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Technical difficulties of mixed culture driven waste biomass-based biohydrogen production: Sustainability of current pretreatment techniques and future prospective

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ABSTRACT

Biohydrogen production from different types of waste biomass using mixed culture inoculum has been an active research area in recent years. Several emerging techniques has been studied targeting to achieve higher efficiency and competitive hydrogen production through the dark fermentation. However, the production of biohydrogen from complex waste biomass meets the setback of lower final product yield due to the low substrate degradation rate. Besides, hydrogen consuming microbes in the mixed culture impede the rate of biohydrogen production. Thus, the waste biomass substrate and microbial inoculum are highly required to pretreat for enhancing system performance. With the aim to gain deeper insight into waste biomass and inoculum pretreatment techniques, this review compiles recently practiced pretreatment techniques with their limitations. The major challenges of these methods and few strategies to overcome these limitations with future directions are discussed in this review. Moreover, the efficiency of a potential nonconventional pretreatment technique such as electroporation (EP) over traditional techniques to degrade the complex waste biomass and reduce the load of hydrogen consuming microbes in the mixed culture consortium was reviewed. This review argued to provide a deeper insight to develop an efficient pretreatment technique by combining traditional techniques and EP for enhancing mixed culture driven biomass-based hydrogen production.

1. Introduction

The increasing energy demand and exhaustion of conventional fossil fuels together with global warming drive the research community to find the eco-friendly, renewable, and sustainable energy sources [1,2]. Among the different types of energy resources, hydrogen (H_2) is regarded as an ideal and sustainable energy carrier as it contains high energy density (~142 kJg^{-1}; 3.5 times higher than other hydrocarbon based fuels); it is totally clean (since carbon-di-oxide (CO_2) is not generated during combustion of H_2); and it can be produced from biomass (a renewable source) [3]. Generally, different techniques such as water electrolysis, methane (CH_4) reforming by steams, coal gasification, oil and natural gas oxidations, and thermal process, *etc.* have been applied to produce hydrogen energy [1]. However, these methods are not considered efficient as they are associated with high temperature (>700 °C) as well as non-renewable [4]. Therefore, the biological production of H_2 (biohydrogen) including photo and dark fermentations of carbon based waste substrates, bio-photolysis of water by bacteria

Abbreviations: Lignocellulosic biomass, (LCB); Anaerobic sludge, (AS); Volatile fatty acids, (VFAs); Electroporation, (EP); Pulsed electric field, (PEF); Direct current, (DC); Treatment intensity, (TI); Volatile solid, (VS); Lignocellulose, (LC); Dry cell weight, (DCW); Carbon/Nitrogen ratio, (C/N); Methane, (CH_4); Carbon dioxide, (CO_2); Hydrogen, (H_2); Carbon monoxide, (CO); Hydrogen sulfide, (H_2S); Adenosine-triphosphate, (ATP).

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and/or microalgae has gained increased interest as the process requires relatively low temperature (25–37 °C) and pressure (atmospheric) [1]. Several techniques have been proposed to enhance system performance as illustrated in Fig. 1. However, a selective pretreatment of the substrate as well as the inoculum is the bottleneck for practical biohydrogen production.

Nowadays, cellulosic biomass is widely used as feed-stocks for producing biohydrogen as they are cost effective and source of outnumber carbon [3,5]. The cellulosic waste biomass known as lignocellulosic biomass (LCB) comprising municipal wastes and agricultural residuals, such as forest residues, stalks, straws, yard clippings, wood chips grass, and so on [3] are mostly used LCB for biohydrogen production due to their availability in nature. However, the conversion of raw LCB into hydrogen is troublesome because the hydrolysis of their complex polymeric (hemicellulose and cellulose) to smaller molecules is the rate-limiting step for biomass degradation [6,7]. Therefore, pretreatment of substrate is indispensable to metabolize crystalline and rigid structure of LCB for reducing the retention time of fermentation and to improve hydrogen yield. Several pretreatment techniques including physical, chemical, biological, or combined have been extensively used to disintegrate the recalcitrant structure of LCB for ease assimilation by cellulolytic bacteria in the anaerobic fermentation [8]. However, the physical methods (*e.g.*, steam explosion, ultrasonication, high-pressure homogenization, bead-beating, osmotic shocks, *etc.*) are associated with increased heat generation and requires high energy input [9,10]. In contrast, the chemical pretreatments such as acid or/and base hydrolysis are widely used methods for degrading cellulose and/or hemicellulose structure in LCB; however, a diverse range of toxic inhibitors such as furan, furfural, and phenolic compounds are common by-products of the chemical pretreatments, which reduce the efficacy of H_2 producing bacteria in fermentation process [11]. The enzymatic pretreatments could resolve these problems since they are usually conducted under mild condition, but high cost of enzyme and sluggish rate of reaction constrain their application in biological hydrogen production [12]. Therefore, further research is emergent to find a suitable pretreatment method for addressing afore-mentioned problems.

In the fermentation process, the mixed microbial communities, particularly anaerobic sludge (AS) are considered more practical and effective inoculum due to their availability in nature, capability of surviving in extreme environmental conditions and accommodating diverse range of substrates [13,14]. However, AS contains a certain composition of H_2 producing and H_2 consuming microbes (*i.e.,* homo-acetogens and methanogens) which mainly impedes their application in biohydrogen production [13,15]. Therefore, H_2 consumers, especially, methanogenic bacteria must be suppressed or inhibited from mixed culture to maximize biohydrogen production [14,16]. In this regard, an effective pretreatment of mixed culture inoculum is emergent to enrich H_2 producers by inhibiting H_2 consumers [14,17]. Numerous pretreatment techniques including chemical (*e.g.*, acidic, alkaline), physical (*e.g.*, heat-shock, ultra-sound, microwave), and others (*e.g.*, enzymatic), *etc.* are currently implemented to reduce the load of hydrogen consuming bacteria from the inoculum [3,17]. However, none of them showed optimum performance in terms of inhibiting hydrogen consuming bacteria; thus, further investigations are highly required to elucidate the mechanism of these pretreatment techniques and their influences on microbial community composition.

Recently, a non-conventional pretreatment method, called electroporation (EP) has been implemented to inactivate the methanogens in AS for increasing the hydrogen yield [14,18]. Generally, a pulsed electric field (PEF) associated with a high-voltage of direct current (DC), which is subjected to the live cells for a short duration of time to disintegrate cell membrane [19,20]. In some recent studies, it was

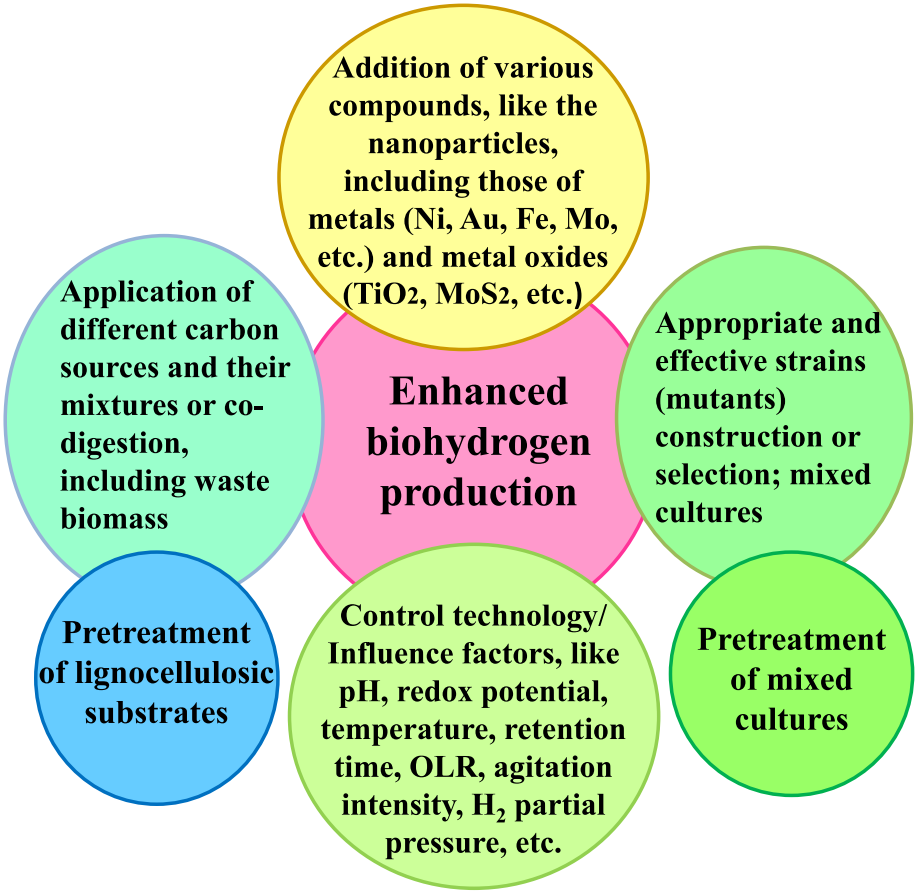

Fig. 1. Key approaches adopted to enhance the production of biohydrogen.

observed that the high electrical field could increase the porosity of biomass and substrate permeability, which enhanced the hydrolysis of cellulose to simple sugar, and consequently, augmented the biohydrogen production [21–23]. In addition, EP could also be used as an efficient pretreatment technique to suppress the H_2 consuming methanogenic microbes from mixed culture inoculum for enhancing the yield of biohydrogen production [14,18]. However, the practicability of EP as a pretreatment method remains unclear for a large-scale application in the cases of waste biomass substrate and mixed culture inoculum to increase biohydrogen production.

Though LCB and mixed inoculum are suggested as potential feedstock and microbial source for biohydrogen production, respectively and pretreatment of such substrate/inoculum considered as step limiting factor, very limited studies have been published in this regard. Therefore, the main objective of this article is to review the technical difficulties of mixed culture driven waste biomass-based biohydrogen production through the dark fermentation and sustainability of current pretreatment techniques. Furthermore, this review presents the state-of-the-art mechanism, prospects, and challenges of a new alternative pretreatment method (*i.e.,* EP) for both substrate and inoculum to enhance biohydrogen production, which has been little discussed in the other reviews, but it is essential to make this technique viable and competitive. This review will facilitate the deeper insights of pretreatment mechanism and further development of processes for future applications.

2. Substrate pretreatment of biomass-based biohydrogen production

2.1. Different pretreatment methods of substrate

The performance of biohydrogen production can be influenced by several process parameters and environmental factors including substrate type and concentration, pretreatment techniques, source and type of inoculum, fermentation conditions (pH, temperature, carbon/nitrogen ratio, etc.), metabolic pathways, by-products, and so on [24]. Among them, the pretreatment of substrate is a crucial factor for efficient biohydrogen production, especially, while the LCB is used as substrate because of the complex structure of cellulose, hemicellulose, and lignin. A longer retention time is usually required to degrade them due to their complex structures. Consequently, the rate of fermentation is slowed down, and the hydrogen yield is reduced [23]. Due to the crystallinity and porosity, the LCB must be pretreated to release its underlying monomeric sugars prior to microbial fermentation. Pretreatment aims to break this recalcitrant heteropolymer structure and increase the amount of monomeric sugars for nourishing H_2-producing microorganisms [25]. The primary objectives of a pretreatment are to disintegrate the elements of the LCB [3,26], to lower the cellulose crystallinity for increasing permeability of the biomass, and to make it more accessible for microbes [8,27]. However, the efficiency of pre-treatment methods could be estimated by several criteria such as energy requirements; sugar formation ability; restrain the properties of carbohydrates; avoiding the inhibitory by-products formation; and economically viability [3,8].

Different types of pretreatment methods (Fig. 2) including chemical, physical, biological, and combined (or integrated) treatment have been employed to disintegrate the rigid structure of LCB for easy assimilation by cellulolytic microbes [3]. Among the different chemical pretreatments, the acid and/or alkali-based hydrolysis have been widely implemented because of high solubilization of feedstocks allowing easier digestion by microbes. Generally, the acid treatment is preferred for treating hemicellulose containing feedstocks [28], while the base or alkali pretreatment technique is commonly employed for treating LCB as it can break down the crystalline structures of cellulose [29]. Furthermore, these treatments are capable of facilitating the dissolution and saponification of ester bonds present in LCB [3]. Tian, Liang [30] reported that the alkali pretreatment (with the addition of 20 mM $CaCO_3$) of sugar cane bagasse enhanced the productivity of biohydrogen by 116.72 %. They obtained high H_2 production due to the significant degradation of recalcitrant crystalline structure of the substrate. Likewise, Fan, Zhang [31] reported 136 times increase in hydrogen production using acid (HCl)-pretreated wheat straw biomass compared to untreated biomass. However, the uses of extensive chemical can lead to a greater complexity to the downstream processing (Table 1). Moreover, in some cases, the acid pretreatment could lead to the losses of fermentable sugars. In a study by Saratale, Kshirsagar [32] reported that the rice husk hydrolysate pretreated by acid (0.4% (w/v) H_2SO_4) led to a drop in hydrogen production (around 1.74 mmol of H_2/g reducing sugar) compared to a raw sample (about 2.93 mmol of H_2/g reducing sugar). The reduction of H_2 yield was ascribed to the inhibition of microbial growth due to the presence of acidic metabolites (*e.g.,* acetate, lactate, butyrate, formate, *etc.*) in the substrates. Nevertheless, a wide variety of toxic by-products (*e.g.,* furan, furfural, and phenol) could be produced in a more acidic environment during the acidification process, resulting in the inhibition of microbial growth [29,33]. Furthermore, additional chemicals are needed to neutralize the acid or base treated substrates, which is neither ecofriendly nor cost effective. In some cases, the corrosion of the equipment (*e.g.*, bioreactors) causes upon long contact time and odorous environment is generated due to the acidic by-products that create complications during the fermentation process [34]. Apart from these, the doses of acid/alkali need to be optimized for maximizing the hydrogen yields. Although acid/base treatment was

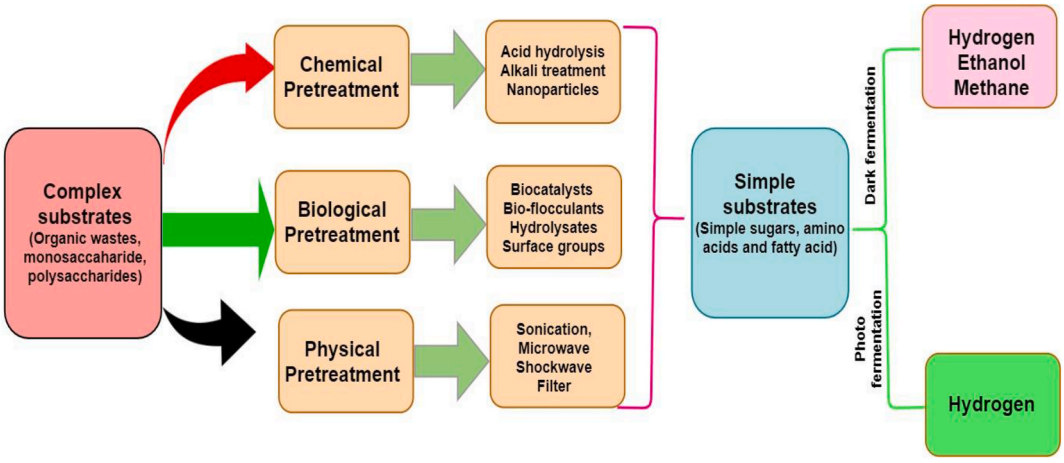

Fig. 2. Different techniques implemented for substrate pretreatment to improve biohydrogen production.

Table 1
Various kinds of pretreatment techniques of lignocellulosic biomass.

Types of pre-treatments	Pretreatment methods	Advantages	Disadvantages	References
Physical pretreatment	Mechanical comminution	- Decreases cellulose crystallinity	- Power intake typically higher than inherent biomass energy	[38]
	Pyrolysis	- Generates liquid and gas products	- High temperature; ash production	[39]
	Ultrasound	- Enhances the extractability of hemicelluloses sugars; decreases extraction time	- This technique is expensive and energy intensive	[40–42]
	Liquid hot water (hydro thermolysis)	- Cleaves hemiacetal linkages and releases acids during biomass hydrolysis; removes hemicelluloses and lignin; no catalyst required	- Formation of inhibitory products effect in microbial fermentation; suffer from low concentration of sugars; excessive energy/water input;	[43]
	Irradiation	- Splits the complex structure of β-1,4-glycosidic bonds, consequently, lowering the crystallinity	- It is costly technique and far from practical application	[44,45]
Chemical pretreatment	Acid hydrolysis	- Convert hemi-cellulose into xylose and other simple sugars via the hydrolysis; modifies lignin formation	- High costs of acids; equipment deterioration; formation of inhibitory substances	[44,46]
	Alkaline hydrolysis	- Removal of lignin and hemi-celluloses; low inhibitor formation	- Long retention time needed; irrecoverable compounds particularly different salts are formed and immersed into the biomass; modification of lignin structure; elevated cost of alkaline catalyst	[45,47,48]
	Ozonolysis	- Lowers lignin constituent; toxic residues are not generated	- Large volume of ozone needed; costly	[49,50]
	Organosolv process	- hemicelluloses and lignin are hydrolyzed	-Solvents require to be removed from the reactor, evaporated, condensed, and recycled; expensive	[49,51]
Biological pretreatment	Fungal pretreatment	- Environmentally friendly method, less energy requirement	- Sluggish hydrolysis rate	[52]
	Microbial consortium pretreatment	- Disintegrates lignin and hemicelluloses; does not require high energy	- Low hydrolysis rate	[49]
	Enzymatic pretreatment	- Require minimal energy in moderate environmental conditions	- Very low hydrolysis rate	[53]
Other pre-treatment techniques	Steam explosion (autohydrolysis)	- Lignin conversion and hemicellulose solubilization; cost-effective; high yield of glucose and hemicellulose in two-step process	- Sometimes damage the part of the xylan ; inadequate modification of the lignin-carbohydrate matrix; toxic compound production; acid catalyst is required to faster degradation of lignin material; partial hemicellulose degradation	[46,54]
	Ammonia (NH_3) fiber explosion	- Enhances accessible surface area, eradicates lignin and hemicellulose to an extent; -Low creation of inhibitors for downstream processes	- Excessive ammonia requires to be recycled content; modifies lignin structure; high cost of ammonia	[55–57]
	CO_2 explosion	- Accessible surface areas are increased; less cost; does not generate toxic substances	- Does not alter lignin or hemicelluloses	[58,59]
	Pulsed electrical field	- Ambient conditions; complete disruption of plant cells; short time; no chemical uses	- Process needs more research	[8,49,60]

efficiently used for treating different types of biomass (*i.e.,* waste activated sludge or algal biomass), the enhanced performance was obtained by combining alkali/acid with heat or enzymatic treatment [3,35]. This is because the longer retention time was required (*i.e.,* ~30 min to 24 h) for both acid and alkali treatment while they were solely applied [3]. In general, the heat treatment in the ranges from 50 to 220 °C with the period from 90s to 90 min is employed for treating LCB [3]. However, the substrate treated with too high temperature could form refractory compounds, that are certainly inhibitory to fermentation process [3]. Apart from the heat treatment, other physical pretreatment methods, such as microwave [36], ultra-sonication [37], bead-beating, and osmotic-shocks [27] have been widely implemented, which are not considered as economical since they are associated with excessive heat generation [10] and high energy input [9].

On the other hand, biological methods could curtail the limitations associated with the chemical and physical treatments since they are conducted in a mild condition (Table 1). Moreover, the efficiency of the biological methods (*i.e.,* enzymatic) was found to be significantly higher than that of some chemical and physical methods [3]. For instance, higher yield of sugar (1.74 mol of H_2/mol sugar) was produced using enzyme (*i.e.*, *Aspergillus niger*) pretreated substrate (rice mill wastewater), however, lower performance (1.40 mol of H_2/mol sugar) was achieved for acid pretreated substrate [61]. In a recent study, Cheng and Liu [62] observed that the cumulative H_2 production of ~195 mL was attained using fungal (*i.e.*, *Trichoderma reesei*) pretreated cornstalk, which was remarkably higher (~209 %) than the untreated substrate. This might be ascribed to the increased cellulase activity by *T. reesei* led to the higher rate of hydrolysis, and thereby, the rate of fermentation of cornstalk was significantly increased. In another study, Leaño and Babel [63] observed that the H_2 production was increased by 29.2 % for ultrasonication (45 min) pretreated substrate (cassava wastewater), while H_2 production was augmented by 51.4 % and 53.5 % for OPTIMASH BG® enzyme and α-amylase pretreatment, respectively. A significant enhancement was obtained by α-amylase treatment due to the efficient hydrolysis of the glucosidic linkages present in polysaccharides, and the increased transformation of polysaccharides into monosaccharides resulted in enhancement of biohydrogen production [29,63]. It is worth noting that the enzymatic pretreatment of biomass could significantly enhance the biohydrogen production; however, this technique is not considered as an attractive option since it involves with higher cost and low production efficiency [63].

Besides, the compatibility of enzymes needs to be carefully estimated since some enzymes could have the possibility of microbial inactivation, which can substantially reduce the hydrogen production [29]. Furthermore, the selectivity of enzymes can vary depending on the type of substrates and their structure. This is because degradation of polymers is not only dependent on the chemical structure of the polymers, like the presence of functional groups and hydrophobicity-hydrophilicity balance, but also on the ordered structure, especially crystallinity, orientation, and other morphological properties [64]. A list of microbial enzymes with their substrate specificities is presented in Table 2. Generally, polymers that are biodegradable include hydrolyzable bonds, such as esters, ortho-esters, glycosides, carbonates, amides, anhydrides, urethanes, ureas, *etc*. The polymers without hydrolyzable groups and with strong covalent bonds in the backbone (*e.g.*, C–C) require longer time to degrade. Hydrolysis

Table 2
Microbial enzymes and their compatibility with different substrates.

Microorganisms	Enzymes	Substrate specificity	References
Funalia trogii	Laccases	Phenolics, aromatic amines and ascorbate	[67]
Bacillus amyloliquefaciencs, Bacillus licheniformis	α-Amylases	Starch	[67,68]
Cryptococcus sp.	Lipases	Poly (ε-caprolactone), poly (L-lactic acid)	[69,70]
Thermomyces lanuginosus,		Poly (trimethylene terephthalate)	[71]
Paenebacillus amylolyticus		Poly (L-lactic acid)	[72]
Thermobifida alba	Esterases	Poly (ethylene terephthalate)	[73]
Roseateles depolymerans		Poly (butylene succinate), poly (butylene succinate)-co-(butylene adipate), poly (ε-caprolactone), poly (ethylene succinate), poly (butylene adipate-co terephthalate, poly (butylene succinate-co-terephthalate), poly (butylene succinate/terephthalate/isophthalate)-co-(-lactate)	[74,75]
Leptothrix sp.		Poly (butylene succinate)-co-(butylene adipate), poly (ε-caprolactone), poly (ethylene succinate), poly (L-lactic acid), poly (butylene adipate-co terephthalate), poly (butylene succinate-co-terephthalate), poly (butylene succinate/terephthalate/isophthalate)-co-(-lactate)	[76,77]
Aspergillus such as *A. niger* and *A. oryzae*	*β*-glucosidase	Plant and lignocellulosic biomass	[78]
Pseudozyma antarctica	Cutinase	Poly (butylene succinate), poly (butylene succinate)-co-(butylene adipate), poly (ε-caprolactone), poly (L-lactic acid)	[79]
Thermobifida cellulosilytica		Poly (ethylene terephthalate)	[80]
Thermobifida fusca		Poly (trimethylene terephthalate)	[71]
Aspergillus oryzae		Poly (butylene succinate)	[70]
Cryptococcus sp.	Protease	Poly (L-lactic acid)	[69]
Pseudomonas lemoignei	Polyhydroxyalkanoates	Poly (3-hydoxy-*co*-3 hydroxyvalterate)	[81]
Bacillus thuringiensis	depolymerases	Poly (3-hydroxybutyrate) and poly (3-hydoxybutyrate-*co*-3 hydroxyvalterate)	[82]
Streptomyces sp.		Poly (3-hydoxybutyrate-*co*-3 hydroxyvalterate)	[83]
Bacillus sp., *Streptomyces* sp., *Pseudomonas* sp.	Xylanases	Xylans	[67,84]
Aspergillus, Trichoderma, Bacillus, Paenibacillus	Cellulases	Polymeric cellulose	[67]

reactions could be catalyzed using enzymes known as hydrolases, which include lipase, esterases, proteases, phosphatases, glycosidases, *etc*. Several other hydrolases such as cellulases, pectinases, and xylanases are countable for the hydrolysis of cellulose, pectin, and xylene, respectively. In this regard, it is expected that some of these enzymes would be interesting in the degradation of organic substrates by catalyzing their hydrolysis [64]. For instance, esterases and lipases are distinguished based on hydrolytic cleavage of acyl glycerols with different acyl chain lengths. Esterases catalyze breakdown of esters with chain lengths <10 carbon atoms while lipases hydrolyze acyl esters with >10 carbon atoms [65]. On the other hand, poly-esterases specially connect the amorphous region of the polymer and consequently, the degree of crystallinity of the polymer increases on enzyme treatment [66]. Thus, the enzyme must be chosen based on substrate types and structure to obtain maximum hydrogen production efficiency.

Over time, the substrate pretreatment methods had further coupled each other to improve the efficiency of the process performance. For instance, Ozkan, Erguder [85] evaluated a combined thermal-alkaline pretreatment on sugar beet pulp substrate for enhancing biohydrogen production and they observed that the combined pretreatment technique (*i.e.*, thermal-alkaline, 148.5 mL cumulative H_2) achieved ~10 % higher hydrogen production compared to an individual technique (*i.e.*, alkaline, 134 mL cumulative H_2). Likewise, Elbeshbishy, Hafez [86] observed that the maximum yield of H_2 (118 mL/g VS) can be achieved from food waste using ultrasonication with acid pretreatment compared to a single pretreatment. Furthermore, a recent study by Li, Zhao [87] stated that the microwave assisted alkali pretreatment of cornstalk could effectively remove lignin, and consequently, increased the accessibility of soluble components by microbes, which in turn enhanced the hydrogen production (by ~54.8 %). Nevertheless, the combined pretreatment of sewage sludge used as feedstock for biohydrogen production also shortened the lag time of hydrogen formation, accelerated the rate of hydrolysis, and increased the utilization of organics, thus contributing to the enhanced hydrogen yield [88]. However, the practical applicability and economic feasibility of combined pretreatment methods need to be further investigated to establish a large-scale biohydrogen production [29].

2.2. Significance and challenges of substrate pretreatment

A pretreatment can promote the hydrolysis of biomass to enhance the efficiency of hydrogen production by facilitating substrate degradation rate. Not only biodegradability of biomass, it can also affect the composition of organic matters in the feedstocks [3]. Consequently, the quantity and quality of final product can vary depending on the pretreatment applied. In a study, Kumar, Sivagurunathan [89] demonstrated the influences of several pretreatment methods such as ultrasonication, autoclave, and electrolysis on wet algal biomass used as feedstocks for fermentative H_2 production at 37 °C, pH 5.5, and 120 rpm. The analysis of cumulative biogas and hydrogen production indicated that the biogas production was at peak with ultrasonication treated biomass while the maximum hydrogen production was obtained for electrolysis pretreated hydrolysate. This might be due to the difference in H_2 content in the biogas, which was 24, 22, and 21 % for electrolysis, ultrasonication, and autoclave pretreatments, respectively. Although there was not much difference among the pretreated hydrolysates, the H_2 content was improved about 3 times compared to the control experiment, which demonstrated only 8 % H_2 content. Beside the improvement in the yield of total biogas and H_2 content, CO_2 content might decrease in the final product for the pretreated substrate than the untreated one [63,90]. Usually, a mixed biogas is produced in the dark-fermentation processes, which predominantly contains H_2 and CO_2 as shown in Table 3. It has been also reported that a lower amounts of CH_4, carbon monoxide (CO), and/or hydrogen sulfide (H_2S) could be found in the total gas [90,91]. However, very few studies have reported CH_4 content in the literatures of biohydrogen production since the methanogenic microbes were eliminated by heat-shock treatment of seed sludge before using it as inoculum [63,92]. Although the pretreatment methods of substrate would have a significant impact on the composition of final product, and it is important to understand the technical difficulties of pretreatment methods based on the final product composition, temperature, and pressure; this fact remains a huge gap in the literature of biohydrogen studies. Therefore, further studies are highly recommended to design in this regard.

Nevertheless, there are several bottlenecks of each pretreatment

Table 3
The impact of substrate pretreatment on the final product composition.

Pre-treatment applied	Pretreatment conditions	Substrate	Inoculum	Fermentation condition	Composition of final product, mol%				References
						H_2	CH_4	CO_2	
Sonication	45 min; 50/60 kHz	Cassava wastewater	Anaerobic sludge (heat treated with 105 °C and 90 min)	37 °C, pH 7.0, 90 rpm, 10 days	Control Treated	0.72 0.78	– –	0.28 0.22	[63]
Enzymatic (OPTIMASH BG®)	0.20 %, pH 4.0, 60 °C, 45 rpm	Cassava wastewater	Anaerobic sludge (heat treated with 105 °C and 90 min)	37 °C, pH 7.0, 90 rpm, 10 days	Control Treated	0.50 0.67	– –	0.50 0.33	
Enzymatic (α-amylase)	0.20 %, 37 °C, 45 rpm	Cassava wastewater	Anaerobic sludge (heat treated with 105 °C and 90 min)	37 °C, pH 7.0, 90 rpm, 10 days	Control Treated	0.44 0.56	– –	0.56 0.44	
Ultrasonic	0.13-kW/20-kHz probe-type; dose: 195 J/mL	Palm oil mill effluent	Anaerobic sludge (heat treated with 105 °C and 90 min)	44 °C, pH 7.0, 90 rpm, 10 days	Control Treated	0.60 0.61	– –	0.40 0.39	[92]

method, which are presented in Table 1 (limitations of different pretreatment methods). The challenges of pretreatment of biomass-based biohydrogen production mainly include three aspects. First, many pretreatment techniques are associated with high energy consumption like microwave, hydrothermal and some other methods such as chemicals and formed hazardous products may be harmful to the environment (Table 4). Thus, they are not considered feasible in terms of energy and environmental aspects [3]. Second, a variety of inhibitory compounds including carbonic acids, furan derivatives, and phenols are usually produced during the pretreatment of LCB (Table 4), thus affecting microbial communities, microbial activities, and overall metabolic pathway [93]. For example, several organic acids (*e.g.*, acetate), furanic compounds (*e.g.*, furfural, 5-hydroxymethylfurfural or 5-HMF), and phenolic compounds (*e.g.*, phenol, vanillin, syringaldehyde) are usually produced from hemicellulose, cellulose, and lignin, respectively [24]. The nano-sized products penetrate the cytoplasmic membrane of those bacteria responsible for hydrogen production to infect the internal structures, and subsequently, impede the synthesis of nucleic acid and protein [94]. Moreover, the weak acids inhibit the transport of sugar within the cell, impede the regeneration of adenosine-triphosphate (ATP) and glycolytic enzymes, thus hinder the growth of hydrogen-producing bacteria [95]. Third, several subsequent treatments may be needed after some pretreatment methods, especially, after the chemical methods. For instance, desalination is typically required after base/acid treatment [3]. Considering the probable adverse impact of biomass pretreatment, further investigations are needed to make the process more efficient, and to recommend the proper direction of choosing a suitable method based on biomass characteristics, economic and environmental efficiency.

2.3. Application of electroporation in substrate pretreatment

EP is considered as a promising technique in which a high voltage electric field is subjected to the substrate placed between electrodes, and commonly used to increase the cell membrane permeability [20], allowing the severe effects on the structure of biological tissues [8]. Generally, an intense electrical potential is applied to the cellular membrane, which subsequently disintegrate the structure of cell wall as well as cell membrane as presented in Fig. 3 [14,97]. However, it can be speculated that the EP treatment with high electrical field could make changes to the structure and size of the biomass, reduce the cellulose crystallinity, and increase their porosity as well as chemical composition [8,97]. Furthermore, the cell conductivity and permeability would be significantly increased due to the strong electrical forces generated by EP [20,98]. The rigid structure of biomass and lignocellulosic bonding could be loosen (Fig. 4) due to the increase of mass penetrability, consequently, enhancing the accessibility of cellulase to cellulose and augmenting the sugar yield from enzymatic hydrolysis of cellulose [8,99]. The simple sugars are formed through the hydrolysis of complex carbohydrates leading to higher yield of hydrogen production. Thus, the retention time could be significantly reduced and boost up the biohydrogen production. Although EP is a well-known technique in some areas of biotechnology and medicine, nowadays, it has been widely investigated in the area of environment and energy [20]. In last decade, EP technique has been applied in various biomass feedstocks and food plants for the recovery and extraction of value-added chemicals [21–23,100,101]. Moreover, the reversible EP has been utilized to take out desired molecules from the cell [102], such as protein from several microorganisms [103,104], in some cases, plasmid DNA from bacteria [105,106], sugars from the sugar beets [107,108], and lipids for

Table 4
Effect of pretreatment on biomass structure and byproducts formation. (Adopted from Ref. [96] with permission of Elsevier. copyright 2021).

Pretreatment method	Major outcome	Surface area	Crystallinity	Hemicellulose removal	Delignification	Chemical usage	Inhibitors/byproducts
Acidic	Conversion of hemicellulose to sugars	Increase	Decrease	Complete	Good	Acids (HCl, H_2SO_4) and acidic gases (SO_2, CO_2)	Aliphatic compounds, carboxylic acids, phenolic compounds, furans
Alkaline	Separation of lignin and hemicellulose	Increase	Decrease	Not complete	Good	Bases (NaOH, KOH, NH_3, Ca $(OH)_2$, NH_4OH	Acetic acid, carboxylic acids, phenolic compounds, hydroxyl acids
Steam explosion	Complete hemicellulosic solubilization	Increase	Decrease	Complete	Poor	CO_2/No other chemicals	Acetic acid, furans, aldehydes, ketones
Green solvent/ Ionic liquid	Solubilization of lignocelluloses residues	Increase	Decrease	Not complete	Poor	Ionic liquids	Dependent on solvent used
Wet oxidation	Lignin and hemicellulose exclusion	Increase	Decrease	–	–	Oxidizing agents (H_2O_2, O_2, O_3)	Carboxylic acids, phenolic compounds, furans
Biological	Separation of lignin and hemicellulose	Increase	Decrease	Complete	Good	Enzymes (lipases)	Aliphatic compounds

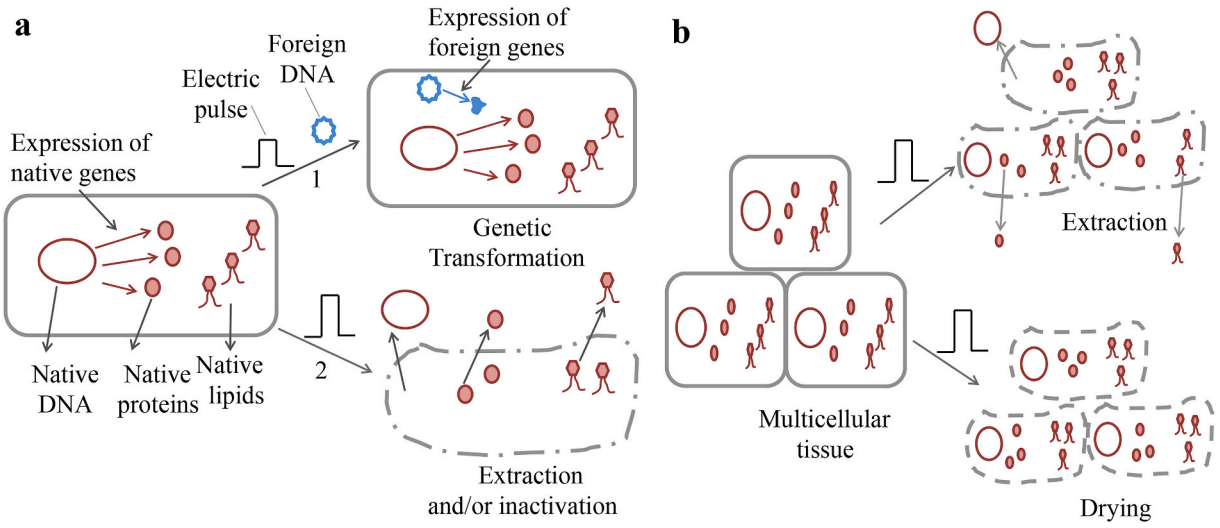

Fig. 3. Application of electroporation in biotechnology, a) application of electroporation to a biological cell, route-1: low electric pulse for medical application and route-2: high electric pulse for biofuel application, b) application of electroporation to multicellular tissue. (Adopted from Ref. [110] with permission of Elsevier. copyright 2015).

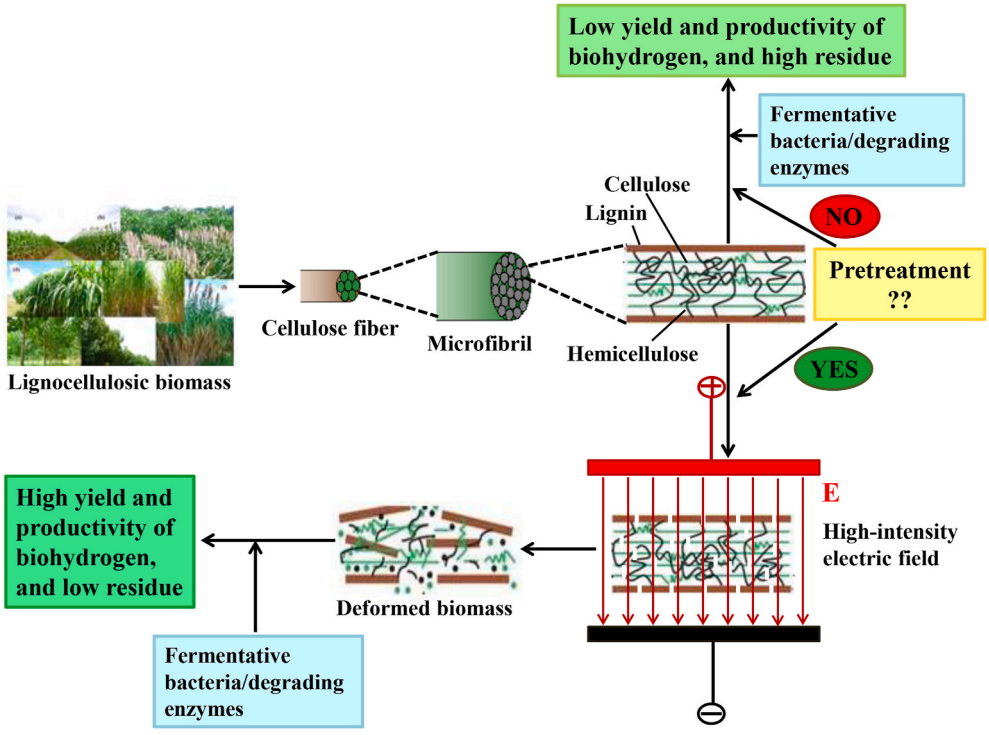

Fig. 4. Conceptual effect of electroporation on lignocellulosic biomass.

biodiesel from oil-producing microalgal cells [104,109].

EP could be an efficient pretreatment technique to treat biomass before employing it as a feedstock for producing biohydrogen and other fermentative products. It has been implemented as a substrate pretreatment technique in few studies [22,23,111]. Kumar, Barrett [112] applied a high electrical field (≥8 kV/cm) to treat the biomass (*i.e.*, switch grass and wood chips) and observed a substantial improvement in the biomass porosity and permeability. The increment in the porosity and permeability of substrate might be useful to enhance the hydrolysis of cellulose to simple sugars [21]. Lindmark, Lagerkvist [23] demonstrated that the digestibility of the substrate could be increased by applying high electric fields of direct current. The production of biogas was increased to 16 % using EP (field strength of 96 kV/cm) pretreated substrate (ley crop silage). In a recent study, Almohammed, Mhemdi [21] employed a high PEF to sugar beet tails as a pretreatment technique to produce bioethanol. The solute yield was significantly enhanced for ~3.75 times (from 16.8 to 79.85 %) and the EP treated juice was more concentrated (~2 times) in sucrose than the untreated one. They also observed that the EP pretreated substrate produced higher ethanol than that of untreated substrate (sugar beet tails). In another study, biomethane production was increased to a maximum of 110 % for EP pretreated algal biomass at a treatment intensity (TI) of 35 kWh/m^3 [22]. This could be attributed to the disintegration of the crystalline structure of molecular constituents of algal biomass and releasing their

polymerization degree as a consequence of the strong electric field [8,23]. As a result, a greater volume of readily fermentable sugars were available for microorganism, which resulted in increased biomethane production [21,23]. Therefore, it can be hypothesized that the biodegradability of cellulosic biomass feedstocks could be increased by applying an external electric field to enhance the fermentative hydrogen production (Fig. 4). However, the application of EP for treating feedstocks in the case of biohydrogen production have been found in very few studies.

In a recent study, Jeong, Cho [111] revealed the feasibility of EP (20–100 V for 30 min) as a pretreatment technique to increase biohydrogen production from marine brown algae (*e.g.*, *Laminaria japonica*) biomass feedstock. They demonstrated that the yield of H_2 was enhanced by 72.6 % under 58.5 V for 30 min, which indicates the potential of the EP as alternative technique for feedstock preparation (Table 5). The maximum hydrogen yield was availed at 58.5 V because of the enhanced biodigestibilty of substrate. This has been ascribed to the fact that the structural bonding of LCB would have been broken due to the implementation of a high voltage electric field (Fig. 4), which in turn could promote the enzymatic accessibility to cellulose and hemicellulose for hydrolysis by fermentative bacteria [8,21]. Moreover, the crystallinity index (CI) of the LCB biomass might be decreased because of the strong electric field which evidenced by the increasing solubility of the substrate and enhanced hydrogen production [8]. The CI, a quantitative indicator of crystallinity has been used to describe the relative amount of crystalline material in cellulose and it is considered an important parameter determining the enzymatic hydrolysis susceptibility of cellulose [99]. It also characterizes the average crystallite size, perfection, and ordering of a crystal [113]. The accessibility of cellulase to cellulose ought to be affected by its crystallinity, however it can also be influenced by some other parameters, including lignin/hemicellulose contents and distribution, porosity, and particle size [114]. In some studies, it is reported that the crystallinity have a superior effect on enzymatic hydrolysis compared to other structural attributes such as the specific surface area or the degree of polymerization of the cellulose [115]. Cellulose with a lower CI is more susceptible to hydrolysis due to its weak structure [99]. For enzymatic hydrolysis of microcrystalline cellulose, it has also been reported that the lower cellulose CI, the higher will be the sugar yield and the faster will be the hydrolysis reaction rate. However, besides the intrinsic crystalline structure of microcrystalline cellulose, the availability of enzymes is also an important factor that determines the reaction rate of enzymatic hydrolysis of microcrystalline cellulose [99,115]. Based on the above findings, it can be mentioned that the EP possesses enormous potential as an alternative pretreatment technique to the traditional methods for feedstocks preparation in biohydrogen production. Thus, EP could reduce the long retention time of fermentation and subsequently, assist easier substrate degradation to enhance the performance of biohydrogen production.

3. Inoculum pretreatment for enhancing biohydrogen production

3.1. Role of microorganisms in fermentative biohydrogen production

Microorganisms can convert organic rich biomass into hydrogen. This conversion process occurs through the different microbial enzymes such as hydrogenases and nitrogenases, as they are greatly influence the metabolism of microorganisms [118]. In this way, microorganisms play a pivotal role for biohydrogen generation during fermentation process [17]. In general, several organics such as saccharides, lipids, and proteins present in the complex substrate are catabolized to generate short chain fatty-acids by fermentative bacteria, and then these acids are further hydrolyzed into acetate, CO_2, and H_2 by acetogens (Fig. 5) [119]. Therefore, the rate of hydrolysis is crucial factor for generating biohydrogen from waste biomass as mentioned in section 2. Generally, the spore-forming bacteria including *Bacillus* sp., *Clostridium* sp., *Thermoanaero bacterium, etc.* are predominant microorganisms to produce hydrogen through the fermentative process [119]. These hydrogen producing microorganisms contain the hydrogenase enzyme, by which energy are produced as ATP and hydrogen during the bacterial metabolism [118]. The metabolism of energy for biohydrogen producing

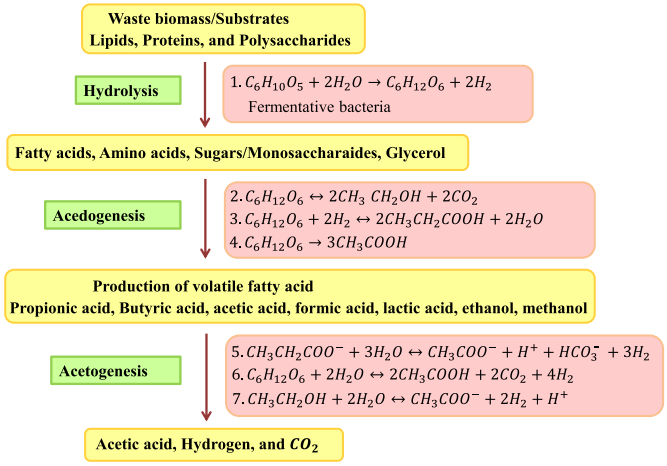

Fig. 5. Steps of hydrogen generation through fermentative conversion of organic substrates.

Table 5
Application of electroporation for substrate degradation to increase the product yield.

Type of substrates	Pretreatment conditions	Characteristics of fermentation	Product	Enhanced yield	References
Sugar beet tails	Total energy (input), Q = 1.91 Wh/kg (intensity, E = 450 V/cm, pulse duration, t = 10 ms)	Anaerobic fermentation, *S. cerevisiae*, pH 5.0, 28 °C @ 160 rpm for 72 h	Bioethanol	2 times (EP: 6.1 % v/v, control: 2.95 % v/v	[21]
Algal biomass	TI = 35 kWh/m^3 (DC power rated at 3 kV and 40 mA)	Anaerobic digestion, pH 7.0, 35 °C @ 150 rpm	Biomethane	110 % (EP: 92 mL, control: 15 mL)	[22]
Algal biomass	TI = 5.4 kWh/m^3 (DC power rated at 3 kV and 40 mA)	Anaerobic digestion, pH 7.0, 35 °C @ 150 rpm	Biomethane	27.6 % (EP: 53.47 mL, control: 15 mL)	[22]
Ley crop silage	Total energy (input) of 259 Wh/kg VS (65 pulses at a field strength of 96 kV/cm)	Anaerobic digestion, pH 9.0, 30 °C	Biomethane	16 % (EP: 785 mL/g VS, control: 677	[116]
LC substrates (corn stover)	EP pretreatment by using voltage, U = 200–365 V	Anaerobic co-fermentation (with cow manure) at 55 °C for 26 days	Biogas and methane yields	Biogas increased by 18 % and methane by 16 %	[117]
LC substrates (soybean straw)	EP treatment by applying voltage, U = 200–365 V	Anaerobic co-fermentation (with cow manure) at 55 °C for 26 days	Biogas and methane yields	Biogas increased by 13 % and methane by 10 %	[117]
Brown algae biomass	EP treatment, 58.5 V for 30 min	Anaerobic digestion, pH 8.0, 37 °C @ 150 rpm	Hydrogen	Increased by 72.6 % (H_2 yield was 102.7 mL H_2/g dcw)	[111]

EP, electroporation; TI, treatment intensity; DC, direct current; VS, volatile solid; LC, lignocellulose; DCW, dry cell weight.

microbes might be affected by the expression activity of hydrogenase [120]. Nonetheless, the yield of H_2 from a substrate depends on the type of microbial inoculum and other physico-chemical conditions. For instance, the metabolic pathways and hydrogenase activities of biohydrogen generating bacteria are strongly influenced by pH because the hydrogenase activity depends on a suitable range of pH during fermentative process [121]. Few studies suggested that, the pH ranging from 5 to 6 is optimal for hydrogen production via fermentation since the methanogenic activity is limited at this pH range [121,122]. The by-products formed during the fermentative hydrogen production are also varied, such as acetate, butyrate, propanoate, lactate, and alcohols, *etc.,* which depends upon the type of bacterium species and its physico-chemical conditions [119]. As can be seen from Fig. 5 reactions, both glucose and hydrogen are produced during the reaction, and subsequently, the glucose is converted into the acetic acid. The bacteria can change their metabolic pathways during the fermentative process according to their physico-chemical properties, such as type of carbon sources and its concentrations, pH, and temperature, *etc.*

In the anaerobic fermentation process, the carbon/nitrogen (C/N) ratio is another critical factor indicating nutrient balance in the fermentation medium, which is important for cell synthesis, growth, and metabolism [123]. The hydrogen productivity of the microbes can drop if nitrogen source is limited because lack of nitrogen source indicates that there were insufficient cells to maintain the growth and metabolism of microbes, thus resulting in lower hydrogen production [123]. Moreover, the metabolic pathway may switch to methane production due to low C/N ratios [29]. The lag time also influenced by the C/N ratio. Reyna-Gómez, Molina-Guerrero [124] observed that the lag time could be 1.95–22 h depending on the C/N ratio. Nevertheless, the buffering capacity of the substrate is important for overall performance of the fermentation process because increased buffering capacity would result in sluggish pH decrease (slow acidification rate) and enhance the efficiency of fermentation process in terms of improved fermentation time and products formation [125]. Reyna-Gómez, Molina-Guerrero [124] used C/N ratio of 20–40 as reference parameter associated with the increased buffering capacity of the system. However, the optimum C/N ratio may vary depending on the substrate types and characteristics, inoculum, and other fermentation conditions. Gomez-Romero, Gonzalez-Garcia [126] studied the adaptation of the most important microbial community including *Bifidobacterium, Klebsiella, Lactobacillus, Citrobacter, Enterobacter, and Clostridium* to produce biohydrogen at different C/N ratios ($5 < C/N < 46$) using milk serum and fruit wastes. A maximal specific biohydrogen production rate (10.68 mmol/h/L) was achieved for a C/N ratio of 21 [126]. Basak, Fatima [127] obtained the highest hydrogen productivity of 118.12 ± 1.05 mmol/L with a C/N ratio of 68 using a feed of fruit and vegetables mixed with cheese serum, however, the predominant species were *Escherichia coli, Clostridium butyricum, and Streptococcus henryi*. Therefore, an optimum C/N ratio is imperative to maximize the production yield. In this context, a suitable pretreatment (as discussed in Section 2) could help to maintain an optimum C/N ratio by increasing carbohydrates availability in the medium.

Beside above mentioned factors, the efficiency of biohydrogen production mostly depends on microbial diversity present in the system, and the microbial diversity is usually varied based on inoculum sources [18,128]. The presence or absence of hydrogen consumers in the mixed culture inoculum and fermentative medium plays an important role to balance the metabolic process, and consequently, shows an impact to the fermentative hydrogen production and by-products formation [129]. The microbial species including *Clostridium* sp., *Bacillus* sp., *Enterobacterium* sp. are the most common genera, which are known as fermentative hydrogen producing bacterium [129]. The anaerobes, like *Clostridium* sp. needs strict anaerobic condition to produce hydrogen [129,130]. However, *Enterobacter* sp., particularly *Enterobacter aerogenes* is a facultative, anaerobic, and mesophilic bacteria, which can consume different substrates, and no special operational parameters are required for removing oxygen from the fermentation system unlike the cultivation of other strict anaerobes [129,130]. Moreover, *E. aerogenes* can doubling themselves within very short time, efficient for hydrogen evolution rate, and tolerable with the high hydrogen partial pressure; however, their production efficiency is lower compared to other strict anaerobes, especially *Clostridia* [131]. The *Clostridium* sp. are highly sensitive to the amount of O_2 in a reactor, hence the L-cysteine is usually added in a medium as a reducing agent for obtaining a stable H_2 production [132]. However, the reducing agent (L-cysteine) is expensive, thus a co-culture inoculum including a facultative anaerobe of *Enterobacter* sp., and strict anaerobes like *Clostridium* sp. [133] has been proposed to produce biohydrogen without addition reducing agent. This is because, the facultative anaerobic consortia can consume O_2 in a medium, and thereby an anaerobic condition would be created for the obligate anaerobes in a reactor. Recently, a co-culture (*E. aerogenes* and *C. butyricum*) inoculum was used by Yokoi, Tokushige [133], they observed that the co-culture inoculum obtained 57 % higher hydrogen production compared to the mono-culture. *Clostridium* sp. and *Enterobacter* sp. are considered to be efficient for producing hydrogen, however, they are not capable of producing a significant amount of H_2 at a low pH (<5.5) [134].

3.2. Impact of pretreatment on microbial community

The efficient H_2 production significantly influenced by the metabolic activities of microorganisms (Fig. 5). In fermentative biohydrogen generation, the mixed and pure cultures are generally used as inoculum, however, mixed inoculums are preferable because the synergistic interactions between the microorganisms provide a broader range of feedstock choice and offered simpler operating conditions [135]. In mixed culture microbial consortia, the hydrogen consuming bacteria co-exist with hydrogen producing bacteria (Table 6). The hydrogenic bacterium (H_2 producers) can use varieties of carbon sources as feedstocks to produce hydrogen [14]. However, the methanogens (H_2 consuming bacteria) compete with the hydrogenic bacterium for substrate degradation, and consequently, reduce the hydrogen productivity. Thus, the deactivation or inhibition of methanogens before the fermentative process could be advantageous to increase hydrogen productivity while mixed cultures are used as inoculum [16]. Naturally, H_2 is produced during the acidogenic process where the acid forming bacteria produce organic acids as by-products along with H_2 and CO_2 (Fig. 5) [136]. Thereafter, H_2 produced during the fermentation can be readily consumed as a source of reducing power by H_2 consuming bacteria (methanogens and other sulfate reducing bacterium) present in the system, and subsequently, leading to the generation of CH_4 and volatile fatty acids (VFAs) [18,137]. Therefore, adopting a proper pretreatment technique is necessary to inactivate H_2 consuming microbes together with conserving the H_2 producers, for obtaining enhanced hydrogen productivity.

The application of various pretreatment techniques, such as chemical, heat treatment on sludge samples for eliminating methanogens is well reported to date. Generally, the H_2 producers have better stability in harsh environmental conditions, such as chemical, heat or pH shock, *etc.* [121]. Perhaps the best-known bacterial adaptation to stress condition is the formation of endospores. Endospores are bacterial survival structures that are highly resistant to many different types of chemical and environmental stresses, and therefore, enable the survival of bacteria in the environments which are lethal for these cells in their normal vegetative form. Endospore formation is limited to several genera of gram-positive bacteria, such as *Clostridium* and *Bacillus* [151]. In a recent study, Yang, Yin [135] observed that, though the *Clostridium* sp. was the predominant genera for hydrogen production, other gram-positive, rod-shaped, and spore-forming genera, such as *Bacillus* sp., *Exiguobacterium*, *Paeniclostridium*, *Romboutsia*, *Paraclostridium*, *etc.* had a great potential in hydrogen production.

In contrast, the methanogens can be considered as inhibitors to the

Table 6
The predominant hydrogen-producing and hydrogen-consuming bacteria present in mixed culture system.

Nature of microbial community	Types of microorganism	Examples of dominant species	General characteristics	Influential roles in fermentation process	References
Hydrogen consumers	Methanogens	*Methanococcus* sp., *Methanobacterium* sp., *etc.*	- Obligate anaerobic bacteria - Shows activity in narrow range of pH - Mesophilic	Produce methane produces by consuming hydrogen	[138,139]
	Homo-acetogenic bacteria	*Clostridium carboxidiyorans, Clostridium ljungdahlii, Clostridium ragsdalei, etc.*	- Obligate anaerobic bacteria - Gram-positive - Spore forming bacteria - Motile	Produce VFAs via the utilization of hydrogen, reduce carboxylic acids reduced to their corresponding alcohols	[137,140]
	Sulfate reducing bacteria	*Campylobacter, Pseudomonas, etc.*	- Gram-negative - Motile	Capable of oxidizing H_2 for hydrogen sulphide generation in the existence of sulfate	[141],
	Hydrogenotrophic methanogens	*Methanococcus maripaludis*	- Anaerobic - Weakly motile - Non-spore-forming - Gram-negative	Utilize CO_2 as a carbon source, and H_2 as a reducing agent	[142]
Hydrogen producers	*Clostridium* sp.	*Clostridium tyrobutyricum, Clostridium butyricum, Clostridium sartagoforme, Clostridium pasteurianum, Clostridium beijerinckii, etc.*	- Gram-positive - Spore-forming - Obligate anaerobes - Mesophilic	High H_2 produces, enable to degrade carbohydrates	[143–145]
	Bacillus sp.	*Bacillus amyloliquefaciens, Bacillus firmus, etc.*	- Mesophilic - Facultative anaerobes - Gram-positive	Create an anaerobic environment, capable of hydrolyzing simple substrates or sugars to produce H_2	[146,147]
	Enterobacter sp.	*Enterobacter cloacae, Enterobacter asburiae, Enterobacter aerogenes*	- Mesophilic - Facultative anaerobes - Gram-negative - Non-spore-forming	Create an anaerobic environment	[145,148, 149]
	Thermophilic bacteria	*Klebsiella pneumonia, Thermoanaerobacterium thermosaccharolyticum, Caloranaerobacter azorensis*	- Obligates or facultative anaerobic bacteria	Produce high rate of hydrogen	[145,150]

hydrogen producers, which could easily be inactivated by the pretreatment because most of the methanogens are gram-negative and non-spore forming [152]. When the mixed inoculums are treated under the harsh conditions, such as high temperature, low pH, *etc.*, only hydrogen producers can be retained as they have a better chance of survival due to their unique feature of endospore formation [153]. In one of recent study, Maintinguer, Sakamoto [154] observed that the gram-positive rods with endospores were predominant in a pretreated anaerobic consortia and they strongly influenced hydrogen production in the system. This could be ascribed that the biohydrogen production substantially depends on the microbial community distribution and their microbial metabolic rate and pathway in the system [17]. The pretreatment method affects the biohydrogen production by influencing the distribution of microbial consortia in the system [17,135]. Hence, the knowledge of interaction between or within the microbes in the microbial community and fermentation environment could provide a new insight about the necessity of inoculum pretreatment for enhancing fermentative H_2 production [135].

3.3. Different methods for pretreating mixed culture inoculum (sludge)

Different pretreatment techniques have been investigated in the mixed microbial flora for improving H_2 production efficiency. This pretreatment can be categorized on the basis of operational process, such as chemical treatment, physical treatment, biological treatment, and combinations of these treatment processes [145]. The physical treatment method mainly includes heat-shock, microwave, ultrasound, UV-light treatment, *etc.*, while the chemical pretreatment method includes alkaline, acid, chloroform, 2-bromoethane sulfonic acid (BESA) and iodopropane, and oxidation pretreatments. Microbial and enzymatic treatments are regarded as the biological treatment and the combination of different treatments like alkaline-ultrasound, alkaline-heat, ultrasound-heat-ozone, alkaline-ionizing radiations, *etc.* are commonly known as combined treatment process [145].

Three predominant methods such as heat (~23 %), alkaline (~35.8 %), and ultrasound (~10.5 %) have been widely used as individual pretreatment techniques [145]. However, the pretreatment conditions such as acid shock (at pH 2–4, from 30 min to 24 h), base (at pH 10–12, from 30 min to 24 h), uses of chemical inhibitors (*e.g.,* iodopropane, BESA, chloroform for 30 min-24 h), heat treatment (from 65 to 121 °C, for 10 min-10 h), microwave treatment (power intensity ranging from 325 to 2450 W, for a duration of 1.5–5 min), ultrasonic treatment (intensity ranging from 20 to 79 kJ/g TS), aeration treatment (from 30 min to 4 days), UV irradiation treatment (15 min–3 h), ionizing radiation treatment (0.5–10 kGy), application of electric current (at 10 V and around 10 min), load-shock treatment (COD range from 50 to 83 g/L, for 2–3 days), *etc.* [145] were reported as efficient for suppressing methanogenic bacteria in the mixed microbial consortia. It was observed from various studies that the H_2 producing microbes evolve from the spores and the number of methane producing microbes is reduced after pretreatment (Table 7). For instance, the dominancy of bacterial species like *Caloramator australicus* and *Clostridium* sp. was reported after applying heat pretreatment to seed sludge in fermentative biohydrogen generation [155]. It was evidenced that the pretreatment of sludge having mixed microbial flora before inoculating to the fermentative medium have better performance for hydrogen production compared to the untreated seed sludge cultures.

The pretreatment techniques are not only helping to suppress the methanogens, but they also have impact both on the physiology of microbes and the performance of reactor [166]. The variation on hydrogen

Table 7
The predominant microbial community of anaerobic sludge after treating with different pretreatment methods.

Treatment method	Treating conditions	Dominant microbial community	Increment of H_2 production efficiency (%)	References
Acid	at pH 3.0, ~24 h	*Clostridium longisporum*; *Clostridium tryobutyricum*	94 %	[156]
Acid	at pH 3.0, ~24 h, 1 mol/L HCl	*C. sensu stricto* 1; *Romboutsia*; *Paeniclostridium*	–	[135]
Acid	at pH 3.0–4.0, ~24 h	*Bacillus coagulans*; *Acetivibrio cellulolyticus*; *Acetivibrio acidocalvarius*	48 %	[157,158]
Base	at pH 11.0, ~24 h	*C. tyrobutyricum*; *C. vincentii*; *Bacteroides vulgatus*	77 %	[156,157]
Base	at pH 10.0, ~24 h, 1 mol/L NaOH	*Paraclostridium; C. sensu stricto* 1; *Terrisporobacter*	–	[135]
Heat-shock	95–100 °C, 1 h	*C. butyricum*; *Klebsiella oxytoca*	99 %	[159,160]
Heat-shock	100 °C, 15 min	*Paraclostridium; Exiguobacterium; C. sensu stricto* 1;	–	[135]
Aeration	Continuous, 7 days	*Bacteriodes* sp.; *Clostridium* sp.; *Propionibacterium* sp.; *Fusobacterium* sp.	24 %	[161,162]
Aeration	Continuous aeration with air for 24 h	*Enterococcus; C. sensu stricto 1; Citrobacter*	–	[135]
Load-shock	50 g COD/L, 3 days	*Firmicutes*	–	[163]
γ - radiation	5 kGy	*Clostridium* sp.	194 %	[160,164]
γ - radiation	5 kGy, ^{60}Co	*C. sensu stricto* 1; *Paeniclostridium; Romboutsia*	–	[135]
Microwave and Base	860 W, 2 min and pH = 11.0, 24 h	*Clostridium* sp.	32%	[165]
Heat-shock and Chemical	121 °C, 20 min and BESA, 10 mmol/L, 24 h	*Bacillus coagulans*	–	[158]
Electric current	10 V, 10 min	*Clostridium* sp.; *C. sardiniense*; *C. saccharobutylicum*; *C. butyricum*; *C. beijerinckii*; *C. saccharobutylicum*;	–	[18]

yield depending on pretreatment methods have been reported in several literatures [17,135]. This can be correlated to the diversity of the prevalent microbial population in the sludge after treating with different pretreatment methods (Table 7). For instance, five different methods of pretreatment (*i.e.,* heat-shock, aeration, γ-radiation, acid and base) have been employed on seed sludge collected from sewage to assess their impact on hydrogen productivity by Yang, Yin [135]. The results of this study suggested that the enrichment of the hydrogen producers for all five pretreatments, however, the diversity in microbial communities was observed for different methods. Consequently, the metabolic pathways were followed by microbial communities, resulting in different process performance for hydrogen production. They deduced that the maximum H_2 productivity was achieved by employing heat pretreatment, while *Paraclostridium, Exiguobacterium,* and *Clostridium sensu stricto* were the most dominated genera. In another study, the methanogenic activity of seed sludge was completely suppressed by the heat-shock, acid, and repeated-aeration pretreatments, but not by the alkaline pretreatment [162]. Nevertheless, the change in fermentation types was also observed based on the different pretreatment methods applied such as mixed-acid type fermentation for acid pretreatment and control, butyric-acid type fermentation for heat-shock and alkaline pretreatments, and ethanol type fermentation for repeated-aeration pretreatment [162]. Consequently, the pretreatment methods would have a strong influence on the quality of final product. Singhal and Singh [167] observed that the production of methane completely avoided by microwave pretreatment of mixed culture using waste biomass of *Benincasa hispida*, however, 28 % methane of total gas was produced for control reactor. Nevertheless, a slight amount of CO_2 was also produced in all reactors. Wang, Zhang [168] demonstrated a two-phase anaerobic fermentation process (at 35 ± 1 °C) for producing H_2 and CH_4 from swine manure using pretreated anaerobic sludge with heat, alkali, and acid treatments as inoculum. It was observed that the production of H_2 and CH_4 were increased by 77.4 and 16.8 % for heat (80 °C and 15 min) and 41.2 and 1 % for alkali (pH 10) treated inocula, respectively. However, the production of CH_4 was decreased by 4 % while the H_2 production was increased by 35.4 % using the acid (pH 3) treated inoculum. The performance of several pretreatment technologies for inoculum is presented in Table 8.

In contrast, the variation on hydrogen productivity have also been sreported by using a similar pretreatment method for the inoculums obtained from different sources [145]. Chen, Chuang [181] have investigated the hydrogen productivity by employing inoculum from different sources (*i.e.,* sludge from municipal wastewater and cow dung compost), but using the similar pretreatment method (*i.e.,* heat pretreatment) for both. They observed the heat-treated sludge form municipal wastewater treatment plant obtained 2.2 times higher hydrogen production compared to the cow dung compost at the same reaction conditions. This can be ascribed to the microbial diversity in different sludge sources based on their origin. This is because the bacterial community composition can be different according to their origins *e.g.,* source of collection, types of sludge, *etc.* and consequently, the predominant fermentation pathways depend on the nature of seed sludge. The heat-treatment of sludge has been employed by various researchers due to its simplicity, cost-effectiveness, and requirement of less time than the acid, alkali, and other chemical pretreatment methods. Athough the heat-shock pretretment has been employed by various researchers [182], it cannot be considered as the most effective pretreatment method as some H_2 producing microbes especially non spore forming bacteria could be eliminated at a high temperature [157]. However, the spore-forming homo-acetogens, which are also an important hydrogen consuming microbe, can barely be impeded by the heat treatment [152]. Moreover, some microbes that are beneficial to H_2 production, such as *Bacillus* sp., *Enterobacter* sp., (cellulose degrading microbes) are destroyed or inhibited at high temperature [17,157]. Energy consumption is also an important factor that limits heat-pretreatment [17]. Some other physical techniques such as microwave, ultrasonication, *etc.* can eradicate methanogenic bacteria but they are associated with high energy [9] and heat generation [10]. Elbeshbishy, Hafez [174] investigated the effects of several pretreatment methods such as heat-shock, ultrasonication, acid, and base on anaerobic sludge used as inoculum for fermentative H_2 production at 37 °C, pH 6.5, and 180 rpm. It was observed that the molar hydrogen yield increased by 45 % to 1.55 mol H_2/mol glucose using sonication with temperature control pretreated sludge over the heat-shock at 70 °C for 30 min (1.04 mol H_2/mol glucose) and acid at pH 3.0 for 24 h (1.04 mol H_2/mol glucose) pretreatments. However, the molar hydrogen yield decreased by 30 % to 1.03 mol H_2/mol glucose for sonication without temperature control treatment. This might be due to the damaging impact of heat on some hydrogen producers, that was produced during sonication. Similarly, the chemical pretreatment using sodium 2-BESA

Table 8
Performance of several pretreatment technologies for inoculum to enrich hydrogen production.

Source of Inoculum	Pre-treatment applied	Pretreatment conditions	Substrate	Operational conditions	H_2 yield	References
Sewage sludge	Acid	pH 3–4 for 24 h	Sucrose (20 g/L)	35 °C, pH 6.7	1.40 mol H_2/mol hexose	[169]
Digested sludge	Acid	pH 3 for 30 min	Sucrose (25 g/L)	35 °C, pH 10	1.55 mol H_2/mol hexose	[170]
Sewage sludge	Acid	pH 3 for 24 h	Sucrose (10 g/L)	35 °C, pH 7.0	0.36 mol H_2/mol hexose	[158]
Waste activated sludge	Heat	100 °C for 45 min	Sucrose (20 g/L)	35 °C, pH 5.8	1.72 mol H_2/mol hexose	[171]
Waste activated sludge	Aeration	Repeated-aerated with air for one week	Glucose (10 g/L)	35 ± 0.5 °C, pH 6.8	1.96 mol H_2/mol hexose	[162]
Pseudomonas sp. GZ1 (EFSS1040) seed bacteria	Ultrasonication	2 w/ml , 2 min	Wastewater sludge	35 °C, pH 7.57	4.68 mL H_2/g TCOD with ultrasonication To 15.02 mL/g TCOD	[172]
Compost	Heat	100 °C, 45 min	Sweet potato solution (COD: 150 g/L)	35 °C, pH 6.5	1.31 mmol H_2/g potato	[173]
Cracked cereal	Heat	100 °C, 30 min	Grass	35 °C, pH 7.0	72.2 mL H_2/g dry grass	[156]
Anaerobic sludge	Ultrasonication	20–79 kJ/g TS	Glucose (8–8.5 g/L)	37 °C, pH = 6.5 e6.8	1.03–1.7 mol H_2/mol hexose	[174]
Anaerobic sludge	Ionizing radiation	0.5–10 kGy	Waste sludge	35 °C, pH 7.9	14.2 mL H_2/g VSS	[163]
Anaerobic sludge	Load-shock	COD: 50 g/L, 3d	Wastewater (COD: 3 g/L)	28 °C, pH 6.0	3.17 mol H_2/mol hexose	[175]
Anaerobic sludge	Electric current	10 V, 10 min	Sucrose (20 g/L)	60 °C, pH 5.5	1.57 mol H_2/mol hexose	[176]
Compost	Microwave	450 W, 1.5 min	Maize straws (20 g/L)	36 °C, pH 7.0	144.3 mL H_2/g substrate	[177]
Mixed culture (sludge from a settling channel)	Heat	100 °C for 30 min	Rice straw	55 °C, pH 7.0	40.04 mL H_2/g VS rem	[178]
Anaerobic sludge (brewery wastewater treatment plant)	Heat	90 °C for 30 min	Rice husk	35 °C, pH 7.0–7.5, 150 rpm	473.1 mL H_2/g biomass	[179]
Anaerobic sludge (wastewater treatment plant)	Heat	121 °C for 10 min	Sugarcane leaf waste	37 °C, pH 6.5, 180 rpm	18.6 mL H_2/g sugar	[180]

and iodopropane have shown to be effective for the suppression of methanogens [158,183], but less desirable to apply in a large-scale [184] owing to its negative impact on the environment. Moreover, pretreatment of inoculum by a chemical shock, acid, or alkali, requires prolonged treatment time and extensive use of chemicals [185,186].

3.4. Application of electroporation in inoculum pretreatment

3.4.1. The state-of-the-art mechanism of electroporation

Inactivation of microorganisms from mixed culture consortia by applying EP could be an alternative approach for treating inoculum to enhance biohydrogen production [14]. The cell disruption mechanism for the EP treatment was defined by Sheng, Vannela [187], where they described that the unequal electrical charges are produced on dipolar molecules *e.g.*, peptidoglycan in the cell wall and diacylglycerols in the cell membrane as soon as a biological cell passes through the high strength and rapidly transforming electric fields. In general, the microbial cells have peptidoglycan and phospholipids composed of polar molecules with ligand groups (Fig. 6) [188], which are vulnerable to the action of strong electric fields [189]. As soon as, the electric field exceeds the critical potential value (typically, ~1 V for bacteria), irreversible pores in the membrane can be formed due to the pressure induced by the attractive forces of unequal electric charges [187]. Loghavi, Sastry [190] reported that the formation of pores on the cell wall can be triggered by EP once the electrical charge exceeds the critical rupturing value. Thus, the electrical charges accumulated on the di-electric plasma membrane and cell wall caused irreversible breakdown of cells. Furthermore, EP possesses a strong effect on the

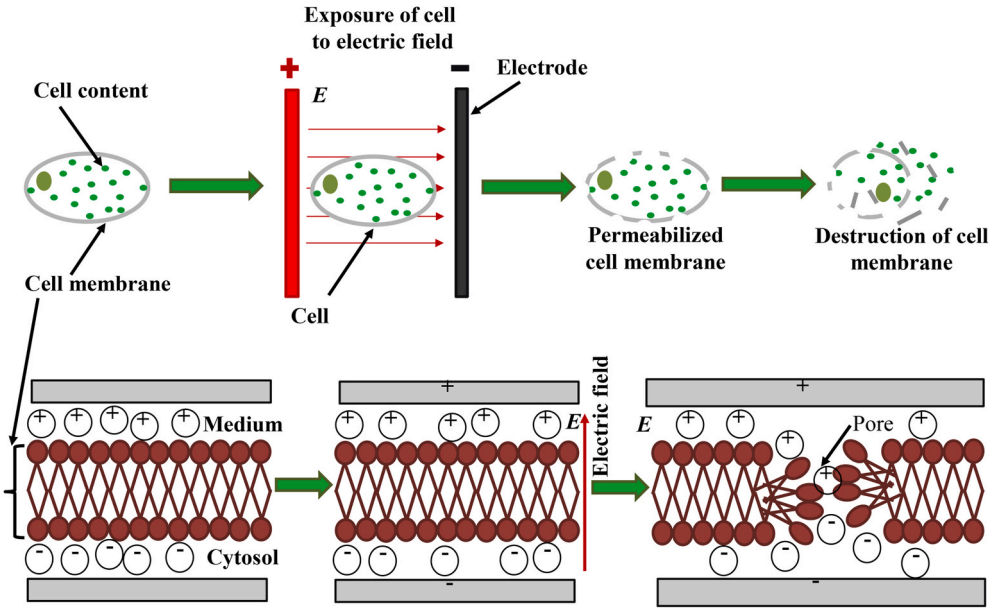

Fig. 6. Schematic diagram of permeabilization and destruction of cell membrane by applying an electric field.

modifications of the cell wall structure, thus resulting in enhanced cell wall porosity [191]. Sheng, Vannela [187] observed that the cell membrane and cell wall of cyanobacteria (*i.e., Synechocystis*) were severely damaged after applying EP, in particular, with a TI greater than 35 kWh/m^3 [187], whereas *Chlorella vulgaris* cells were seriously disrupted at a TI of 30 kWh/m^3 [22]. In another study, Dutreux, Notermans [192] stated the breakdown of the *E. coli* cell wall by the application of EP. Likewise, the inactivation of different pure culture microbes via various electric fields has been reported in some studies [193–195]. For example, Spilimbergo, Dehghani [195] demonstrated that the cell viability of several bacteria like *E. coli, Staphylococcus aureus, etc.* was reduced with the increasing of electric field intensity and pulse number. A similar trend was demonstrated by Karim, Islam [14], where they found a substantial reduction (~48 %) of anaerobes within the 30 s (TI = 30 kWh/m^3) while ~96 % cell was inactivated by applying EP of 2 min (equivalent TI of 120 kWh/m^3). However, *E. coli* cells were observed to be reduced rapidly by using EP at initial pulses (30 pulses with 12.34 kV/cm) when a lesser effect on the cell inactivation was observed with the consequent pulses (30 pluses with 30 kV/cm) [193,194]. Thus, it can be postulated that the EP could potentially be applied to eliminate the hydrogen consuming microbes of AS during the fermentative hydrogen production.

The sensitivity of the EP particularly depends on the characteristics of microbes such as the size, shape, and types of microorganisms and on the cell wall thickness. For example, gram-positive bacteria (mainly H_2 generating microbes) are more resistant or impervious to the electric field than the gram-negative bacteria (mainly H_2 expending microbes) [196]. In a study by Pothakamury, Vega [197] stated that the time of inactivation was different for gram-negative and gram-positive bacterial species while EP was employed to the cell. This might be due to the thicker and stiffer cell walls of gram-positive bacteria (Fig. 7), which possess strong protection against an external electric field [198]. Generally, gram-positive cell walls are thick, and the peptidoglycan layer constitutes almost 95 % of the cell wall in some species of gram-positive bacteria [199] in contrast to a thin peptidoglycan layer (as little as 5–10 % of the cell wall) of gram-negative bacterial species (Fig. 7) [200]. However, methanogens are archaea which differ from bacteria regarding the cell membranes and cell walls characteristics. Unlike hydrogen producing bacteria, archaean cell walls do not have peptidoglycan, but they may have pseudopeptidoglycan, polysaccharides, glycoproteins, or protein-based cell walls [201]. Thus, it can be speculated that the EP treatment with a particular range of intensity, would degrade the pseudopeptidoglycan, polysaccharides, glycoproteins, or pure protein in cell wall of methanogen archaea, and consequently, the methanogens of mixed culture could be inactivated.

3.4.2. Prospects and challenges of electroporation

Peptidoglycan (also known as murein) is an important element of bacterial cell wall structure, which supports to retain the shape of the cell and protects the cell from bursting [202]. The architecture of the peptidoglycan in the cell wall network is organized in the cables perpendicular to the long-axis of the cell for a vegetative form of bacteria [202]. The network of the cables should be disorganized via an electric field to disrupt the cell. A latest report has shown that about 14 % of the cell membrane can be degraded during the PEF application (intensity 5.7 kV/cm) for a 2 min duration [203]. Therefore, the EP treatment with a certain range of TI would easily destroy the polysaccharides and phospholipids of cell wall in methanogenic archaea (Fig. 7) and leads to the disturbance of their cell walls. Such disruption of the cell walls as a result of EP could have a destructive effect on the cell viability. Karim, Islam [14] observed that the predominant methanogenic archea, such as *Methanocullous thermophilies*, *Methanobacillus arboreus*, *Methanothermous fervidus*, *etc.* were inactivated from AS after being treated with TI of 120 kWh/m^3 (for 1 min EP treatment). The outcomes of their study showed that the predominant methanogens could be eliminated from AS through EP treatment, which ultimately reduced the formation of methane, and resulted in increased biohydrogen production in the fermentation system [14]. Moreover, EP treatment required several minutes to remove methanogens depending on TI (Table 9), whereas the time of treatment can be 10 min to 3 days for other pretreatment techniques [14,17]. These observations suggest the potential application of EP to inactivate methanogens which can be a promising alternative to the commonly used techniques for preparing the inoculums in biohydrogen production (Fig. 8).

The hydrogen producing microorganisms can be retained by eliminating hydrogen consuming bacteria using EP on mixed culture inoculum as reported in few recent studies [14,18]. In this way, the application of EP can substantially increase the fermentation-based hydrogen production. In a recent study, an electric field with variable voltages (*i.e.*, 5–20 V) was employed by Jeong, Cho [18] to observe the feasibility and efficiency of the EP as an alternative pretreatment technique in fermentative H_2 production (Table 9). The optimum hydrogen yield of 1.43 mol H_2/mol hexose and the maximum H_2 production rate of 101.4 mL H_2/L/h was obtained for the EP treated (voltage of 10 V for 10 min) inoculum. Moreover, the production of H_2 was observed to be significantly higher (~22 %) than the heat treated (90 °C for 20 min) inoculum. This was due to the co-existence of the H_2 consumers with the H_2 producing bacteria in heat treated inoculum which was confirmed by microbial analysis. However, only H_2 producing bacteria but no H_2 consuming bacteria were detected in the EP treated (at 10 V for 10 min) inoculum [18]. They also observed that the EP treatment with a relatively low voltage (5 V) was inefficient to inactivate all H_2 consumers

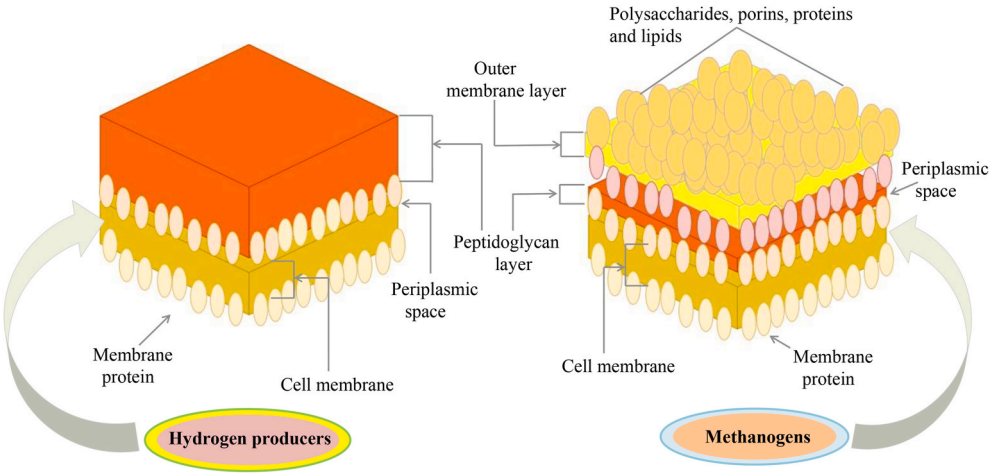

Fig. 7. Typical differences between methanogens and non-methanogens in cell wall structure.

Table 9
Application of electroporation pretreatment to the seed inoculum and its influence in biohydrogen production.

Method	Inoculum	Condition	Substrate	Characteristics of fermentation	Cumulative H_2 production (mL)	H_2 production rate (mL/L/hr.)	References
Control (Heat)	Anaerobic sludge (45 mL)	90 °C, 20 min	Glucose	Batch fermentation; working volume, 150 mL (total volume 250 mL); 37 ± 1 °C; 150 rpm; initial pH 8.0	392 ± 13.5	88.2	[18]
EP	Anaerobic sludge (45 mL)	DC current, 5 V, 10 min	Glucose	Batch fermentation; working volume, 150 mL (total volume 250 mL); 37 ± 1 °C; 150 rpm; initial pH 8.0	480 ± 9.6	92.2	[18]
EP	Anaerobic sludge (45 mL)	DC current, 10 V, 10 min	Glucose	Batch fermentation; working volume, 150 mL (total volume 250 mL); 37 ± 1 °C; 150 rpm; initial pH 8.0	501 ± 11.3	101.4	[18]
EP	Anaerobic sludge (45 mL)	DC current, 15 V, 10 min	Glucose	Batch fermentation; working volume, 150 mL (total volume 250 mL); 37 ± 1 °C; 150 rpm; initial pH 8.0	441 ± 10.1	97.1	[18]
EP	Anaerobic sludge (45 mL)	DC current, 20 V, 10 min	Glucose	Batch fermentation; working volume, 150 mL (total volume 250 mL); 37 ± 1 °C; 150 rpm; initial pH 8.0	357 ± 12.7	88.1	[18]
Control (No treatment)	Anaerobic sludge (100 mL)	–	Citrus wastewater	Batch fermentation; 700 mL working volume (in a total volume of 1000 mL); 36 ± 1 °C; 120 rpm; initial pH 6.0	290 ± 14	–	[14]
EP	Anaerobic sludge (100 mL)	DC current, 4 kV for 0.5 min corresponding to a TI of 30 kWh/m^3	Citrus wastewater	Batch fermentation; 700 mL working volume (in a total volume of 1000 mL); 36 ± 1 °C; 120 rpm; initial pH 6.0	589 ± 36	–	[14]
EP	Anaerobic sludge (100 mL)	DC current, 4 kV and 1 min (equivalent to a TI of 60 kWh/m^3)	Citrus wastewater	Batch fermentation; 700 mL working volume (in a total volume of 1000 mL); 36 ± 1 °C; 120 rpm; initial pH 6.0	896 ± 26	–	[14]
EP	Anaerobic sludge (100 mL)	DC current, 4 kV and 2 min corresponding to a TI of 120 kWh/m^3	Citrus wastewater	Batch fermentation; 700 mL working volume (in a total volume of 1000 mL); 36 ± 1 °C; 120 rpm; initial pH 6.0	240 ± 19	–	[14]

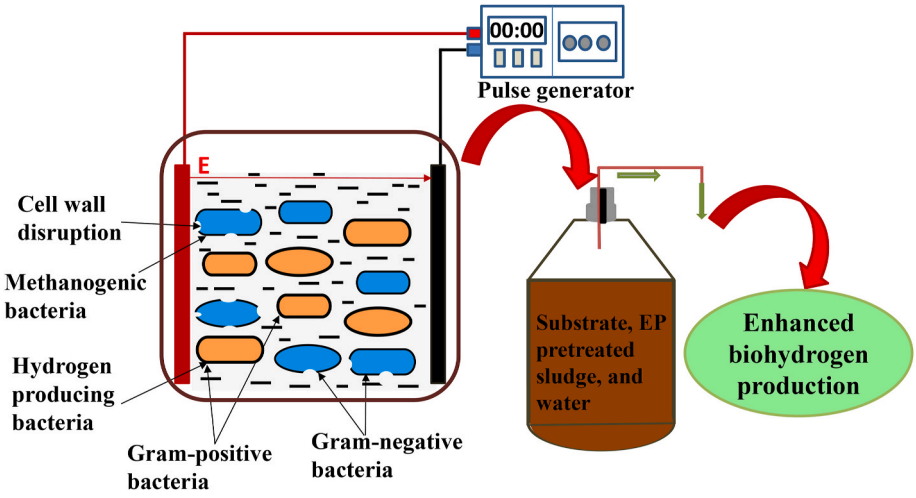

Fig. 8. Conceptual diagram of inactivating methanogens in anaerobic sludge by the electroporation pretreatment.

(~480 mL cumulative H_2) while a moderate voltage (10 V) could eliminate a maximum H_2 consumers (~501 mL cumulative H_2). Conversely, the EP treated inoculum with a higher voltage (20 V) obtained a lower hydrogen production (~357 mL cumulative H_2). This might be due to the elimination of some hydrogen producing microbes by a stronger electric field. Comparable observations to this study were described by Karim, Islam [14], and they demonstrated that the EP treatment with different TI (such as 30 kWh/m^3, 60 kWh/m^3, and 120 kWh/m^3) have shown different effects on the microbial community of AS, which resulted in variable performance in H_2 production. Among the various TIs, the mild intensity *i.e.*, 60 kWh/m^3 showed a drastic increase (about 315 % higher than control) in H_2 yield compared to the other EP treatments (30 kWh/m^3 and 120 kWh/m^3). This was due to the greater ability to enrich hydrogen producers in the AS with that EP treatment (*i.e.*, 60 kWh/m^3). They also compared the results with other conventional treatment methods, and found that the maximum H_2 production (around 896 mL) was obtained with EP treatment for 1 min (~60 kWh/m^3), followed by a treatment with a probe type sonication for 20 min (~678 mL), 40 min treatment in a bath type sonication (~563 mL), and 60 min heat treatment with 100 °C (~545 mL) [14]. Therefore, EP technique could be an attractive and alternative pretreatment method to inactivate H_2 consumers from mixed culture consortia for enhancing biohydrogen production in the fermentation system. This pretreatment method possesses the advantages of being mild (hence less possibility of inhibitory by-products formation) and relatively selective. Moreover, it is quick (few seconds time scale, hence less time required) and can be used in a continuous or batch mode [204]. However, it can be expensive if it is applied on a large scale or need to process a large number of samples [191]. This limitation could be overcome using a flow type camber in continuous mode. In this regard, designing the flow rate and the geometry of the chamber to ensure that the cells in the mixed culture are exposed to the required number of

pulses or appropriate electric field to deactivate methanogens while passing through the chamber is a huge challenge [205].

4. Concluding remarks

Biohydrogen production through the fermentation of renewable biomass particularly waste biomass could be a sustainable and eco-friendly technology. However, biohydrogen production in large scale is still challenging due to lack of appropriate bioprocess engineering. In this regard, to make the biohydrogen production technically feasible, industrially practicable, financially attractive, and ultimately sustainable; it is very essential to understand the influence of pretreatment over metabolic function of hydrogenic bacteria (bioengineering) and their roles in fermentation process (process engineering). However, the pretreatment of complex substrates and natural inoculum (*e.g.*, AS) is a step limiting factor in biohydrogen production.

The conversion rate of raw cellulosic biomass is challenging due to their complex polymeric backbone, especially cellulose and hemicellulose structure. Therefore, the pretreatment of substrate isemergent for reducing the retention time and maximizing system performance. A number of pre-treatment techniques such as acid, alkali, microwave, ultrasound, biocatalysts, *etc.* have been employed to enhance the degradation rate of substrate in waste biomass based biohydrogen production. However, the final yield of hydrogen production is still not satisfactory by practicing afore-mentioned methods. Recently, several combined pretreatment techniques have been proposed as novel and efficient, however, some difficulties still remaining in the context of optimization.

Besides, the composition of microbial archaea in a complex microbial community system plays a crucial role for the degradation of biomass-based substrates as well as system performance since the process is considerably controlled by the metabolism rate and pathway of microorganisms. Generally, the use of natural mixed culture consortium, especially AS, could be a potential inoculum to achieve an optimum production efficiency of biohydrogen. However, the presence of hydrogen consumers in mixed culture consortia restricts the application of AS in biohydrogen production. Therefore, several pretreatment techniques such as chemical, physico-chemical, heat, sonication, microwave, UV-irradiation, *etc.* have been used to inhibit hydrogen consumers in mixed microbial flora, but no agreed conclusion has been found regarding the most suitable pretreatment method till date. Nevertheless, the efficiency of hydrogen production can be different for an individual pretreatment, even though the inoculum source and the fermentation conditions are same.

In this context, EP can be a promising non-conventional pretreatment technique for both inoculum and substrate pretreatment. EP pretreatment in a certain range of TI according to the substrate type (since EP is a more selective technique) could help to shorten the adaptation time of microbes and thereby enhance the biohydrogen production through better substrate utilization. However, the intensity and treatment time of EP are yet to be studied to optimize the process for biohydrogen production with a minimum production cost and energy consumption. Numerous efforts have been implemented to examine the influence of pretreatments on the microbial diversity as well as in hydrogen production. However, there are still lack of experimental justification that suppress the hydrogen consuming microbes (or methanogens) by retaining hydrogen producers. In this context, EP could be a promising technique for eliminating hydrogen consuming microbes while mixed cultures are used as inoculum. This is because a certain intensity and time of EP pretreatment can eradicate H_2 consumers, especially methanogens, present in AS. However, the underlying mechanisms of EP for the survival of hydrogen producers and suppression of hydrogen consumers are rarely discovered. Thus, rigorous investigations are highly required to elucidate the exact mechanisms of hydrogen consumers inhibition by EP including optimization of time and TI.

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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