 mL/g COD was obtained at 35°C. Similar values were obtained with the 55°C under the same conditions (HY of 58.8 mL/g COD).

6.4. Use of co-substrates

The use of co-substrates is motivated by other objectives being pursued concomitantly, including (a) combined treatment of different waste streams, (b) ability to treat residues otherwise difficult to manage individually, (c) dilution of potentially toxic/inhibitory compounds, (d) optimization of the conditions for hydrogen production and (e) optimization of the carbohydrate/protein ratio [16].

The literature also reports that simple substrates, such as glucose, have been used in mixtures with other complex substrates in the search for optimal conditions for hydrogen production: mixture of sugarcane stillage and glucose [30, 43]; glucose and cheese whey [21]; glucose and cassava wastewater [25]; and corn starch wastewaters [44]. The strategy of using mixed substrates demonstrates the high interest among researchers in evaluating the feasibility of hydrogen production through waste fermentation in the presence of glucose.

Wang et al. [44] using corn starch wastewaters exhibited an efficient H₂ yield which was found to be 76.0 and 31.7% higher than that of using corn starch and cassava starch, respectively. Moreover, in the study of Ferreira Rosa et al. [21] showed that the use of mixed substrates also favoured the production of hydrogen, when compared using glucose as an individual substrate. The co-fermentation of the cheese whey and glucose mixture was favourable for the concomitant production of hydrogen and ethanol, with yields of up to 1.7 mmol H₂/g COD and 3.45 mol EtOH/g COD in AFBR.

Most studies of co-fermentation focused on the performance of hydrogen production in AFBRs. It is interesting to note that there was a variation in biogas composition when the carbon source was changed from a mixture of glucose/wastewater [21, 30, 43]. Even with different operating conditions and wastewater, the same pattern of behaviour was observed, indicating that the substrate mixtures are a preferable carbon source compared with glucose.

Chen et al. [73] reported the inhibition of anaerobic processes, suggesting that to effect a better adaptation of microbial community, prior to use more complex substrate is placed on a simpler carbon source until their total consumption. Acclimation of anaerobic microorganisms both increases their tolerance to the toxicants shock and enhances toxicant biodegradability.

Co-fermentation from wastewaters with glucose and adaptation of microorganisms to inhibitory substances can significantly improve the wastewater treatment efficiency and hydrogen production. Possibly a favourable environment for the development of microorganisms has been created, with the presence of simple substrates and nutrients. However, the costs for pure carbohydrate sources are high for practical-scale hydrogen production, which can only be viable when based on renewable and low cost sources [6]. Studies to analyse the nutrients of different wastewaters, in order to get a better rate C:N and C:P, could make viable the combination of two complex wastewaters. This would make it feasible to process hydrogen production, due to the lower cost of substrates.

7. Microbial diversity

Table 4 shows that a limited number of reports co-exist on microbial communities producing hydrogen from complex wastewaters. These studies analysed the composition of microbial communities by cloning and sequencing the 16s rRNA from sugarcane vinasse, 454-pyrosequencing data analysis from sugarcane vinasse, fluorescent *in situ* hybridization (FISH) from glycerol and affiliation of band sequence from denaturing gradient gel electrophoresis (DGGE) from beverage wastewater.

Substrate	Microorganisms	GenBankaccess	Relative abundance	Microbiological analysis	Reference
Beverage wastewater	<i>Clostridium</i> sp.	NR_042144.1 NR_044718.2 NR_042144.1 NR_026100.1 NR_104822.1NR_074511.1JF4 99889NR_074482.1		Affiliation of band sequence (retrieved from DGGE gel)	Sivagurunathan et al. [29]
	<i>Klebsiellaoxytoca</i>	NR_102982.1			
	<i>Selenomonas lactificifex</i>	AF373024.1			
Sugarcane vinasse	Uncultured <i>Prevotella</i> sp.	JX575984.1	7	Cloning and sequencing the 16s rRNA	Reis et al. [30]
	Uncultured Prevotellaceae bacterium	JF806757.1	55		
	<i>Megasphaera</i> sp.	HM990965.2	28		
	Uncultured bacterium	JQ072158.1	7		
	Uncultured <i>Clostridia</i> bacterium	EU887973.1	13		
Glycerol	<i>Enterobacter</i> sp.		27.1	Fluorescent in situ hybridization (FISH)	Reungsang et al. [38]
	Firmicutes bacteria		18.88		
Sugarcane vinasse	<i>Pectinatus</i>		54.1	454-pyrosequencing data analysis	Ferraz Junior et al. [39]
	<i>Clostridium</i>		12.8		
	<i>Megasphaera</i>		3.3		
	<i>Propionispora</i>		3.2		
	Order Burkholderiales		3.6		
Family Comamonadaceae		18			
Textile wastewater	<i>Clostridium butyricum</i>			PCR-DGGE) with partial 16S rRNA genes followed by their sequencing	Li et al. [40]
	<i>Klebsiella oxytoca</i>				
	<i>Clostridium</i> sp.				
Sugar cane stillage	<i>Clostridium cellulosi</i>	NR044624.1	7	Cloning and sequencing the 16s rRNA	Santos et al. [43]
	<i>Thermoanaerobacterium</i>	JX442957.1JX984979.1JX984974.1HM585225.1AF247003.1	62		
	Uncultured bacterium clone D8-50C-C4-3	HQ266872.1	20		
	<i>Lactobacillus</i> sp.	AB016864.1DQ523489.2	4		
	<i>Moorella</i> sp.	AB086398.1	2		
Soft-drink wastewater	<i>Clostridium</i> sp.	DQ196630		Amplified and sequenced from the DGGE samples	Peixoto et al. [47]
	<i>Klebsiella</i> sp.	EU196756			
	<i>Enterobacter</i> sp.	EU430750			

Substrate	Microorganisms	GenBankaccess	Relative abundance	Microbiological analysis	Reference
Condensed molasses fermentation solubles	<i>Clostridium butyricum</i>			Affiliation of band sequence (retrieved from DGGE gel)	Chu et al. [48]
	<i>Megasphaera</i> sp.				
	<i>Corynebacterium glutamicum</i>				

Table 4. Microbial diversity from complex wastewaters.

Hydrogen can be efficiently and economically obtained from dark fermentation by hydrogen-producing bacteria (HPB) [74]. *Clostridium* and *Enterobacter* were the most widely used microorganisms for fermentative hydrogen production in mesophilic conditions, and *Thermoanaerobacterium* genus under thermophilic conditions [75]. The members of genus *Clostridium* are Gram-positive, and contain endospore-forming rods that produce hydrogen. Already, *Enterobacter* are Gram-negative, rod-shaped and facultative anaerobes [63].

Among the fermentative anaerobes, *Clostridia* have been well known and studied extensively, not for their hydrogen production capability but for their role in the production of industrial solvent from various carbohydrates [7]. This is a common sense, and numerous studies have already been conducted considering the investigation and identification of *Clostridium* sp. with hydrogen yield productive capacity. However, in the production of hydrogen from complex wastewater it has been shown that it is possible to produce hydrogen from other bacteria beyond the genus *Clostridium*. Ferraz Júnior et al. [39] showed by 454-pyrosequence analysis, organisms affiliated with the *Clostridium* and *Pectinatus* genera were dominant in the sample associated with hydrogen production from sugarcane vinasse. In contrast, from the same wastewater, Reis et al. [30] showed by cloning and sequencing the 16s rRNA that 55% belonged to the phylum Bacteroidetes and uncultured Prevotella, and 28% belonged to the phylum Firmicutes genus Megasphaera. Also, the presence of 3% of uncultured *Clostridia* also belonged to the phylum Firmicutes. Under thermophilic conditions, both *Thermoanaerobacterium* sp. and *Clostridium* sp. were efficient hydrogen producers [43].

Many studies reported in the literature that evaluated the microbial community did not report a direct association between microorganisms found and hydrogen production. The likely cause for this is due to the diversity of other organisms found, different *Clostridium*. In contrast, Sivagurunathan et al. [29] reported that the *Clostridium* species dynamics were not significantly affected, but total microbial community structure changed with respect to HRT variation as evident from PCR-DGGE analyses. Moreover, the appearance of *Selenomonas* spp. in a CSTR at low OLR improved the HY, whereas the disappearance of *Selenomonas* spp. at high OLR improved the HPR, but gave a drop in HY from beverage industry wastewaters.

Other organisms have also been found in complex wastewater, such as *Klebsiella oxytoca* and *Enterobacter* sp., indicating the presence of predominant hydrogen-producing bacteria from textile wastewater and glycerol [38, 40].

8. Conclusions and perspectives

The analysis of over 35 literature references on fermentative hydrogen production from complex wastewater has shown that numerous process parameters have the potential of affecting the evolution of the metabolic pathways involved, in turn affecting the process kinetics and the conversion yield.

The production of hydrogen from wastewaters should contribute technologically to the fate of some wastewater, opening the possibility for them to be used as raw material to produce bioenergy. Thus, the discovery of new raw materials for the production of a sustainable fuel contributes to the consolidation of the sector. However, this review showed that there usually existed some disagreements on the optimal condition of a given factor for fermentative hydrogen production from complex wastewaters, thus more researches in this respect are recommended.

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