Evaluating hydrogen production from glucose using graphite felt beads as a solid matrix in immobilized mixed cell reactor at thermophilic fermentation

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Abstract

This study has successfully evaluated graphite felt (GF) beads as a solid matrix to immobilize or trap the mixed cultures in an immobilized mixed-cell reactor (IMcR). The anaerobic sludge of palm oil mill effluent was used as an inoculum source in the IMcR with mixed culture. Here, glucose, sucrose, and starch were used as the model substrates to evaluate the performance of IMcR with GF beads for producing bio-hydrogen (BioH$_2$). BioH$_2$, effluent, and surface morphology of GF beads were analyzed by using gas chromatography equipped with a thermal conductivity detector, high-performance liquid chromatography, and scanning electron microscopy, respectively. The highest H$_2$ yield (Y_{H2}) and production rates were obtained at 304.0 ± 13.2 mL g$^{-1}_{COD}$ (corresponding to 2.26 mol mol$^{-1}_{glucose}$) and 1403 ± 61 mL L^{-1} day^{-1}, respectively. IMcR with GF beads is a new approach for generating high Y_{H2}, which can be used for more than two months in an experimental run.

Keywords: Biohydrogen; graphite felt; dark fermentation; immobilized mixed cell reactor; palm oil mill effluent

1. Introduction

Energy is a critical issue humanity faces at present and in the future [1]. Approximately 95% of energy resources are derived from fossil fuels [2,3]. This phenomenon is a critical problem faced worldwide as fossil fuels can trigger a number of environmental effects, such as global warming and climate change, thereby harming human health and ecosystem. At the same time, finding new and alternative energy resources is deemed highly critical to address the increased energy demand worldwide. The future ideal is a world without pollution with the energy source derived from renewable and sustainable resources [4]. The use of hydrogen as a green fuel is one of the best scenarios in future to replace fossil fuels [5].

Hydrogen (H$_2$) is a promising energy carrier and environmentally friendly fuel source in the universe. It is an attractive fuel as it can be burnt or combined with O$_2$ in the fuel cell system, producing water as the byproduct. H$_2$ is also a sustainable energy source with a high energy content [6,7]. In general, H$_2$ is naturally absent in a single molecule but combined with other elements, primarily O, C, and N, in fossil fuel and living materials. Also, it is absent in a considerable quantity. Therefore, H$_2$ must be generated from various primary resources, including biomass, wastewater, and organic substrates [8]. The chemical and biological approaches can be applied to produce H$_2$ from various resources. In terms of effect on the environment, the biological approaches are more interesting and recommended methods compared to those chemical [9,10].

Several substrates, such as glucose [11–13], sucrose [14], xylose [15], honey [16], whey [17], starch [18], sugarcane molasses [19] and cellulose [1,20], glycerol [6], and wastewater [21,22], have been used as models to produce biogas by using dark fermentation (DF) with suspended cell. Briefly, DF is a fermentation process occurred in the absence of O$_2$ without additional light energy [23]. In general, suspended pure cultures produce high volumetric H$_2$ from organic matter; unfortunately, pure cultures in comparison to mixed cultures are more sensitive to environmental conditions. DF with suspended pure cultures is uneconomical now that it is a complex process requiring additional costs (i.e. isolating cells); in contrast, DF with mixed culture is an easy process with low sensitivity to environmental conditions and it is applicable with a wide range of substrates and without any additional cost to collect the cells. In addition, immobilized mixed culture can be

used as a biological approach to treat industrial effluents [24]. This approach, therefore, becomes a good option and a promising method to generate biogas and/or biohydrogen.

The immobilized mixed cell is a promising technique for a long-term operation to enhance the DF performance. The DF using the immobilized mixed cultures is applicable to generate H_2 from various substrates such as biomass [25] and wastewater [26]. Sodium or calcium alginate is the most common material used to immobilize either single or mixed cells in the bioreactor thus far. Unfortunately, alginate beads are unsuitable for long-term bioreactor operation, especially under thermophilic (55 to 60°C) and hyper-thermophilic (70°C) conditions where their performance gradually drops after one month of operation [12,27]. It is well known that the DF processes under thermophilic conditions can generate high H_2 compared to mesophilic (30 to 50°C) and hyper-thermophilic conditions [23,28]. In this study, the gaps were used to overcome the performance decline by introducing C-based materials as a solid matrix for long-term operation under thermophilic conditions.

Generally, solid organic supports, such as graphite felt (GF), are used as electrode material in bio-electrochemical technology (BET) for both generating H_2 and treating wastewater [26,29]. In the BET system, especially in the microbial electrolysis cells (MEC) with GF anode and platinum-catalyzed GF, approximately $17.8 - 50.0 \ LL^{-1}d^{-1}$ of H_2 production rate can be generated [30,31]. The good performance of GF is associated with their physiochemical and electrical properties. The GF material has high porosity, good chemical stability, good thermal and electrical conductivities, and no adverse effects on microorganisms, all of which can allow the microorganisms to grow well, and maintain the temperature in a bioreactor. In addition, there is no chemical reaction occurred between GF and any compound in a DF bioreactor. GF is a cheap material, environmentally friendly, reusable, and commercially available as well [32]; for this reason, it becomes a good option as a solid matrix to immobilize or trap fermentative bacteria (H_2-producing bacteria) in a DF bioreactor as it has an appropriate characteristic for biofilm formation [33]. The H_2-fermentative bacteria can grow well on the GF matrix, so the most of organic substrates will be consumed to generate the high hydrogen [34].

Based on the references above, the main objective of the present study is to evaluate the performance of GF as a solid matrix in the immobilized mixed cell reactor (IMcR) in terms of H_2 production from various substrates under a thermophilic condition. This work would be focused on improving H_2 production from glucose because the performance of IMcR with alginate was deficient ($37 \ mmol \ mol^{-1}_{glucose}$) in our previous study [12]. The IMcR with GF is a promising approach to improve biogas production.

2. Materials and Methods

2.1. Anaerobic sludge of Palm oil mill effluent (POME) as an inoculum source

Anaerobic sludge of POME was collected from the effluent treatment pond located at Seri Ulu Langat Dengkil, Selangor, Malaysia. In this work, it was used as a mixed culture source. The mixed culture was enriched in a rich nutrient medium (RNM) in a bottle (DURAN, 500 mL) for 7 days. Once the

mixed culture solution produced biogas, it was used as an inoculum in the dark fermentation (DF) process. The RNM contained (all per liter $[L^{-1}]$) 5.0 g glucose, 2.0 g yeast, 2.0 g tryptone, 1.0 g NH_4Cl, 1.0 g NaCl, 3.0 g K_2HPO_4, 1.5 g KH_2PO_4, 0.5 cysteine-HCl, 0.5 g $MgCl_2.6H_2O$, 1.0 mL of vitamin and mineral solutions. Sodium hydroxide (NaOH) was used to adjust the pH of RNM (pH = 7) [12,35]. The RNM was stirred and heated at 60°C to obtain a homogenous mixture. Table 1 presents the selected characterizations of the anaerobic sludge of POME.

Table 1. The characteristics of the POME sludge

Parameter	Value
Temperature (ºC)	$15.9 \pm 0,1$
Dissolved oxygen (DO, % sat)	62.2 ± 0.2
pH	4.6 ± 0.1
Water content (%)	93 ± 2
Total suspended solids (TSS, gL^{-1})	65.0 ± 5.9
Volatile suspended solid (VSS, gL^{-1})	10.2 ± 1.3
Biological oxygen demand (BOD, gL^{-1})	77.2 ± 0.1
Chemical oxygen demand (COD, gL^{-1})	44.0 ± 3.1
Ash (gL^{-1})	54.9 ± 5.9

2.2. Immobilized cell with GF beads as a solid matrix

The GF material was supplied by Sigma Aldrich Malaysia SDN.BHD. The GF beads were prepared by cutting off GF with a surface area of 25 mm^2 and were immersed in 1 M HCl solution for 30 min prior to be washed with deionized water (DW). They were subsequently immersed in 1 M NaOH for 30 min before being washed with DW. The treated GF beads were then enriched with fermentative bacteria using an enriched mixed culture solution for a week. Lastly, the enriched GF beads were placed in a reactor and purged with pure N (99.9%) for 5 min. Immobilization processes were conducted at room temperature. This study used only GF beads as a solid matrix because alginate has already been reported in our previous work [12]. As a comparison, this study also cited some references such as fermentation by using free (suspended cell) and immobilized mixed cells.

2.3. Immobilized mixed cell reactor design and gas analysis

The tubular bioreactor or IMcR had an internal diameter and a height of 8 and 15 cm, respectively. The bioreactor was filled with a fresh substrate (80 vol. %) and enriched GF beads with a ratio of 1: 3. Meanwhile, a total of 20% extra space was prepared to anticipate bead expansion [11,12]. IMcR was conducted at different pH levels (i.e. 7.5, 7.0, 6.5, and 6.0), various substrates (i.e. glucose, sucrose, and starch), and at different fermentation times in batch mode operation. Biogases, such as H_2, CH_4, and CO_2 compositions, were monitored at each stage. The produced gases were collected via a water displacement method, and analyzed using gas chromatography equipped with a thermal conductivity detector (GC-TCD, HP 6890, USA).

2.4. Substrate and effluent analyses

The chemical oxygen demands (CODs) of fresh substrates

and fermentation effluent (FE) were analyzed based on the American Public Health Association (APHA) standard method by using an instrument set for COD analysis (DRB 200 and DR 2800 spectrometer, HACH, USA) [36]. Approximately 2 mL of the sample was put into the centrifuge (FORCEMICRO model) and was then operated at 13000 g for 10 min. 0.2 mL of the sample was then extracted and dropped into a vial containing standard potassium dichromate solution (0.042 M). The sample, later on, was placed in a heating plate (DRB 200 instrument) at 150°C for 2 h. Finally, the sample was cooled at room temperature before measuring the COD. The pH changes in fresh substrates and FE were determined by a pH meter (Trans Instrument, BP3001).

2.5. Mixed culture colony characterization

Scanning electron microscopy (SEM) analysis was performed to characterize mixed culture colonies in the GFs. In this analysis, the beads were collected under high biogas production (in the 3rd week of the experimental run). The dried GF beads were mounted on SEM stubs before being coated with gold. Different matrix sections were determined and imaged using SEM equipment (JEOL JSM 5800) operated with an accelerated voltage of 20 kV at a distance of 10 mm. The digital images with the resolution of 1280 × 960 were captured [11,12]. H_2 can be produced from organic substrates (i.e. glucose, sucrose, and starch) using mixed culture fermentative bacteria. To characterize the microbial distribution of biofilm on the GF beads, a total of 1.0 cm^2 (1.0 cm x 1.0 cm) of the GF was excised using a sterile cutter. The biofilm was scratched from the GF surface using autoclaved micropipette tips. Following that, the genomic DNAs were identified through the manufacturer's protocol as described by Jafary et al. [37].

2.6. Calculations

The changes in chemical oxygen demand *(ΔCOD)* can be determined in accordance to the APHA standard method as described by Mishra et al. [36]. The ΔCOD was calculated based on the difference of the COD_s of feed COD_0 and effluent (COD_t); $\Delta COD = COD_0 - COD_t$. The percentage of COD removal (COD_{rem}) is the amount of the change in COD divided by the COD of feed. The COD_{rem} was calculated using Eq. (1):

$$COD_{rem} = \frac{\Delta COD}{COD_0} \times 100\% \qquad (1)$$

Whereas, the volume of H_2 (V_{H2}) was calculated based on its composition in the total gas produced from IMcR (V_t). The V_{H2} was determined using Eq. (2):

$$V_{H2} = V_t \times [g] \qquad (2)$$

Where $[g]$ is the gas compositions (%) based on the GC-TCD analysis. The number of H_2 moles (n_{H2}) divided by the number of substrate moles (n_S) added to the reactor was calculated to determine the H_2 yield (Y_{H2}) produced from the reactor. The Y_{H2} was calculated using Eq. (3):

$$Y_{H2} = \frac{n_{H2}}{n_S} \qquad (3)$$

The energy efficiency of IMcR (η_E) was determined based on the energy produced by H_2 (W_{H2}, kJ mol^{-1}) over the energy produced by the substrate (W_S, kJ mol^{-1}) as described by Satar et al. [12]. The η_E was determined using Eq. (4):

$$\eta_E = \frac{W_{H2}}{W_s} \qquad (4)$$

where n_{H2} refers to the number of mole gases; n_S is the amount of the substrate moles; MW_s is the molecular weight of the substrates (i.e. glucose = 180.15 g mol^{-1}, sucrose = 340.29 g mol^{-1}, and starch = 692.66 g mol^{-1}); g_s is the weight of substrate (g); t is the time of the experiment, and ΔH_{H2} and ΔH_S are the heat combustion values for H (285.83 kJ mol^{-1}) and substrate (glucose = 2800 kJ mol^{-1}, sucrose = 5644.17 kJ mol^{-1}, and starch = 5758.74 kJ mol^{-1}), respectively. The n_{H2} can be determined using Eq. (5):

$$n_{H2} = \frac{PV_{H2}}{RT} \qquad (5)$$

where P (1 atm) and T (273.15 K) are the pressure and temperature at the standard condition, respectively; R is the ideal gas constant (0.08206 L atm mol^{-1}·K^{-1}), and V_{H2} is the volume of H_2 gas produced from IMcR. The H_2 production rate (Q) is the V_{H2} divided by the volume of substrate (V_s) added to IMcR over time (t). The Q was calculated using Eq. (6):

$$Q = \frac{V_{H2}}{V_s \times t} \qquad (6)$$

3. Results and Discussion

3.1. H_2 production from different substrates

The IMcR performance is closely related to the microorganisms, substrates, time, pH, temperature, bioreactor configuration, and mode of operation used [35,38]. As shown in Figure 1, the maximum Y_{H2} values from glucose, sucrose, and starch were 304.0 ± 13.2 g^{-1}CODinitial (corresponding to 2.2 ± 1 mol mol^{-1}glucose), 240.6 ± 21.9 mL g^{-1}CODinitial (3.3 ± 0.2 mol mol^{-1}sucrose), and 196.9 ± 6.6 mL g^{-1}CODinitial (0.9 ± 0.0 mol mol^{-1}glucose), respectively. This study showed a considerable increase in H_2 composition (36.6 %) compared with the one reported in our previous study (8.7 % by using 5 gL^{-1}glucose) [9]. This considerable increase may be due to the mixed culture's successful enrichment in the GF beads.

Theoretically, 1mol of glucose can produce 12 moles of H_2, while 1 mol of glucose, practically, can generally produce 3 to 4 moles of H_2 [39]. The maximum Y_{H2} values of 3.1 and 8.7 mol mol^{-1}glucose were produced using mixed (digested sludge) [40] and single culture (*Citrobacter amalonaticus Y19*) [41], respectively. However, the Y_{H2} from this study (2.257 ± 0.098 mol mol^{-1}glucose) was slightly lower than the reported values because the source of inoculum, mode of operation, and pretreatment methods used in this study were different from those used in the literature. According to the obtained result,

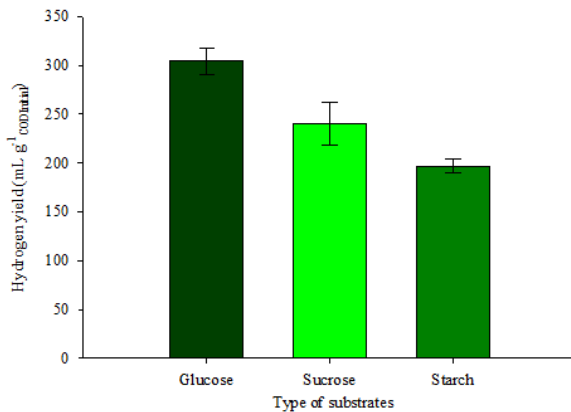glucose was assumed as a suitable substrate in IMcR for producing H_2.

Fig. 1. H_2 production from different substrates by using the IMcR system. IMcR was performed in the fed-batch mode at 60°C by using 5 g substrates with a $pH_{initial}$ of 7 for 24 h

3.2. H_2 production at different fermentation times

Fermentation time (t) significantly impacts the bioactivity of H_2-producing bacteria and fermentative H_2 production. A suitable range of fermentation time needs to be applied as it can significantly affect the ability of H_2-producing bacteria to produce high H_2 yield [8]. Figure 2 shows that H_2 composition gradually increased from 25.0% ± 0.8% to 36.6% ± 0.9%, while CO_2 composition decreased from 74.90% ± 0.84% to 63.9% ± 2.6% with the increase in operation t.
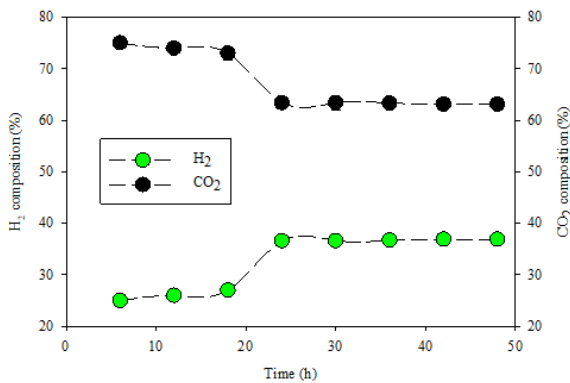

Fig. 2. Typical trends for H_2 and CO_2 compositions during 48 h of the experimental run. The data were collected using IMcR performed using 5 g L^{-1} glucose at 60°C with $pH_{initial}$ of 7.0

The H_2 and CO_2 compositions were stable after 24 hours of the experimental run. This result might be due to the optimum ability of H_2-producing bacteria during the experimental run. These results were consistent with our previous reports [12] in which the gas compositions increased along with the increase in operation t. However, the H_2 and CO_2 compositions decreased after 48 hours of operation. This behavior was expected due to the release of both H_2 and CO_2 gases from the reactor.

3.3. Effect of initial pH on the H_2 production

The pH is a critical factor that can affect the bioactivity of H_2-producing bacteria and H_2 production by fermentation as it has a significant effect on the hydrogenase activities and metabolism pathways. The present study was performed in a fed-batch mode without a pH controller. The initial pH of substrates had a significant effect on Y_{H2} and Q, which are low at either high or low pH values of the substrate [42]. In the present study, we found that H_2 production was high at the initial pH of 7 (Figure 3).

Meanwhile, some studies showed that the optimum initial pH was in the range of 4.2–6.0 by using different substrates and inoculums [42]. The initial pH of each substrate was dependent upon the substrate type, inoculum, and initial pH range used in a specific study. Figure 3 shows the H_2 production trend from glucose with an initial pH range of 6.0–7.5. The H_2 production decreased when the pH was either higher or lower than 7. These results indicated that the pH of 7 was a suitable condition for producing H_2 from all substrates in this study.
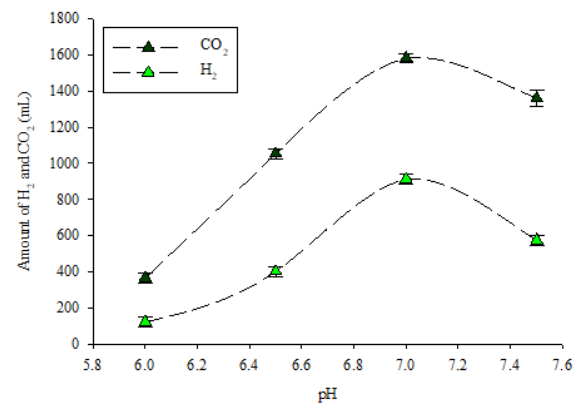

Fig. 3. Trends of H_2 production from glucose at different pH values

3.4. Effect of initial pH on the H_2 production

The efficiency of a bioreactor is a critical factor in H_2 production from an industrial point of view. Low efficiency indicates that the fermentation reactor is unsuitable for real-life applications. During a fermentation process, factors determining efficiencies, such as substrate type, mode of operation, inoculum, and bioreactor configurations, must be considered. This study showed that the efficiency of IMcR by using glucose (23.0%) was higher than that of by using starch (18.4%) and sucrose (16.7%), as shown in Figure 4(a). The higher efficiency of IMcR using glucose was due to the higher degradability of glucose than those of starch and sucrose. Substrate degradability affected the bioactivity of H_2-producing bacteria and fermentative H_2 production. These results were further supported by a higher $COD_{removal}$ of glucose (59.8%) than those of sucrose (33.6%) and starch (14.9%). Therefore, glucose is the simplest substrate, which can easily be degraded and consumed by H_2-producing bacteria.

The stability of IMcR performance during long-term operation is another important factor that needs to be considered [12,43]. IMcR can effectively be operated for >1 month without a significant decrease in H_2 production and composition (Figure 4(b)). During this time, the maximum Y_{H2} were 304.0 mmol $g^{-1}_{CODinitial}$, 240.6 mmol $g^{-1}_{CODinitial}$, and 196.9 mmol $g^{-1}_{CODinitial}$ from glucose, sucrose, and starch, respectively. Although the Y_{H2} values were slightly lower than the theoretical one, in terms of durability, the IMcR system can

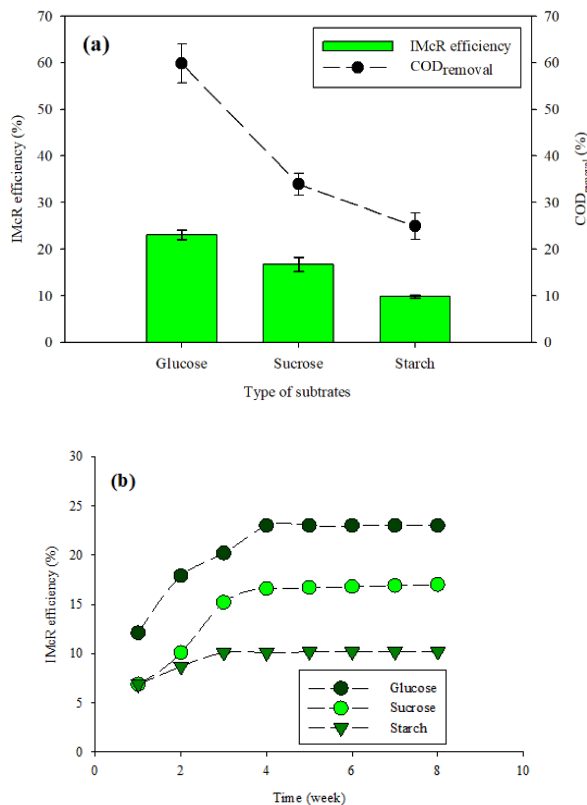be considered as an alternative method for the fermentation process.

Fig. 4. (a) IMcR efficiency and COD removal by using different substrates. (b) IMcR efficiency trend by using different substrates for 8 weeks.

3.5. Fermentation effluent (FE) properties: pH and conductivity

FE conductivity and pH are the two important indicators to facilitate H_2 production, especially when FE is used as a substrate in the microbial electrolysis cell (MEC). The FE and/or other wastes (i.e. the pyrolysis of switchgrass waste) can be used as substrates in the MEC system to generate high H_2 because the effluent contains a high organic matter concentration [44,45]. Hence, in this study, it became logical to evaluate the FE conductivity and pH. In general, the conductivity and pH of the substrate decrease after the fermentation process. Conductivity is the amount of charged species in the solution, while pH indicates the number of proton ions present in the solution. The initial and final conductivities of the solution were in the ranges of 15.2–15.5 and 10.2–10.9 mS cm^{-1}, respectively. A decrease in conductivity may be because a part of charged ions is converted into other compounds or gases.
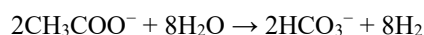
Meanwhile, a decrease in pH may be caused by proton ion accumulation resulting from the presence of organic acids [12,46]. VFAs were produced when H_2-producing bacteria consumed the substrate. As shown in Table 2, the conductivity of glucose effluent was relatively similar to those of sucrose and starch. In terms of MEC application, the pH of substrates played a critical role compared to conductivity. Generally, EAB decreases at low pH (<5.5), decreasing the MEC performance. Overall, the pH of FE was <5.5; hence, pretreatment processes were needed before FE could be used in the MEC system.

However, the performance of IMcR with GF using glucose was lower than that of MEC using FE in which 1 mol of glucose could only produce 4 moles of H_2 by using IMcR (Eq. 7), while FE produced 8 moles of H_2 by using MEC (Eq. 8). This was probably that the reactions in the IMcR and MEC systems were completely different. The fermentation reaction was spontaneous ($\Delta G = -184$ kJ mol^{-1}), while the electrolysis reaction was nonspontaneous ($\Delta G = +91.1$ kJ mol^{-1}). This phenomenon illustrates that the IMcR and MEC systems can be combined to enhance the H_2 production from glucose.

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

$$\Delta G = -184 \text{ kJ mol}^{-1} \tag{7}$$

$$2CH_3COO^- + 8H_2O \rightarrow 2HCO_3^- + 8H_2$$

$$\Delta G = +91.1 \text{ kJ mol}^{-1} \tag{8}$$

3.6. Comparison of IMcR to some studies

Most fermentation studies have been performed in batch mode operation because the processes are easy to handle and require no pH controller and pump to feed into the reactor. As a comparison, this research was also performed in a similar operation mode. Table 2 presents all data of this study.

Overall, the performance of IMcR was found better than that of other reports, except for the results reported by Li et al. [48]. The logical reason for these facts was related to the difference among the fermentation processes in terms of the inoculum types, substrates, and reactor designs [43]. The bioactivity of the H_2-producing bacteria was at the optimum under appropriate conditions. Glucose, for instance, was a suitable substrate for high Y_{H2} production in this study, while Li et al. [48] showed that crude glycerol is a suitable substrate for high Y_{H2} production.

This study demonstrated that glucose was a better substrate than sucrose and starch. This fact was also supported by Marone et al.[47] in which various substrates produced different Y_{H2} values even at the same temperature. Based on this result, it can be concluded that high IMcR performance is closely related to factors such as substrate type, inoculum, mode of operation, matrix type, and bioreactor design. Therefore, all factors must be controlled at appropriate conditions to reach the optimum IMcR performance.

3.7. Identification of microorganism colonies with SEM

The GF beads of the immobilized mixed culture were obtained at the beginning and 3rd week of the IMcR run (Figure 5). As shown in Figure 7(a), the SEM images showed that the GF beads surface remained clean and poor in microorganism colonies at the beginning of the experiment (before the enrichment process), thereby leading to low biogas production on the 1st week (347 ± 15 mL). The highest biogas production of 1401 ± 16 mL was obtained using 5 g glucose on the 3rd week of the experimental run owing to enriched GF and matrix by the fermentative bacteria colonies. As shown in Figure 5(b), the GF surfaces were covered by cylindrical-shaped colonies.

Table 2. Summary of H_2 production from various substrates by using an immobilized mixed cell reactor (IMcR)

Inoc.	Subs.	pH_{final}	T (°C)	CondF (mScm⁻¹)	CODrem (%)	YH_2 mL g⁻¹$_{CODinit}$	YH_2 mmol mol⁻¹$_{Sub}$	QH_2 (mLL⁻¹d⁻¹)	H_2 Comp (%)	ηIMcR (%)	Sources
AS	Glu.	5.3 ± 0.0	60	10.9 ± 0.0	59.8 ± 4.2	304.0 ± 13.2	2257 ± 98	1403 ± 61	36.6 ± 0.5	23.0 ± 1.0	This study
AS	Sucr.	5.4 ± 0.1	60	10.3 ± 0.0	33.6 ± 2.4	240.6 ± 21.9	3306 ± 245	1081 ± 98	29.5 ± 0.2	16.7 ± 1.5	This study
AS	Star.	5.4 ± 0.1	60	10.2 ± 0.1	24.9 ± 2.9	196.9 ± 6.6	962 ± 32	934 ± 20	23.6 ± 4.1	9.8 ± 0.1**	This study
ADS[a]	Glu	4.1 ± 0.1	60	NA	30.3 ± 1.0	0.9*	37 ± 0	12.0 ± 0.0	6.2	NA	[11]
ADS[b]	CW	5.5	37	7.4	78.5 ± 5.7	95.1	NA	250	13.8	NA	[11,47]
ADS[b]	FJW	4.8	37	5.9	71.8 ± 1.6	57.6	NA	150	12.1	NA	[11,47]
CDC[b]	CS	NA	36	10.9*	44 ± 2	168.2*	NA	1730*	46.3-54.2	NA	[48]
SS[b]	CG	NA	35	12.1	40.6 ± 4.9	124.1	NA	NA	NA	NA	[49]
AMM[b]	CMS	NA	29	NA	NA	0.71*	NA	NA	NA	NA	[50]

Inoc. = Inoculum, AS = Anaerobic sludge of POME, ADS = Anaerobic digested sludge, AMM = Anaerobic mixed-microflora, CDC= Cow dung compost, CG = crude glycerol, CS = corn stalk, CW = Chees whey, FJW = fruit juice wastewater, Glu. = Glucose, NA = not available, SS = Sediment sample, Sta. = Starch, Subs. = Substrate, Sucr. = Sucrose, WAS = waste activated sludge, * = calculated, ** = calculated based on heat combustion of glucose, [a] = immobilized mixed-cell with alginate, [b] = suspended mixed-cell.
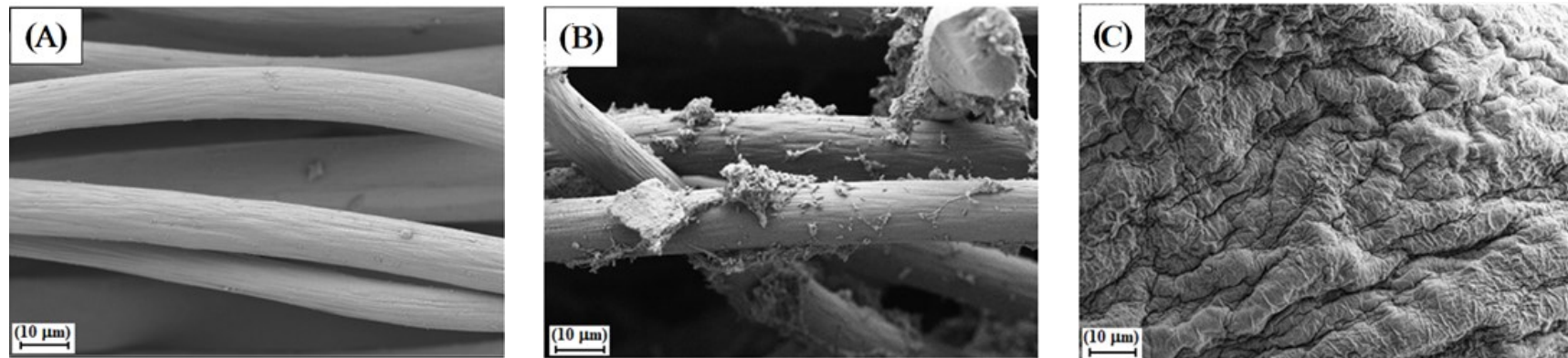

Fig. 5. SEM images of the GF bead surfaces; (a) image of GF before enrichment process (at the start), (b) image of enriched GF after the 3rd week, and (c) image of alginate beads after 1 month of experimental run

As revealed in a previous work, alginate beads were damaged after one month of the experimental run, and the fermentative bacteria colonies were detached from the beads (Figure 5(c)) [12]. This result indicated that alginate could not be applied in the long-term fermentation operation, especially under thermophilic conditions. Hence, the enriched GF can be used to overcome this problem. In this study, IMcR with GF could be performed for more than 2 months. Also, the performance of IMcR with GF was higher than that of IMcR with alginate, as reported in our previous work [12].

The taxonomic composition distribution at the phylum level for immobilized mixed culture on GF bead surfaces is shown in Figure 6. Four major phyla attached to the GF beads consisted of *Firmicutes* (51.12 %), *Chlamydia* (20.13 %), *Bacteroidetes* (18.02 %) and *Proteobacteria* (6.14 %), as listed in Table 3. Three of those four phyla such as *Firmicutes, Bacteroidetes,* and *Proteobacteria* contributed to H_2

production. At the class level, *Firmicutes* was divided into two sub-classes namely *Bacilli* and *Clostridia*. Based on these results, the fermentation of glucose in the bioreactor was dominantly facilitated by these four phyla. Furthermore, the distribution and dominance in class level were found different in which *Clostridia* (47.29 %), *Bacteroidia* (13.00 %), *Flavobacteria* (5.01 %), and *Bacilli* (2.93 %) were involved in the fermentation of glucose to produce H_2, carbon dioxide (CO_2), volatile fatty acids (VFAs) and/or alcohols (i.e. ethanol and butanol) [51]. The different abundance and distribution of phyla are well known, resulting in different compositions of products [52]. Whereas less than 4.00 % of abundance for another phylum consisted of *Actinobacteria, SR1, Spirochaetes,* and *Tenericutes*. These results described that the four genera might have no significant contribution to the generation of main products such as H_2 and VFAs.
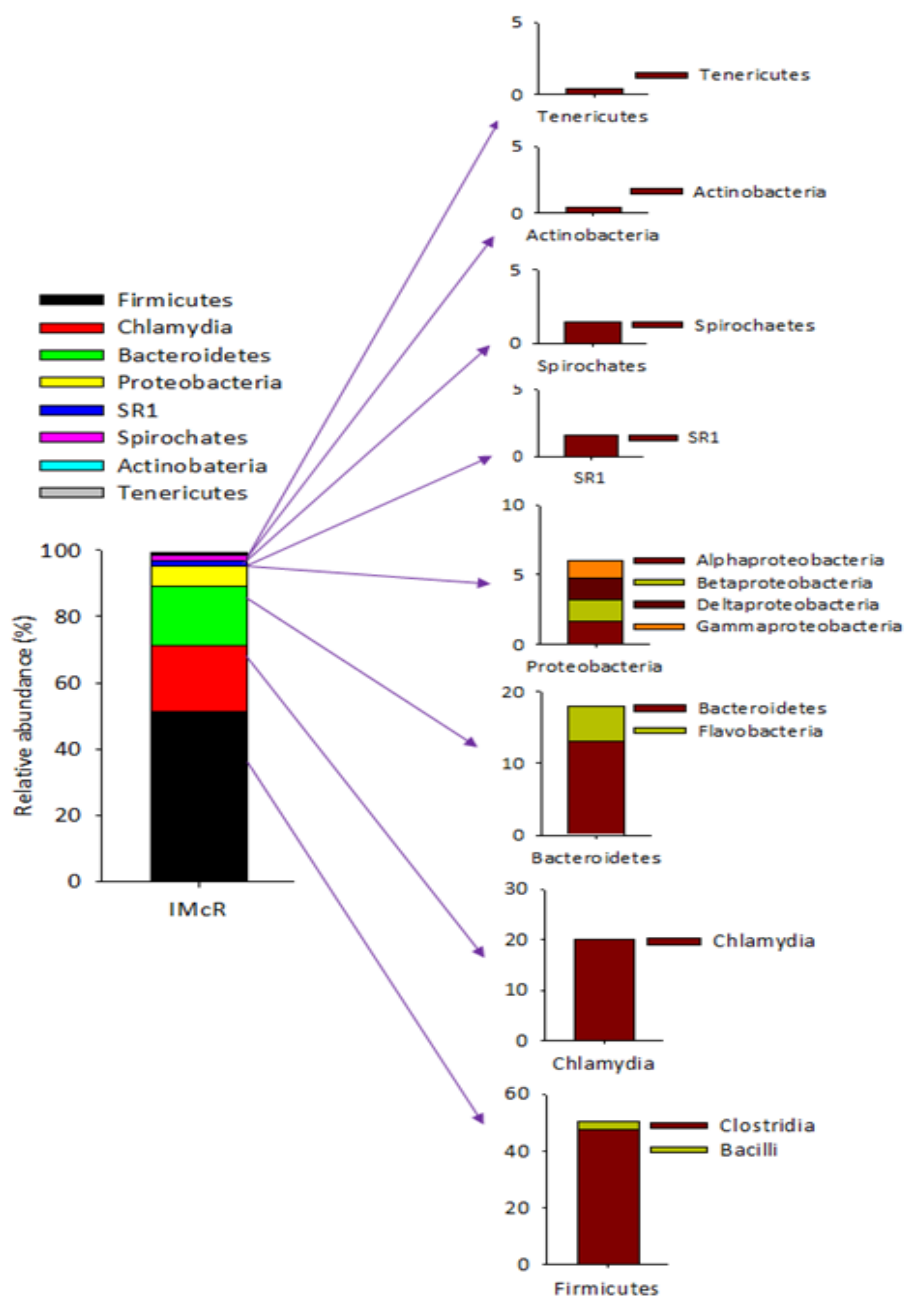

Fig. 6. The histogram of taxonomic composition distribution for biofilm in GF surface at phylum levels

Table 3. Microbial community distribution of biofilm in GF at the phylum, class, order, family, and genus levels

Phylum	%	Class	%	Order	%	Family	%	Genus	%
Firmicutes	51.12	*Bacilli*	2.93	*Lactobacillales*	2.93	*Carnobacteriaceae*	1.48	*Granulicatella*	1.48
						Enterococcaceae	1.45	*Enterococcus*	1.45
						Clostrridiales_[F-1]	2.14	*Clostridiales_[F-1][G-2]*	2.14
		Clostridia	47.29	*Clostridiales*	47.29	*Lachnospiraceae Peptostreptococcaceae*	2.77	*Lachnospiraceae_[XIVa]*	2.77
						Syntrophomonadaceae	3.42	*Peptostreptococcaceae [.]*	3.42
						Unclassified	3.38	*Syntrophomonadaceae [.]*	3.38
							35.58	*Unclassified*	35.58
Chlamydiae	20.13	*Chlamydia*	20.13	*Chlamydiales*	20.13	*Chlamydiaceae*	20.13	*Chlamidia*	20.13
Bacteroidetes	18.02	*Bacteroidia*	13.01	*Bacteroidales*	12.97	*Bacteroidaceae*	6.56	*Bacteroides*	6.52
		Flavobacteria	5.01	*Flavobacteriales*	4.98	*Prevotellaceae*	2.55	*Prevotella*	2.43
Proteobacteria	6.14	*Alphaproteobacteria*	1.62	*Rhizobiales*	1.62				
		Betaproteobacteria	1.60	*Burkholderiales*	1.60	*Commamonadaceae*	1.60	*Commamonas*	1.15
								Leptotrix	0.45
		Deltaproteobacteria	1.52	*Desulfovibrionales*	1.52	*Desulfobulbaceae*	1.42	*Desulfobulbus*	1.42
		Gammaproteobacteria	1.40	*Pseudomonadales*	1.30	*Moraxellaceae*	0.66	*Acinetobacter*	0.66
						Pseudomonadaceae	0.64	*Pseudomonas*	0.64
SR1	1.52	*Saccharibacteria-SR1*	1.52	*Saccharibacteriales*	1.52	*Candidatus Saccharibacteriaceae*	1.52	*Candidatus Saccharibacter*	1.52
TM7	1.44	*Saccharibacteria-TM7*	1.44	*TM7-1*	1.44	*Unclassified*	1.44	*Unclassified*	1.44
Chloroflexi	0.42	*Unclassified*	0.42	*Unclassified*	0.49	*Unclassified*			
Tenericutes	0.40								
Others	0.72								

4. Conclusion

The solid matrix of GF beads has been successfully used as an alternative material to immobilize the mixed culture in IMcR. The IMcR with GF generated high H_2 from glucose under thermophilic conditions. The maximum Y_{H2} value was 304.0 mL $g^{-1}_{CODinitial}$ (corresponding to 2257 mol $mol^{-1}_{glucose}$) from glucose. The Y_{H2} from IMcR with GF was more than 38-fold higher than that of IMcR with alginate. The maximum Q obtained from the IMcR with the GF system was 1403 mL $L^{-1} \cdot day^{-1}$ of H_2. Hence, IMcR with GF is a new approach to enhance H_2 production during fermentation that can be operated for more than 2 months without any significant changes in efficiency, Y_{H2}, and Q. This approach can also be applied to produce H_2 from agroindustrial wastewater.

Acknowledgements

References

1. W. Li, C. Cheng, G. Cao, and N. Ren, *Enhanced biohydrogen production from sugarcane molasses by adding Ginkgo biloba leaves*, Bioresour. Technol. 298 (2020) 122523.

2. T. Emanuele, M. Raul, V. Wouter, W. Thomas, L. Jean-Christophe, and B. Frederic, *Hydrogen from Renewable Power: Technology Outlook for the Energy Transition*. IRENA. (2018) 1–52.

3. D. D. T. Ferraren-De Cagalitan and M. L. S. Abundo, *A review of biohydrogen production technology for application towards hydrogen fuel cells*, Renew. Sust. Ener. Rev. 151 (2021) 111413.

4. B. P. Raj, C. S. Meena, N. Agarwal, L. Saini, S. H. Khahro, U. Subramaniam et al, *A review on numerical approach to achieve building energy efficiency for energy, economy and environment (3E) benefit*, Energies. 14(15) (2021) 1–26.

5. A. Pareek, R. Dom, J. Gupta, J. Chandran, V. Adepu, and P. H. Borse, *Insights into renewable hydrogen energy: Recent advances and prospects*, Mater. Sci. Energy Technol. 3 (2020) 319–327.

6. T. Chookaew, S. O-Thong, and P. Prasertsan, *Biohydrogen production from crude glycerol by immobilized Klebsiella sp. TR17 in a UASB reactor and bacterial quantification under non-sterile conditions*, Int. J. Hydrog. Energy. 39(18) (2014) 9580–9587.

7. P. Sivagurunathan, G. Kumar, and C. Y. Lin, *Hydrogen and ethanol fermentation of various carbon sources by immobilized Escherichia coli (XL1-Blue)*, Int. J. Hydrog. Energy. 39(13) (2014) 6881–6888.

8. P. Khongkliang, A. Jehlee, P. Kongjan, A. Reungsang, and S. O-Thong, *High efficient biohydrogen production from palm oil mill effluent by two-stage dark fermentation and microbial electrolysis under thermophilic condition*, Int. J. Hydrog. Energy. 44(60) (2019) 31841–31852.

9. N. Akhlaghi and G. Najafpour-Darzi, *A comprehensive review on biological hydrogen production*, Int. J. Hydrog. Energy. 45(43) (2020) 22492–22512.

10. M. Samer, *Biological and Chemical Wastewater Treatment Processes*, in Wastewater Treatment Engineering, Intechopen. (2015) 1–50.

11. I. Satar, M. Ghasemi, S. A. Aljlil, W. N. R. W. Isahak, A. M. Abdalla, J. Alam et al., *Production of hydrogen by Enterobacter aerogenes in an immobilized cell reactor*, Int. J. Hydrog. Energy. 42(14) (2017) 9024–9030.

12. I. Satar, W. R. W. Daud, B. H. Kim, M. R. Somalu, and M. Ghasemi, *Immobilized mixed-culture reactor (IMcR) for hydrogen and methane production from glucose*, Energy. 139 (2017) 1188–1196.

13. Y. K. Oh, S. H. Kim, M. S. Kim, and S. Park, *Thermophilic biohydrogen production from glucose with trickling biofilter*, Biotechnol. Bioeng. 88(6) (2004) 690–698.

14. D. M. Fontes Lima, W. K. Moreira, and M. Zaiat, *Comparison of the use of sucrose and glucose as a substrate for hydrogen production in an upflow anaerobic fixed-bed reactor*, Int. J. Hydrog. Energy. 38(35) (2013) 15074–15083.

15. S. K. Bhatia, S. S. Jagtap, A. A. Bedekar, R. K. Bhatia, K. Rajendran, A. Pugazhendhi et al., *Renewable biohydrogen production from lignocellulosic biomass using fermentation and integration of systems with other energy generation technologies*, Sci. Total Environ. 765 (2021) 144429.

16. J. Thipraksa, P. Michu, A. Kongthong, and P. Chaijak, *Exploring the impact of co-fermentation Saccharomyces cerevisiae and Lactobacillus sp. on stingless bee-honey cider fermentation*, Commun. Sci. Technol. 8(1) (2023) 93–99.

17. R. Moreno, A. Escapa, J. Cara, B. Carracedo, and X. Gómez, *A two-stage process for hydrogen production from cheese whey: Integration of dark fermentation and biocatalyzed electrolysis*, Int. J. Hydrog. Energy. 40(1) (2015) 168–175.

18. F. Vendruscolo, *Starch: A potential substrate for biohydrogen production*, Int. J. Energy Res. 39(3) (2015) 293–302.

19. T. C. Chaves, G. N. S. B. Gois, F. S. Peiter, D. V. Vich, and E. L. C. de Amorim, *Biohydrogen production in an AFBR using sugarcane molasses*, Bioprocess Biosyst. Eng. 44 (2021) 307–316.

20. M. Quéméneur, J. Hamelin, A. Barakat, J. P. Steyer, H. Carrre, and E. Trably, *Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures*, Int. J. Hydrog. Energy. 37(4) (2012) 3150–3159.

21. A. K. M. K. Islam, P. S. M. Dunlop, N. J. Hewitt, R. Lenihan, and C. Brandoni, *Bio-Hydrogen Production from Wastewater: A Comparative Study of Low Energy Intensive Production Processes*, Clean Technol. 3(1) (2021) 156–182.

22. Preethi, T. M. M. Usman, J. Rajesh Banu, M. Gunasekaran, and G. Kumar, *Biohydrogen production from industrial wastewater: An overview*, Bioresour, Technol. Rep. 7 (2019) 100287.

23. P. Dessì, E. Porca, N. R. Waters, A. M. Lakaniemi, G. Collins, and P. N. L. Lens, *Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: Biohydrogen production and active microbial community*, Int. J. Hydrog. Energy. 43(11) (2018) 5473–5485.

24. K. Ainiyah, F. Andriyani, S. Soemargono, and N. K. Erliyanti, *Isolation of clove essential oil by fermentation process*, Konversi. 10(1) (2021) 58-64.

25. M. Youssef, E. H. El-Shatoury, S. S. Ali, and G. E. El-Taweel, *Enhancement of phenol degradation by free and immobilized mixed culture of Providencia stuartii PL4 and Pseudomonas aeruginosa PDM isolated from activated sludge*, Bioremediat. J. 23(2) (2019) 53–71.

26. H. Zhang, G. Chen, Q. Zhang, D. J. Lee, Z. Zhang, Y. Li et al., *Photosynthetic hydrogen production by alginate immobilized bacterial consortium*, Bioresour. Technol. 236 (2017) 44–48.

27. Y. A. Bustos-Terrones, E. R. Bandala, G. E. Moeller-Chávez, and V. Bustos-Terrones, *Enhanced biological wastewater treatment using sodium alginate-immobilized microorganisms in a fluidized bed reactor*, Water Sci. Eng. 15(2) (2022) 125–133.

28. H. Gao, E. Khera, J. K. Lee, and F. Wen, *Immobilization of multi-*

biocatalysts in alginate beads for cofactor regeneration and improved reusability, J. Vis. Exp. 110 (2016) e53944.

29. O. García-Depraect, R. Lebrero, S. Rodriguez-Vega, R. A. Börner, T. Börner, and R. Muñoz, *Production of volatile fatty acids (VFAs) from five commercial bioplastics via acidogenic fermentation,* Bioresour. Technol. 360 (2022) 127655.

30. J. H. Kim, E. Jeong, and Y. S. Lee, *Preparation and characterization of graphite foams,* J. Ind. Eng. Chem. 32 (2015) 21–33.

31. S. Cheng and B. E. Logan, *High hydrogen production rate of microbial electrolysis cell (MEC) with reduced electrode spacing,* Bioresour. Technol. 102(3) (2011) 3571–3574.

32. A. W. Jeremiasse, H. V. M. Hamelers, M. Saakes, and C. J. N. Buisman, *Ni foam cathode enables high volumetric H_2 production in a microbial electrolysis cell,* Int. J. Hydrog. Energy. 35(23) (2010) 12716–12723.

33. E. Griškonis, A. Ilginis, I. Jonuškiene, L. Raslavičius, R. Jonynas, and K. Kantminiene, *Enhanced performance of microbial fuel cells with anodes from ethylenediamine and phenylenediamine modified graphite felt,* Processes 8(8) (2020) 1–15.

34. P. Pérez-Rodríguez, V. M. Ovando-Medina, S. Y. Martínez-Amador, and J. A. Rodríguez-de la Garza, *Bioanode of polyurethane/graphite/polypyrrole composite in microbial fuel cells,* Biotechnol. Bioprocess Eng. 21 (2016) 305–313.

35. G. Antonopoulou, I. Ntaikou, K. Stamatelatou, and G. Lyberatos, *Biological and fermentative production of hydrogen,"* in *Handbook of Biofuels Production:* Processes and Technologies. Elsevier Inc. (2011) 305–346.

36. C. Li and H. H. P. Fang, *Fermentative hydrogen production from wastewater and solid wastes by mixed cultures,* Crit. Rev. Environ. Sci. Technol. 37(1) (2007) 1–39.

37. P. Mishra and D. Das, *Biohydrogen production from Enterobacter cloacae IIT-BT 08 using distillery effluent,* Int. J. Hydrog. Energy. 39(14) (2014) 7496–7507.

38. T. Jafary, W. R. W. Daud, S. A. Aljlil, A. F. Ismail, M. Al-Mamun, M. S. Baawain et al., *Simultaneous organics, sulphate and salt removal in a microbial desalination cell with an insight into microbial communities,* Desalination. 445 (2018) 204–212.

39. D. B. Levin, L. Pitt, and M. Love, *Biohydrogen production: Prospects and limitations to practical application,* Int. J. Hydrog. Energy. 29(2) (2004) 173–185.

40. H. Yokoi, R. Maki, J. Hirose, and S. Hayashi, *Microbial production of hydrogen from starch-manufacturing wastes,* Biomass Bioenergy. 22(5)

(2002) 389–395.

41. J. Wang and W. Wan, *The effect of substrate concentration on biohydrogen production by using kinetic models,* Sci. China B. Chem. 51 (2008) 1110–1117.

42. Y. Goyal, M. Kumar, and K. Gayen, *Metabolic engineering for enhanced hydrogen production: A review,* Can. J. Microbiol. 59(2) (2013) 59–78.

43. S. K. Khanal, W. H. Chen, L. Li, and S. Sung, *Biological hydrogen production: Effects of pH and intermediate products,* Int. J. Hydrog. Energy. 29(11) (2004) 1123–1131.

44. J. Wang and W. Wan, *Factors influencing fermentative hydrogen production: A review,* Int. J. Hydrog. Energy. 34(2) (2009) 799–811.

45. L. Lu and Z. J. Ren, *Microbial electrolysis cells for waste biorefinery: A state of the art review,* Bioresour. Technol. 215 (2016) 254–264.

46. A. J. Lewis, S. Ren, X. Ye, P. Kim, N. Labbe, and A. P. Borole, *Hydrogen production from switchgrass via an integrated pyrolysis-microbial electrolysis process,* Bioresour. Technol. 195 (2015) 231–241.

47. H. H. P. Fang and H. Liu, *Effect of pH on hydrogen production from glucose by a mixed culture,* Bioresour. Technol. 82(1) (2002) 87–93.

48. A. Marone, O. R. Ayala-Campos, E. Trably, A. Carmona Martinez, R. Moscoviz, E. Latrille et al., *Coupling dark fermentation and microbial electrolysis to enhance bio-hydrogen production from agro-industrial wastewaters and by-products in a bio-refinery framework,* Int. J. Hydrog. Energy. 42(3) (2017) 1609–1621.

49. X. H. Li, D. W. Liang, Y. X. Bai, Y. T. Fan, and H. W. Hou, *Enhanced H_2 production from corn stalk by integrating dark fermentation and single chamber microbial electrolysis cells with double anode arrangement,* Int. J. Hydrog. Energy. 39(17) (2014) 8977–8982.

50. T. Chookaew, P. Prasertsan, and Z. J. Ren, *Two-stage conversion of crude glycerol to energy using dark fermentation linked with microbial fuel cell or microbial electrolysis cell,* New Biotechnol. 31(2) (2014) 179–184.

51. S. Venkata Mohan, V. Lalit Babu, and P. N. Sarma, *Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate,* Bioresour. Technol. 99(1) (2008) 59–67.

52. V. Müller, *Bacterial Fermentation,* Encyclopedia of Life Sciences. (2002) 1–7.

53. K. Kampmann, S. Ratering, I. Kramer, M. Schmidt, W. Zerr, and S. Schnell, *Unexpected stability of Bacteroidetes and Firmicutes communities in laboratory biogas reactors fed with different defined substrates,* Appl. Environ. Microbiol. 78(7) (2012) 2106–2119.