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# BIOHYDROGEN PRODUCTION IN SEMI-CONTINUOUS SYSTEM USING IMMOBILIZED CELL MEMBRANE

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***ABSTRACT:*** Hydrogen is considered to be the fuel of the future because of its high energy content (122 kJ/g), and water is the only byproduct of its use. Moreover, the production of hydrogen via fermentation of organic wastes is carbon neutral. This study was conducted to evaluate the performance of immobilized cells on PVDF membrane for biohydrogen production using a sequencing batch reactor by varying the hydraulic retention times (HRT) of the system and to compare the efficiency between suspended and attached systems on the production of biohydrogen. It was found that the biohydrogen fermentation performance was improved in a semi-continuous system, especially with immobilized cells. The optimum HRT that supports the highest biohydrogen yield was for an HRT of 12 hours, where the performance of hydrogen production was improved and in which the maximum hydrogen yield was achieved at 2.43 mol $H_2$/mol and maximum hydrogen production rate (HPR) of 2.46 L $H_2$/L.d as compared to other HRT for both systems. Therefore, the result of this study can be applied as the benchmark for scaling up the process.

***ABSTRAK:*** Hidrogen boleh dianggap sebagai sumber tenaga penting pada masa hadapan kerana kapasiti tenaga yang tinggi (122 kJ/g) dan hanya air terhasil dari tindak balas hidrogen. Tambahan, sisa pengeluaran hidrogen melalui proses fermentasi sisa organik adalah bersifat semula jadi. Kajian ini dijalankan bagi mengkaji prestasi sel tidak bergerak pada membran PVDF bagi penghasilan biohidrogen menggunakan reaktor kelompok turutan dengan mengubah sistem masa pengekalan hidraulik (HRT) dan dengan membuat perbandingan kecekapan antara sistem yang tergantung dan sistem yang bersambung pada penghasilan biohidrogen. Dapatan kajian mendapati prestasi fermentasi diperbaharui di bawah sistem separa turutan terutama dengan sel tidak bergerak. Nilai optimum HRT yang mempunyai hasil biohidrogen tertinggi adalah pada ketika HRT 12 jam di mana prestasi penghasilan hidrogen dapat diperbaharui dan menghasilkan hidrogen tertinggi pada 2.43 mol H2/mol dan kadar penghasilan hidrogen maksimum (HPR) pada 2.46 L H2/L.d berbanding sistem HRT lain pada kedua-dua sistem. Oleh itu, dapatan kajian ini boleh digunakan sebagai penanda aras bagi kenaikan proses.

***KEYWORDS:*** *biohydrogen, immobilized cell membrane, semi-continuous system*

# 1. INTRODUCTION

Hydrogen is considered the future's alternative fuel to replace fossil fuels. It offers a lot of possibilities to generate a valuable renewable energy carrier that is cleaner and has high-energy content. Biological hydrogen production is more favorable as compared to conventional physico-chemical methods of hydrogen production such as steam reforming. This is due to its potential as a carbon-neutral and cost-effective fuel that is inexhaustible and versatile, as it can use various types of carbon sources for biological hydrogen production [1-3]. Besides, biological hydrogen production is more feasible for practical application, which includes the integration with fuel cell technologies, since it can produce high rates of hydrogen at lower capital cost [4, 5].

Biological hydrogen production can be categorized into four processes: photo fermentation, dark fermentation, bio-photolysis, and sequential fermentation (an integrated process between dark and photo fermentation) commonly used in hydrogen generation [6]. However, dark or anaerobic fermentation is more favorable as it is much simpler and inexpensive, which makes it suitable for small-scale production facilities [7, 8]. It has been proven that both pure cultures and mixed cultures can be used for dark fermentation, and they are able to utilize a wide range of carbon sources, including waste [9, 10].

Despite its great potential, biological hydrogen production still cannot compete with the thermochemical processes of generating hydrogen as the latter process is more stable and reliable to support the growing demand for energy [11]. Thus, current research on biological hydrogen production, particularly anaerobic processes, has focused on improving the hydrogen conversion and volumetric production rates. One of the attractive ways to achieve a stable and high conversion rate is through the immobilization processes of hydrogen-producing bacteria. A previous study found that immobilized cells are able to increase hydrogen production by up to 20% compared to free cells or suspended cells [13]. Similarly, the stability of hydrogen production with higher hydrogen production was also proven with the increasing value between 15 to 20% when microbial cells were immobilized on the support material compared to suspended cells [3,13,14]. This is because immobilization helps retain the bacteria inside the reactor and prevents it from washing out, especially in a continuous system as compared to a suspended cell system [13,15,24].

A continuous system is preferable to a batch system since it generates a continuous and stable production of hydrogen gas and, thus, improves productivity over batch systems [15,17]. Basically, there are two types of reactors that are commonly used for continuous and semi-continuous hydrogen production. The first type is the suspended system, where the bacteria is suspended and might form bacterial flocks in the bottom of the reactor. Mixing is needed to achieve a homogenous system and ensure that the substrate is well-distributed throughout the reactor. The second type is attached systems, in which the bacteria grow on the surface of support material and form biofilm. The bacteria will remain retained in the system during the effluent's flushing-out process and thus maintain the active biomass inside the reactor.

Various modes of operation have been used for fermentative hydrogen production, including batch, semi-continuous, and continuous systems. It is known that batch hydrogen fermentation will produce lower hydrogen production rates than continuous or semi-continuous modes. Semi-continuous mode, with proper dilution rates, can enhance the fermentation rate. Valdez-Vazquez et al. [18] found that when the dilution rate was controlled during the fermentation of municipal solid waste, the degradation of non-degradable waste improved, and in the meantime, biogas production increased. Lin and Chou [17] found that a sequencing batch reactor run at a hydraulic retention time (HRT) of 8 hours with the organic loading rate (OLR) of 80 kg COD/l was able to produce 2.8 mol of hydrogen (39 mmol H2/ kg COD/d). They concluded that the hydrogenic activity of mixed bacteria was HRT dependent, with the lower HRT having the effect of deteriorating hydrogen productivity. In addition, control of pH was important to ensure a stable operation of the reactor, as pH can be an indicator for monitoring bacterial activity. The advantages of sequencing batch reactors include greater biomass retention (hence the ability to decouple solids retention time (SRT) from HRT), a higher degree of process flexibility with respect to changes in organic loading rate (OLR), relative ease of operation and lower capital investment. The semi-continuous mode operation of this process is also considered more feasible for potential real applications and commercialization [15, 20].

Nowadays, most fermentative hydrogen production is conducted continuously. Continuous stirred tank reactors (CSTR) are widely used for fermentative hydrogen production. Bacteria inside the CSTR accumulate inside the reactor to form a suspension and reduce the mass transfer resistance as compared to the batch system. However, HRT still plays a crucial factor in CSTR operations. Since higher OLR will reduce the efficiency of the fermentation process inside the CSTR, suitable HRT needs to be determined, or biomass washout will deteriorate the hydrogen production rates [18-21].

Later, in order to achieve higher cell density retained inside the reactor, an immobilized system was developed either through entrapment [23,24] or attachment [13,24]. The studies found that cells immobilized were able to increase the hydrogen production rate with decreasing HRT, which would not be achievable under the suspended CSTR system. Cell immobilization on support material improved mass transfer efficiency and cell stability. A longer operation time will allow the formation of granules, where the bacteria formed a dense flock that settled in the bottom of the reactor, reducing the cell washout problem while improving the substrate conversion to hydrogen [25]. The operational conditions, such as pH and HRT, still play an important role in the process, especially when the reactor needs to be maintained for a long time in order to achieve process stability, which can be a major economic barrier in terms of maintenance [26,27].

Even though the continuous system promotes a better hydrogen production process due to process stability, high organic content, and ability to run at shorter HRT, the performance of each mode of operation is still dependent on other factors such as type of substrate used, type of reactor, and operational condition selected. However, in order to develop a new fermentation process, such as the integrated fermentation process, it is found that fermentative hydrogen production via batch system, particularly by sequencing batch mode, offered some advantages, such as better control of the microbial population due to the cyclic operation [28, 29]. Thus, the objective of this study was to evaluate the potential use of immobilized cells on PVDF membrane for energy production using a sequencing batch reactor. The influence of hydraulic retention times (HRT) (36, 24, and 12 h) and the comparison between suspended and attached systems on the production of hydrogen were especially investigated.

# 2. METHODOLOGY

## 2.1 Inoculum Preparation

The immobilized membrane with bacterial cells attached to it was developed with some modifications from Engliman et al. [5]. The membrane underwent the acclimatization step using an HRT of 2 days via repeated batch cultivation in a 500ml modified bottle reactor. Nitrogen gas was purged periodically to maintain the anaerobic condition inside the reactor.

The pH of the fermentation medium was only set initially at pH 5.5, and the seed was cultivated at 55°C inside the shaking water bath at 150 rpm. The microbial cell was acclimatized under sequencing batch mode, and the experiments were run at an HRT of 2 days with a 500 ml working volume. Sequencing batch operation was carried out by the removal of 50% of the culture medium and replacement with a new medium after each cycle. The process cycle was continued until the hydrogen production rate achieved consistency. Samples of fermentation products were taken during each cycle and sent for analysis. The seed was acclimatized until a stable and consistent hydrogen production was achieved before being applied to the semi-continuous reactor.

### 2.2 Fermentation Medium

The synthetic media was used as a fermentation medium in this study. The growth medium used in this study consisted of glucose (10 g/l) as the main carbon source and was supplemented with other nutrients such as yeast extract (3 g/l), peptone (5 g/l), sodium chloride (1 g/l), sodium acetate (1 g/l) and Wolfe's mineral solution (1ml/l). This growth medium will be used throughout the study of the effect of HRT in sequential batch systems.

### 2.3 Experimental Set-Up for Semi-Continuous Fermentation Systems

Fig. 1 shows the schematic diagram of the semi-continuous system used in this study. The fermentation experiments were carried out using 400ml synthetic medium as a feedstock, with 15% inoculum seed added into a 500ml modified Scott Duran bottle. The temperature was controlled at 60ºC using a shaking water bath. The pH was only maintained initially at pH 5.5 using 1M NaOH and 1M HCl. The total volume of gas collected was measured by using a gas meter (MiliGascounter, Ritter, Germany). Another tube was connected to the cylinder for biogas sampling and gas composition analysis.

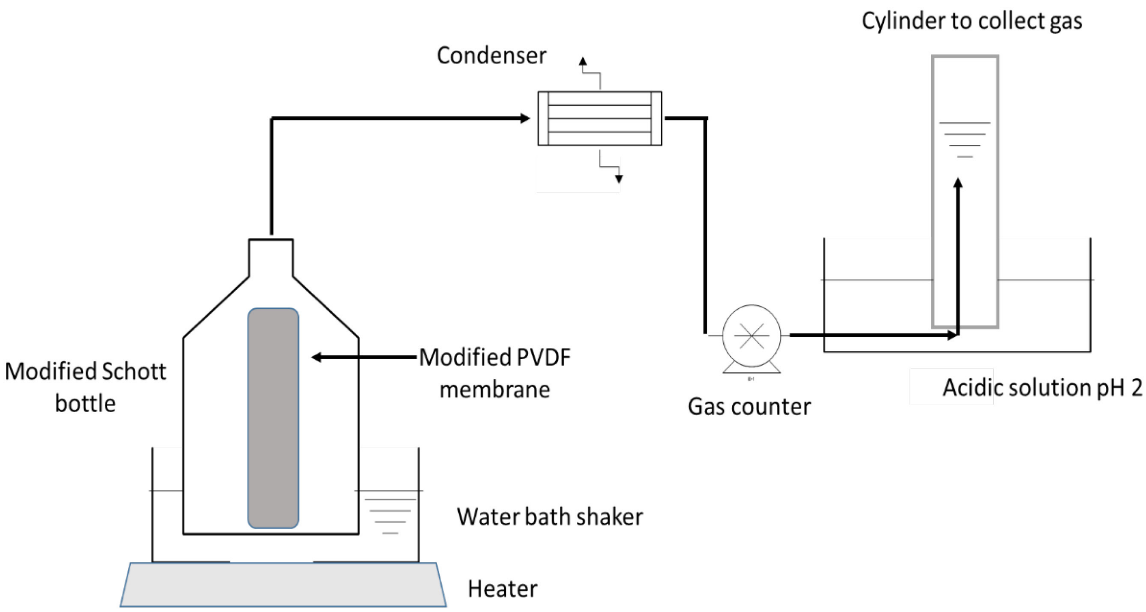


Fig. 1. Schematic diagram of the experimental set-up for this study

### 2.4 Experimental Operation

The experiments were operated in sequential batch mode using HRT of 72, 36, and 24 hours, which corresponded with the feed flow rate of 167, 334, and 500 ml per day, respectively. The influent feed consisted of 10 g/l glucose that was purged with nitrogen gasses for 15 minutes to ensure an anaerobic condition inside the reactor. The feeding cycle was calculated using Eq. (1) according to the respective HRT (Table 1). The analyses were conducted in triplicate to ensure the consistency of the experiment.

$$Feeding\ Cycle\ \left(\frac{cycle}{day}\right) = \frac{24\ hour\ per\ day \times 1\ cycle}{HRT\ (h)} \tag{1}$$

Table 1. Operational parameters involved in this study

| HRT (hr) | Feeding cycle (cycle/day) |
|---|---|
| 72 | 3 |
| 36 | 2 |
| 24 | 1 |
| 12 | 0.5 |

### 2.5 Hydrogen Concentration Analysis

The percentage of hydrogen and carbon dioxide present in the evolved gas was determined using gas chromatography. The gas chromatograph (model SRI 8600C, USA) consisted of two detectors: a helium ionization detector (HID) and a thermal conductivity detector (TCD). High purity helium gas (99.99%) was used as the carrier gas at 25 mL/min. A volume of 1 mL of the gas samples was taken using a 1-mL gas-tight syringe, and the samples were injected into the GC immediately at a temperature of 43ºC and a pressure of 2.7 psi initially, then followed by a ramping of 30ºC per minute, which was maintained for 10 min once the temperature reached 220ºC [5].

### 2.6 Energy Analysis

The hydrogen energy production rate ($EPR$), KJ/L/d was calculated using Eq. (2) [30].

$$EPR = \frac{HPR}{22.4} \times HVH2 \tag{2}$$

where $HPR$ is the hydrogen production rate (L/L/d) calculated from the experimental result, and $HVH2$ is the heating value of hydrogen (286 J/mmol).

## 3. RESULTS AND DISCUSSION

### 3.1 Effect of HRT on Immobilized Membranes Compared to Suspended Systems

The modified bottle reactors with a working volume 500ml were operated via sequential batch mode for fermentative hydrogen production. The effects of hydraulic retention time (HRT) on biogas production rate, biogas composition, and soluble metabolites produced were evaluated throughout the fermentation process. Four different HRTs, including 72, 36, 24, and 12 hours, were evaluated in order to determine the stability of the continuous process using the designated system. The experiment was carried out at 60ºC with the initial pH of the fermentation medium being set at pH 5.5 and a sugar concentration of 10g/l. The experiment was started with acclimatization of the seed before changing the HRT value. The HRT was changed once the daily biogas and biohydrogen production reached a pseudo-steady state. The feeding strategy used for this purpose is shown in Table 1.

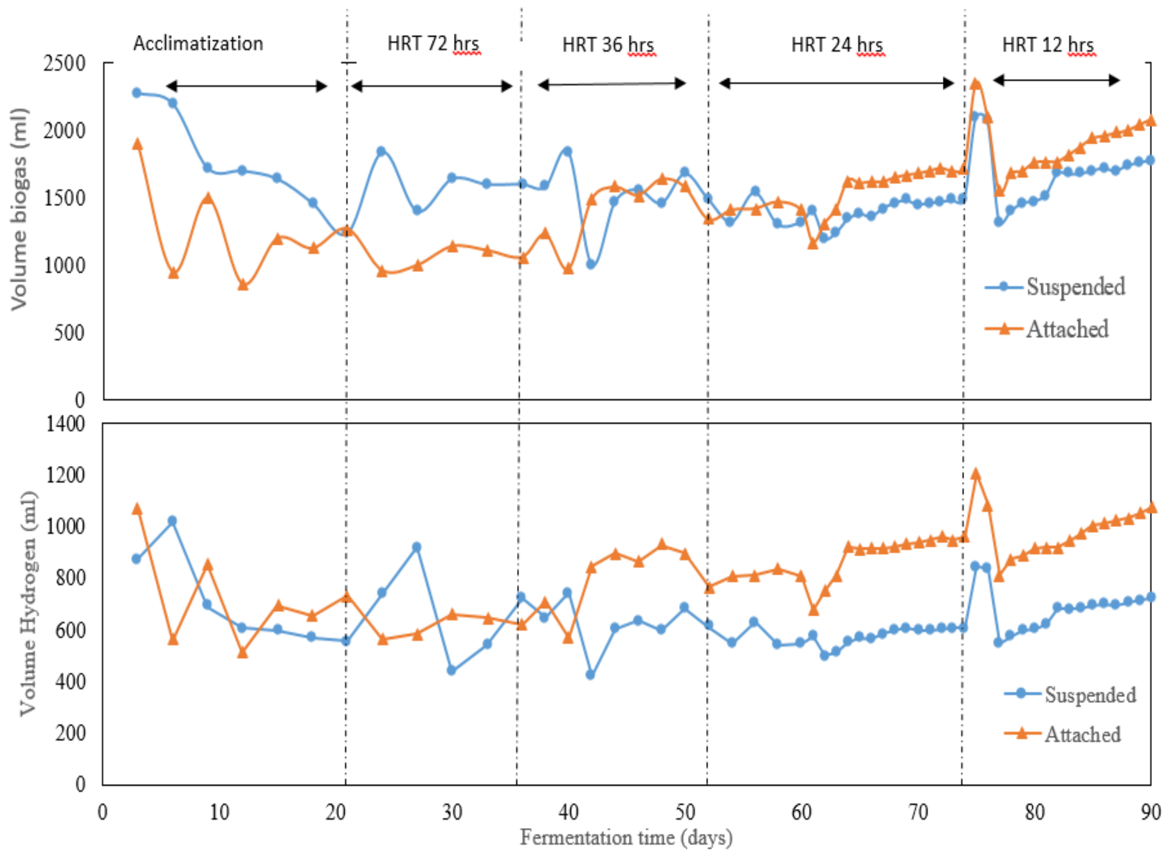


Fig. 2. Biogas and hydrogen production of two different systems when different HRTs were used (HRT of 72, 36, 24, and 12 hours, respectively)

Fig. 2 shows the biogas and hydrogen produced for two different systems: the suspended system, where bacteria was left to be suspended inside the reactor, and the attached system, in which the bacteria was immobilized on the PVDF membrane. It was found that during the early stage of each HRT value, the biogas and biohydrogen produced fluctuated due to the shift in dominant bacteria governing the fermentation process. After an average of three feeding cycles, the biogas and biohydrogen production gradually reached a quasi-steady state with negligible difference from previous cycles. The maximum hydrogen produced was found during the HRT of 12 hours, which was about 1035 ± 26 ml of hydrogen produced daily with the composition of 48% of hydrogen for the attached system. For the suspended system, the HRT of 12 hours showed better hydrogen production than other HRT, with the average daily production during HRT 12 hours being 706 ± 13 ml of hydrogen (average hydrogen composition was 42%). The trend shows that as the HRT became shorter, the hydrogen produced increased up to 10% from the previous HRT. However, the concentration of hydrogen produced in the biogas was not affected significantly by the changes in HRT, still reaching quite high values in a range of 37 – 48% when HRT was decreased from 72 hours to 12 hours (Table 2).

The performance of both systems was evaluated based on hydrogen production rate (HPR) and hydrogen yield (HY). The results showed that the were no significant differences in HPR during HRT 72 and 36 hours, but the HPR started to improve during the middle of HRT 24 hours and kept increasing at HRT 12 hours (Fig. 3). A similar trend was observed from both systems, but attached system shows better HPR during HRT 12 hours, which is about 2.68 L of H2/L of influent/day. However, the hydrogen yield (HY) for all HRT showed little difference between the suspended and attached systems, which might be due to the reduction of sugar utilization by the bacteria. The highest hydrogen yields for both systems were achieved during late HRT 12 hours, which were 1.41 mol H2/mol glucose for the suspended and 2.43 mol H2/mol glucose for the attached system. Thus, the attached system with bacteria immobilized on the surface of the PVDF membrane showed better performance during the fermentative hydrogen production under thermophilic conditions. The designated immobilization system proved that it can be used to improve hydrogen production in continuous systems.

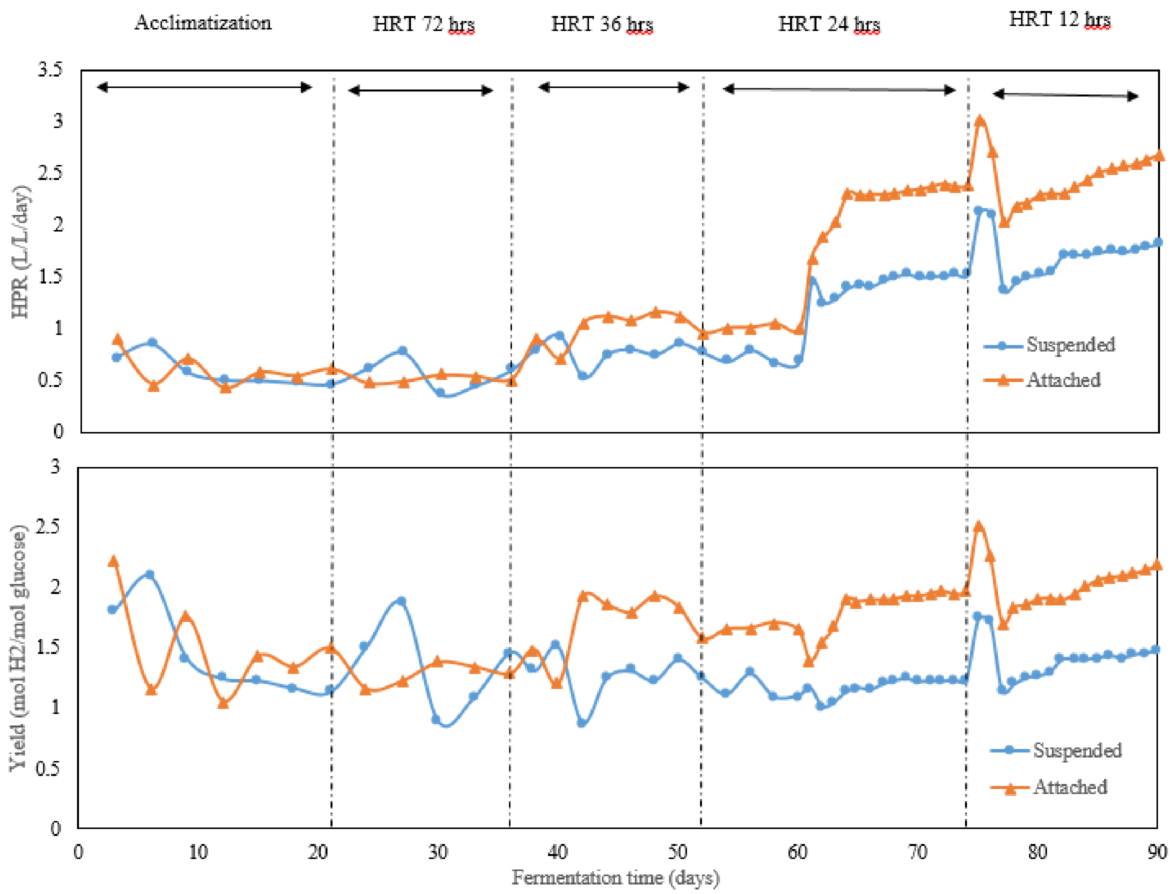


Fig. 3. Performance of suspended and attached systems under different HRT values.

Even though the immobilization system might have a limitation in terms of mass transfer within the system, this limitation can be overcome by modification of support materials used for immobilization systems. The modification of support materials can contribute to improved surface roughness, porosity, and hydrophobicity of the support materials [14]. In this study, the PVDF membrane used had already undergone surface modification that improved its performance to promote better cell immobilization as well as biofilm formation [5,13]. Biofilm formation produced along a cell-surface/interface attachment, known as extracellular polymeric substances (EPS), was responsible for keeping cells together in a three-dimensional structure. EPS mediates in mass transfer through biofilm adsorption of xenobiotics, metals, or inorganic ions and provides physical support for the formation of biofilm [21,23]. The biofilm helped sustain cell viability which would prevent the cells being washed out from the system, thereby enhancing cell density as well as encouraging a consistent biohydrogen production in the system. Similar observations were also obtained from previous studies done by Lutpi et al. [35] and Jamali et al. [24] using granulated activated carbon (GAC) as support materials for cell immobilization to improve hydrogen production in which the highest hydrogen yield of 5.6 mol H2/ mol substrate and maximum hydrogen production rate of 4.3 mmol H2/l.h and 1.77 mol H2/mol substrate consumed and hydrogen production rate (HPR) of 2.0 mmol H2/l.h were achieved via cells immobilization system respectively.

### 3.2 Effect of HRT on pH and Sugar Consumption

Later, the effects of HRT on pH and sugar utilization were observed. As summarized in Tables 2a and 2b, it was found that the final pH for all HRT in both systems was in a range of 4.86 to 5.07. Surprisingly, the lowest final pH resulted in the highest hydrogen production rate for the system. This can be seen during the HRT 12 hours for both systems, the final pH dropped to pH 4.9 for the suspended system, but the HPR increased up to 1.71 liters of hydrogen per liter influent per day. Similarly, for the attached system, where the pH dropped to 4.86, but the HPR value improved to 2.46 liters of hydrogen per liter of influent per day. A previous study by van Ginkel et al. [26] also observed similar results and reported that the maximum hydrogen production was achieved as long as the pH depletion did not occur drastically so as to inhibit the whole process.

The results also showed that at the decreased HRT of as low as 12 hours, the accumulation of total volatile fatty acids was increased, and the pH value was dropped. This trend was similar for both systems, and this is probably due to the fermentation process having already reached an unstable state. The depletion of pH value is the biggest indicator for the instability of the process. Furthermore, the sudden pH drop will cause a slow reactor recovery rate and may lead to system failure [27]. However, since the initial pH selected for the process was the optimum value to support the growth of the bacteria, the bacteria were able to adapt to the environmental changes slowly, and thus, no rapid acid by-product was produced. A rapid drop in fermentation pH indicates that the hydrogen was produced rapidly, causing rapid production of acid by-products that increased to inhibitory levels and, thus, spontaneously depleted the buffering capacity [26,27].

On the other hand, the decrease in HRT value affected the sugar uptake by the microbes. The initial glucose concentration was fixed at 10 g/l, and it was found that as the HRT decreased, the substrate conversion increased, but this trend was applicable only for the attached system, whereas for the suspended system, the substrate conversion decreased when the HRT decreased from 72 hours to 12 hours. This situation happened due to the stability and adaptation of the bacterial community inside the system. Since the bacteria was already stable inside the system, it was able to adapt and maintain itself against any changes in hydraulic dilution due to the decreased HRT. However, to conclude this statement, it is better to relate the event with the hydrogen yield and final biomass concentration [28,29]. For the suspended system, hydrogen yield decreased with decreasing HRT, like the biomass concentration (VSS, g/l) (Table 2a), which might be because of the rapid loss of bacterial cells due to dilution when the HRT was shorter. However, for the attached system, where bacteria were immobilized on the PVDF membrane surface, the hydrogen yield was improved when the HRT was shortened to 12 hours, while the bacterial composition (VSS, g/l) was still high inside the reactor (Table 2b). The retention of bacterial cells inside the reactor improved the substrate utilization rate, and thus, more hydrogen could be produced in this designated system. The shorter HRT was not so efficient when applied to the suspended system, as the bacterial growth was unable to compete with the dilution effect due to the rapid rate of feeding, which led to cell washout. Therefore, among these two systems, the best substrate utilization rate was achieved at HRT 12 hours for the attached system, which is about 91% sugar conversion with a hydrogen yield of 2.48 mol H2/mol glucose.

Table 2: a) Fermentation parameters in various HRT for the suspended system

| HRT (hrs) | $H_2$ content (%) | Final pH | VSS (g/l) | Substrate Consumption (%) | Yield (mol $H_2$/mol glucose) | Energy production rate, EPR (KJ/L/d) | HPR (L/L/d) |
|---|---|---|---|---|---|---|---|
| 72 | 37 | 4.97 ± 0.13 | 3.52 ± 0.33 | 89 ± 0.7 | 1.21 ± 0.36 | 7.35 ± 1.8 | 0.58 ± 0.14 |
| 36 | 40 | 5.07 ± 0.11 | 3.30 ± 0.24 | 89 ± 0.6 | 1.53 ± 0.16 | 9.61 ± 1.3 | 0.75 ± 0.10 |
| 24 | 40 | 4.98 ± 0.13 | 3.16 ± 0.11 | 89 ± 0.5 | 1.88 ± 0.07 | 18.45 ± 1.1 | 1.45 ± 0.09 |
| 12 | 42 | 4.90 ± 0.18 | 2.95 ± 0.18 | 84 ± 0.8 | 1.62 ± 0.16 | 21.81 ± 2.6 | 1.71 ± 0.21 |
| HRT (hrs) | Volatile fatty acids (VFAs) | | | | Soluble Metabolites molar ratio | | |
| | HAc (mM) | HBu (mM) | HPr (mM) | HFr (mM) | B/A | TVFA (mM) | |
| 72 | 20.9 ± 0.18 | 11.4 ± 0.14 | 8.8 ± 0.37 | 7.6 ± 0.51 | 0.65 | 48.7 ± 1.2 | |
| 36 | 21.2 ± 0.22 | 10.2 ± 0.31 | 12.5 ± 0.11 | 9.0 ± 0.08 | 0.48 | 52.9 ± 0.72 | |
| 24 | 21.9 ± 0.09 | 12.0 ± 0.41 | 10.3 ± 0.21 | 10.7 ± 0.15 | 0.43 | 54.9 ± 0.86 | |
| 12 | 26.2 ± 0.70 | 12.7 ± 0.12 | 11.7 ± 0.08 | 7.6 ± 0.28 | 0.49 | 58.2 ± 0.93 | |

Table 2: b) Fermentation parameters in various HRT for the attached system (immobilized PVDF membrane)

| HRT (hrs) | $H_2$ content (%) | Final pH | VSS (g/l) | Substrate Consumption (%) | Yield (mol $H_2$/mol glucose) | Energy production rate, EPR (kJ/L/d) | HPR (L/L/d) |
|---|---|---|---|---|---|---|---|
| 72 | 39 | 5.00 ± 0.09 | 3.96 ± 0.22 | 88 ± 0.6 | 1.41 ± 0.32 | 7.24 ± 1.6 | 0.57 ± 0.13 |
| 36 | 41 | 4.92 ± 0.05 | 4.11 ± 0.41 | 87 ± 2.4 | 1.70 ± 0.21 | 13.00 ± 1.5 | 1.02 ± 0.12 |
| 24 | 45 | 4.94 ± 0.09 | 4.08 ± 0.33 | 89 ± 0.3 | 1.85 ± 0.18 | 28.60 ± 2.7 | 2.24 ± 0.21 |
| 12 | 48 | 4.86 ± 0.17 | 3.50 ± 0.27 | 91 ± 0.9 | 2.48 ± 0.16 | 31.45 ± 3.1 | 2.46 ± 0.24 |
| HRT (hrs) | Volatile fatty acids (VFAs) | | | | Soluble Metabolites molar ratio | | |
| | HAc (mM) | HBu (mM) | HPr (mM) | HFr (mM) | B/A | TVFA (mM) | |
| 72 | 22.9 ± 0.27 | 9.7 ± 0.24 | 9.1 ± 0.15 | 8.6 ± 0.20 | 0.42 | 50.3 ± 0.86 | |
| 36 | 24.3 ± 0.17 | 10.5 ± 0.06 | 8.2 ± 0.12 | 9.2 ± 0.09 | 0.43 | 52.2 ± 0.44 | |
| 24 | 23.9 ± 0.07 | 12.4 ± 0.21 | 10.4 ± 0.24 | 7.1 ± 0.22 | 0.52 | 53.8 ± 0.74 | |
| 12 | 27.7 ± 0.24 | 16.3 ± 0.33 | 9.1 ± 0.11 | 6.4 ± 0.16 | 0.59 | 59.5 ± 0.84 | |

Energy production analysis shows that the amount of energy produced was increased proportionally with the increase of HPR (Table 2). The energy generated for the optimum HRT of 12 hours for both systems was about 21.81 KJ H2/L/d for the suspended and 31.45 KJ H2/L/d for the attached system. This indicates that both systems achieved efficient hydrogen production with stable biomass retained inside the reactor, even though the suspended system suffered a small decrease in hydrogen yield under the high dilution rate.

### 3.3 Effect of HRT on Metabolites Production

Every fermentation process not only produced the desired product, but it also produced biproducts which might subsequently inhibit the process. It was found that the major metabolites produced from these two systems were similar, with acetate and butyrate being the dominant metabolites. Surprisingly, there was no production of ethanol in both reactors, but there was the appearance of a small amount of formic acid in the fermentation effluent. This is probably due to the catalytic hydrogenation of carbon dioxide that accumulated inside the reactor, especially when the fermentation achieved a homogenous state [31]. Besides that, as the initial bacterial seeds used for this study contained a mixed culture of bacteria from the effluent of the CSTR reactor of POME. Cyanobacteria species were probably present inside the seed. Cyanobacteria can oxidize sugar to form formic acid [33].

In Table 2, the total volatile fatty acids (TVFA) increased as HRT decreased from 72 hours to 12 hours, and the patterns were similar for both suspended and attached systems. Within the production of major acids in hydrogen fermentation, acetate and butyrate contributed about 40 – 45% and 19 – 23%, respectively, for the suspended system, while about 44 – 50% of acetate and 19 – 23% of butyrate were produced for the attached system. Ethanol was not detected in any effluent of different HRT for both systems. The high production of acetate and butyrate without any production of ethanol indicates that the system was able to produce hydrogen efficiently, as both acids were correlated with hydrogen production [31,32,34].

The B/A ratio (HAc/HBu) is one of the indicators for hydrogen fermentation, especially in anaerobic processes. Production of acetate and butyrate will always be accompanied by hydrogen generation, as shown in Eq. 3 and 4 [34].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (3)$$

$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2CO_2 + 2H_2 \quad (4)$$

These equations show that the production of acetic acid led to the creation of more hydrogen than butyric acid. Thus, a lower butyric to acetic acid (B/A) ratio was desired for higher hydrogen yield and productivity [36,37]. At optimum HRT, HRT 12 hours, about 26.2 mmol/L acetate and 12.7 mmol butyrate were produced in a suspended system with a B/A ratio of 0.49 accompanied by high HPR and yield. Meanwhile, for the attached system, about 27.7 mmol/L acetate and 16.3 mmol/L butyrate available in the effluent with the B/A ratio of 0.59 resulted in the maximum hydrogen yield and productivity.

Even though the major metabolite produced was the main metabolite for hydrogen production, the accumulation of propionic acid may lead to lower hydrogen. This is because some of the substrates might consume the accumulated hydrogen produced for further reduction to propionic acids (Equation 5). This happened when the condition inside the reactor suffered a low pH, high hydrogen partial pressure, and imbalance in the catabolic reaction of bacteria involved in the NAD+/NADH ratio [33,34]. Increasing NADH level during the fermentation led to butyric acid formation; in order to balance the NAD+/NADH ratio, propionic acid was spontaneously produced to generate enough NAD+, thus resulting in a shift in the metabolic pathway as the bacteria inside the reactor needed to adapt to the changes happening around them [33]. Therefore, it is suggested to constantly remove any forms of stressors from the system to ensure that the high yield and productivity of the fermentation process can be maintained.

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_5COOH + 2H_2O \qquad (5)$$

The soluble metabolites ratios can serve as qualitative indicators of substrate metabolism as well as metabolic pathways involved in fermentative hydrogen production. The linear correlation between the B/A ratio with respect to HPR and hydrogen yield is illustrated in Fig. 4.

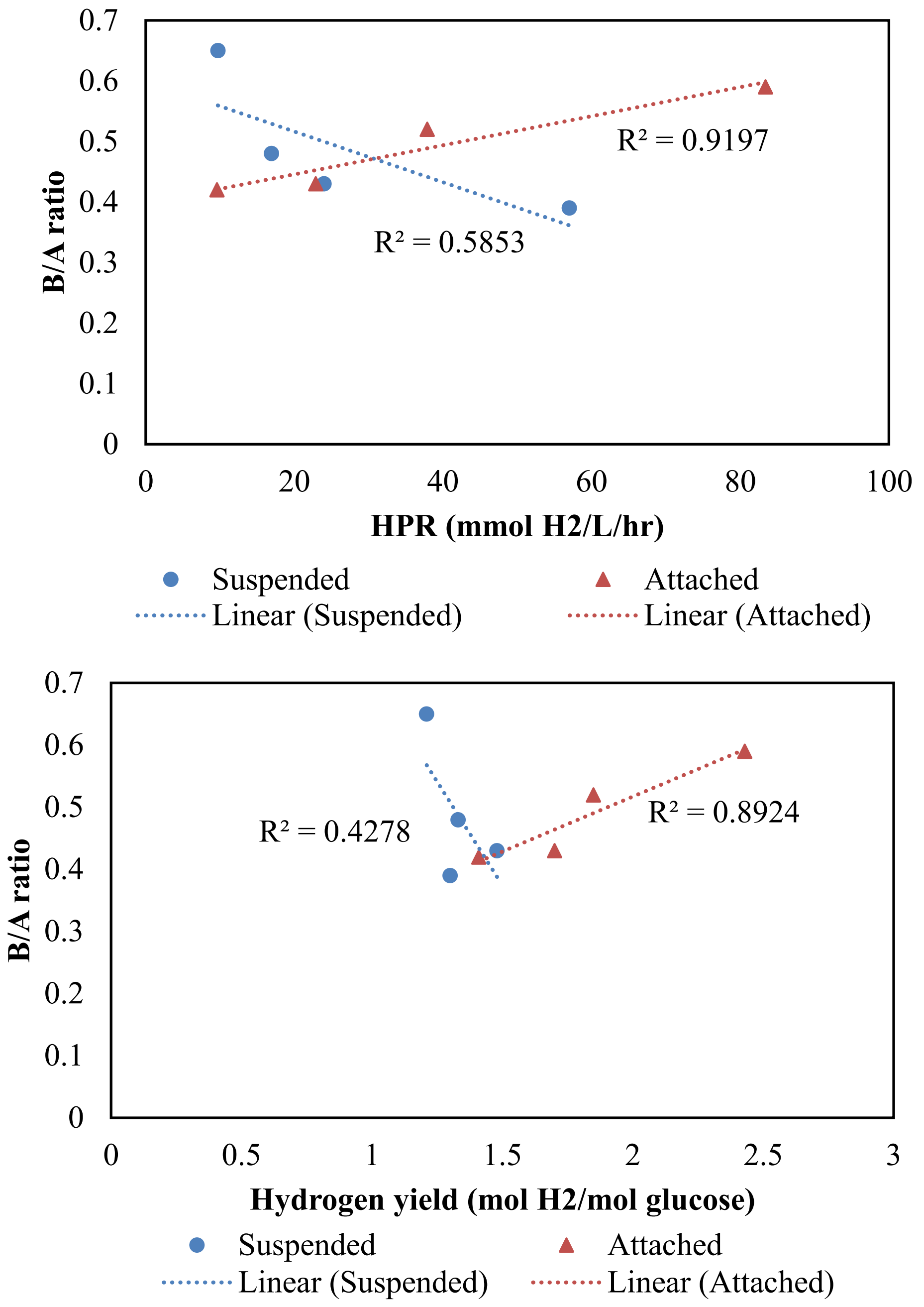


Fig. 4: Linear correlation between B/A ratio with respect to HPR and hydrogen yield

The attached system showed a good linear correlation with respect to HPR and yield with R2 = 0.92 and 0.89, respectively. This indicates that the increment of the B/A ratio near 1 showed that the metabolic pathways favored the acetic pathways for the attached system. However, for the suspended system, the decrement of the linear correlation showed that the production of hydrogen was not so efficient, which correlated with the smallest hydrogen yield and HPR. Therefore, it is proven that the attached system provided better bacterial retention and stability of the fermentation process, which was able to improve the fermentation process and, hence, increase yield and productivity.

## 4. CONCLUSION

To sum up, it was found that the biohydrogen fermentation performance was improved under a semi-continuous system, especially with immobilized cells. From the observation, it was found that the immobilized system promotes better performance than the suspended system. This is due to immobilized cell technology offering numerous advantages, including higher cell density, higher metabolic activity, prevention of interfacial inactivation, better productivity, and protection against changing environmental conditions. Under the immobilization system, the hydrogen yield increased to 54%, while HPR increased to 64% when compared with the suspended system. For all systems, it was found that the optimum HRT was during HRT 12 hours, where the performance of hydrogen production was improved as compared to other HRTs and in which the maximum hydrogen yield was achieved at 2.43 mol H2/mol and maximum hydrogen production rate (HPR) of 2.46 L H2/L.d. However, it is suggested that further observation, especially using a much lower HRT, is needed to conclude the performance of these two systems.

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