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BIOETHANOL PRODUCTION VIA SYNGAS FERMENTATION OF *CLOSTRIDIUM LJUNGDAHLII* IN A HOLLOW FIBER MEMBRANE SUPPORTED BIOREACTOR

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ABSTRACT

The production of ethanol via syngas fermentation obtained from lignocellulose gasification provides a method for completely utilizing all of the carbon content from lignocellulosic feedstock. The low mass transfer rate of less soluble CO and H_2 gas to liquid has been considered a major bottleneck in the overall process; however, microporous membrane has been proposed as a gas diffuser to improve gas-to-liquid mass transfer. In this study, a liquid batch of syngas fermentation employing *Clostridium ljungdahlii* with continuous gas supply was obtained using the configuration of a bioreactor connected to microporous hydrophobic polypropylene hollow fiber membrane (HFM) as a gas diffuser. Liquid recirculation between the fermentation vessel and membrane module was applied to enhance the gas–liquid contact as well as cell-recycle. The fermentation performance with and without HFM was compared and evaluated by cell growth, CO utilization, ethanol yield, and productivity. A higher ethanol yield, 0.22 mol/mol, was achieved using the system of an HFM-supported bioreactor with a higher ethanol titer of 1.09 g/L and an ethanol-acetate molar ratio of 1.43 mol/mol. The obtained result demonstrates that an HFM-supported bioreactor is the best fermentation system compared to stirred tank reactor (STR) without a membrane.

Keywords: *Clostridium ljungdahlii*; Ethanol; Hollow fiber membrane; Mass transfer; Syngas fermentation

1. INTRODUCTION

Syngas fermentation has been widely studied as an alternative means of completely obtaining and further converting all of the carbon content from lignocellulosic feedstock generated from the gasification process. It is possible to generate a cleaner and higher quality of syngas from gasified lignocellulose compared to the gas product derived from coal and other fossil fuels (Sulaiman et al., 2012). Most of the acetogenic species from more than 20 genera as the employed biocatalyst on syngas utilization are identified to produce solely acetate via the Wood–Ljungdahl pathway with acetyl-CoA as the intermediate product, such as *Acetobacterium woodi, Alkalibaculum bacchi, Butyribacterium methylotrophicum,* and

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Eubacterium limosum (Kopke et al., 2011). Some strains of *Clostridium sp.* have been studied as CO-fixing microorganisms able to further convert acetate into solvent products such as ethanol, butanol, and 2,3-butadienol, otherwise known as the carbodixotrophic microorganism, through the syngas fermentation process.

Syngas fermentation presents several advantages, such as greater biocatalyst specificity, lower energy costs, and no fixed ratio requirements in respect of CO: H_2 (Camacho et al., 2014). However, the efficient mass transfer of sparingly soluble gases such as CO and H_2 is a known bottleneck in the overall fermentation performance since CO acts as the main substrate for the growth of bacteria (Mohammadi et al., 2011).

Studies on bioreactor configuration aimed at addressing this mass transfer limitation have been conducted by evaluating the volumetric mass transfer coefficient (k_La) of the system as the key parameter. Such studies have been conducted in various bioreactor configurations, including the Continuous Stirred Tank Reactor (CSTR) (Riggs & Heindel, 2006), Bubble Column Reactor (BCR) (Datar et al., 2004), Gas Airlift Reactor (Munasinghe & Kanal, 2010), Trickle Bed Reactor (TBR) (Orgill et al., 2013), and Hollow Fiber Membrane Bioreactor (HFMBR) (Shen et al., 2014). High CO mass transfer in CSTR is normally achieved by increasing the agitation speed to increase the interface area (Orgill et al., 2013). Microbubble sparging has been used as one tool to enhance the dispersion of gas in the CSTR system, although no significant difference in the k_La values compared to a conventional bubble system has been demonstrated (Munasinghe & Khanal, 2010). Meanwhile, TBR offers the potential for a more efficient mass transfer system without mechanical agitation by applying a low gas and liquid flow rate to maintain the liquid holdup and retention time in packed columns. However, compared to other systems, low productivity fermentation is possible when a high liquid flow rate, if applied, is accompanied by decreasing CO mass transfer due to the bubble regime (Lee et al., 2012).

The Stirred Tank Reactor (STR) is a conventional and the most commonly used type of fermenter in microbial bioethanol production. The approach frequently used to attain high gas–liquid mass transfer is by increasing the agitation speed; however, this is economically infeasible due to the high-power consumption required for upscale fermentation. The application of hollow fiber membrane (HFM) as an external gas–liquid contactor connected to the bioreactor as the reservoir is able to significantly increase the CO mass transfer rate since it provides a large ratio of surface area to volume as the syngas flowing through the lumen of the membrane diffuses through the microporous membrane without forming bubbles (Orgill et al., 2013). The high gas–liquid mass transfer offered by this system enables the application of a low gas flow rate for higher gas conversion. The membrane could also serve as the biofilm support as the microbes grown on the outer wall of the membrane pass by the liquid stream (Shen et al., 2014).

Despite the potential of HFM for syngas fermentation application, no comparative studies on an HFM-supported bioreactor and conventional STR have been undertaken using the same strain of Clostridium sp. and the same scale of reactor. Therefore, the objective of this study is to evaluate the performance of both systems by investigating the profile of cell growth, carbon conversion, ethanol yield, and productivity. Two experiments will be carried out using the same strain of bacteria, working volume fermentation, and applied operating condition to enable a direct comparison of the performance of both systems with no effect from other factors.

2. METHODS

2.1. Set-up of the Bioreactor System

A custom membrane contactor from GDP Filter (Bandung, Indonesia) was used as the HFM gas–liquid contactor. The module contains 591 microporous hydrophobic polypropylene hollow

fibers with a pore size of 0.05 µm and an effective length of 14 cm, providing a total surface area of 0.46 m^2. The membrane module was connected to a 1.5 L working volume fermentation vessel. The fermentation broth was recirculated between the module and the vessel. The fermenter was equipped with a pH controller, temperature controller, and DO monitoring system in the form of a Bioflo 110 benchtop fermenter. The set-up of the bioreactor system is shown in Figure 1.

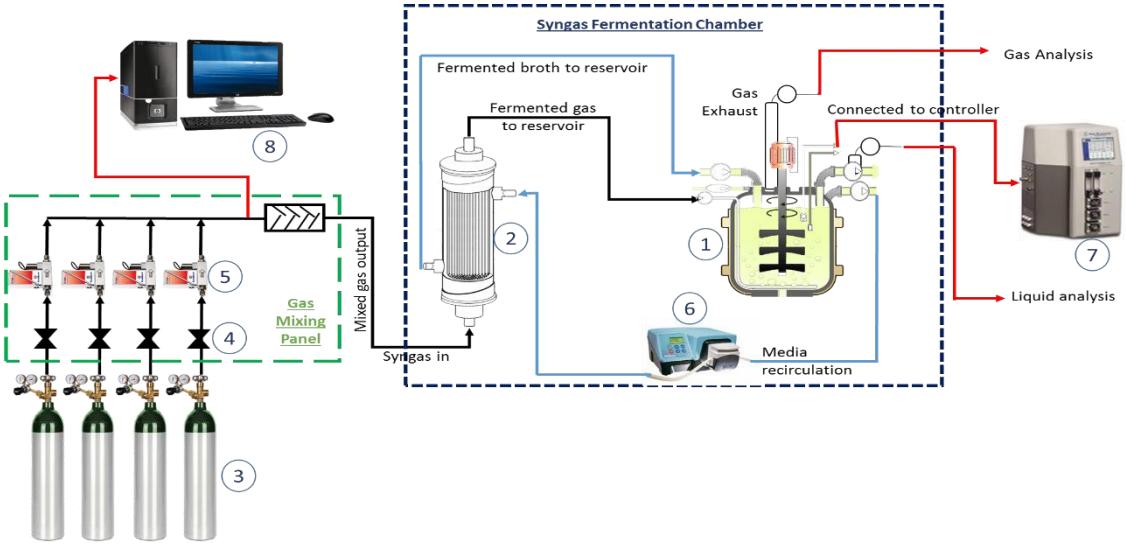


Figure 1 Set-up of the bioreactor system for syngas fermentation. (1) Bioreactor (as a reservoir), (2) Hollow fiber membrane, (3) Syngas cylinder, (4) Valve, (5) Mass flow controller (MFC), (6) Peristaltic pump, (7) Bioreactor controller, (8) PC unit (MFC controller)

The DO probe was used to ensure there was no O_2 present in the bioreactor during syngas fermentation. The artificial gas used in this study had a molar composition of 40% N_2, 25% CO, 20% CO_2, and 15% H_2. The flow rate for each gas was controlled using a mass flow controller (Bronkhorst, USA).

2.2. Strain, Medium, and Inoculum Preparation

Clostridium ljungdahlii DSM 13258 was acquired from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH culture collection (Braunschweig, Germany) in a freeze-dried culture. The culture was activated anaerobically in 10 mL growth medium with 5 g/L fructose as the carbon source and further cultivated and maintained in 50 mL liquid medium. The growth medium was prepared based on DSMZ medium 879 (Najafpour & Younesi, 2006). The medium contained (per L) mineral components that were mixed straight with the other components such as 10 mL trace element, 5 g mercaptoethanosulfonic acid sodium salt (MESNA), 1.5 g yeast extract, 5 mL cysteine 4% w/v, and 0.1 mL resazurin 0.1% w/v solution. Prior to medium sterilization at 121°C for 15 minutes, media in bottle serums equipped with butyl rubber stoppers and aluminum sealed were purged with ultra-high-purity N_2 gas. After sterilization, 10 mL vitamin solution was added into the medium before the pH of all fresh media was adjusted to 6.0 using 2 M NaOH and 2 M HCl prior to inoculation in an anaerobic chamber (N_2 atmospheric).

Starter culture as the inoculum was prepared in 150 mL growth medium with 5 g/L fructose by inoculating 10% active culture grown in the medium with the same composition. Starter culture incubation was carried out inside the anaerobic chamber in order to maintain the anoxic condition at 37°C for 2 days without agitation. Once the cell density had reached an optical density (OD_{600}) of 0.3–0.6, the culture was ready to be inoculated into the bioreactor vessel.

2.3. Syngas Fermentation in a Stirred Tank Reactor (STR)

The configuration of the bioreactor system used in the experiment is shown in Figure 1. Fermentation was carried out in an STR with no HFM as a gas–liquid contactor. The composition of the fermentation medium was prepared based on that used in a previous study by Anggraini et al. (2018), to obtain an optimum condition for acclimatization with a higher concentration of Ni, Se, and W in the trace elements solution and a lower yeast extract concentration (0.05% w/v). The bioreactor containing 1.5 L medium was sterilized at 121^{o}C for 15 minutes. Prior to inoculation, the medium was purged with 100% N_2 for 30 minutes, followed by the artificially mixed syngas for another 30 minutes.

The fermenter was inoculated with 10% v/v inoculum of total working volume by using a peristaltic pump. The pH was controlled at the optimum growth condition of pH 6.0 using 2 M NaOH and 2 M HCl for the initial 2 days of fermentation, while the temperature was also maintained at 37^{o}C (Najafpour & Younesi, 2006). During the first 2 days of fermentation, the agitation speed was kept at 300 rpm in order to increase the CO and H_2 mass transfer rate and obtain optimal cell density. Over the following 7 days, the agitation speed was decreased to 200 rpm and pH was no longer controlled. Artificial syngas was fed continuously and directly into the fermenter at a gas flow rate of 80 mL/min.

2.4. Syngas Fermentation in an HFM-supported Bioreactor

To enable a comparison, a similar experiment was carried out in an STR fitted with HFM as a gas–liquid contactor and medium circulator. Fermentation also took place over a 9-day period. The same operating conditions were applied with respect to the medium composition, initial pH, temperature, and gas flow rate. Artificial mixed syngas with a similar composition was continuously fed into the fiber lumen of the HFM module, directed to the reactor headspace, and expelled from the condenser. The bioreactor was first operated with no membrane-reservoir recirculation for 2 days in order to obtain the optimum cell density of the actively growing culture. Following this, liquid recirculation between the reservoir and membrane module was started at a rate of 120 mL/min and continued for the remaining 7 days of fermentation.

2.4. Analyses

Four millilitres of fermentation broth was withdrawn every day to monitor cell growth and fermentation products. Cell density was determined from an optical density (OD_{600}) measurement using a spectrophotometer at 600 nm. The concentrations of ethanol and acetate as metabolite products were determined by HPLC (Waters, Milford, MA, USA) with an Aminex HPX 87H column (Bio-Rad). The HPLC buffer was 5 mM sulfuric acid in water and the flow rate was 0.6 mL/min (Richter et al., 2013). The CO and H_2 concentration were determined from the inlet gas and headspace reactor gas. The gas sample was withdrawn using a 1 mL Hamilton Gas-Tight Syringe (USA) to measure the CO and H_2 concentrations using Gas Chromatography with Thermal Conductivity Detector (Shimadzu 2014, Japan). The sample was injected with a split ratio of 20:1 at an injector temperature of 200^{o}C. Argon was used as the carrier gas with a total flow of 50 mL/min. The detector temperature was 150^{o}C. The oven temperature was controlled at 35^{o}C for 5 min, ramped to 130^{o}C at 10^{o}C/min, then further to 210^{o}C at 30^{o}C/min (Shen et al., 2014).

The rate of cell growth was characterized by the net specific growth rate, calculated by the following equation (Shuler & Kargi, 2002):

$$\mu_{net} = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

where X is cell mass concentration (g/L), t is time (h), and μ_{net} is the net specific growth rate (h^{-1}). The value was calculated as the slope of the ln cell concentration vs. time curve during the exponential growth phase.

Total CO consumption (mL) was calculated using the following approach:

$$\text{CO consumption} = \int r_{CO}\ \Delta t \tag{2}$$

In which r_{CO} (mL/min) is

$$r_{CO} = \text{CO consumption (\%)} \times F_{CO} \tag{3}$$

Further CO consumption can be converted into moles using the ideal gas assumption.

The overall stoichiometric equations for ethanol synthesis from CO, H_2, and CO_2 are:

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \tag{4}$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \tag{5}$$

With the lump equation as follows:

$$6CO + 6H_2 \rightarrow 2C_2H_5OH + 2CO_2 \tag{6}$$

The ethanol yield (mol/mol) from CO was calculated based on Equation 6 as follows:

$$\text{Ethanol yield} = \frac{\dfrac{\text{Total moles of ethanol produced}}{\text{Total moles of CO consumed}}}{\dfrac{\text{2 moles of ethanol produced}}{\text{6 moles of CO consumed}}} \tag{7}$$

The carbon conversion (%) was calculated as follows:

$$\text{Carbon conversion} = \frac{\text{Total moles of CO consumed}}{\text{Total moles of CO supplied}}\ \text{x } 100\% \tag{8}$$

3. RESULTS AND DISCUSSION

3.1. Syngas Fermentation Performance in STR without HFM Support

The performance of syngas fermentation by *C. ljungdahlii* in an STR liquid batch fermentation without HFM support is shown in Figure 2.

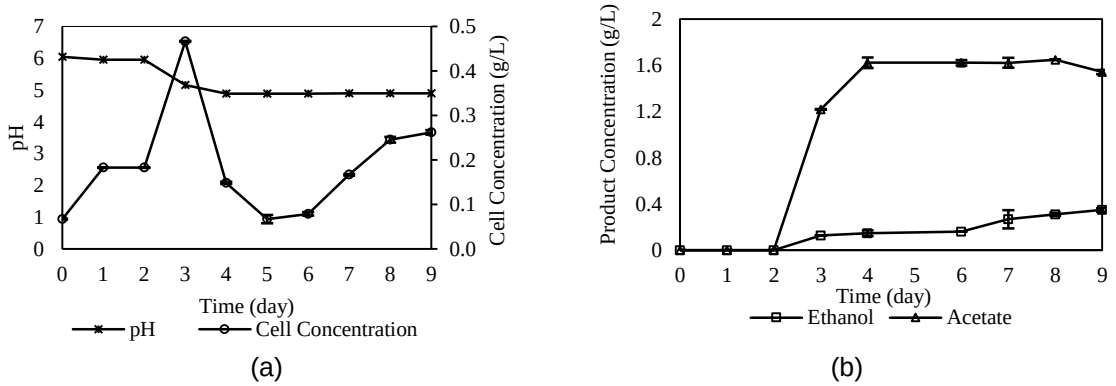


Figure 2 Syngas fermentation performance in STR without HFM support: (a) medium pH and cell concentration; (b) ethanol and acetate concentration

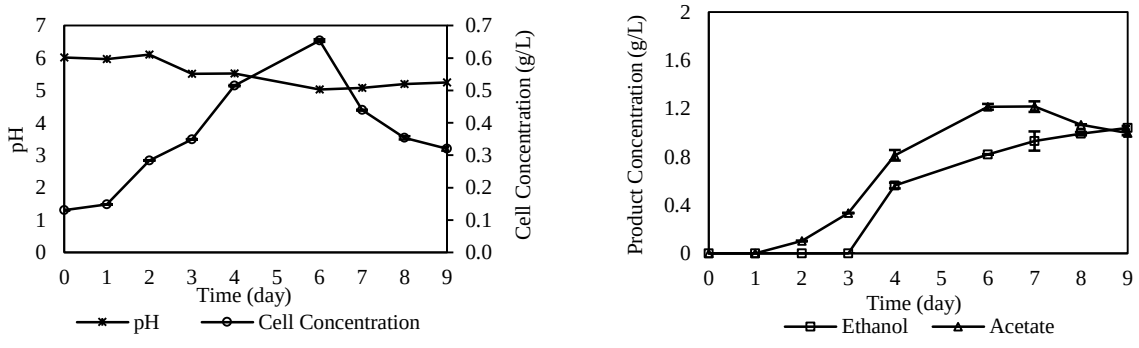
As demonstrated in Figure 2a, the cells continued to grow after 2 days of inoculation until they reached a peak cell density on Day 3 (0.467 g/L). Following the increasing concentration of cells as they started to grow, acetate also began to accumulate as a growth-associated product, which led to a fall in the pH of the medium to 5.0 since it was no longer being controlled at 6.0 for optimum cell growth. The pH value fell continuously prior to remaining steady at 4.8, which corresponded with a steady increase in acetate to a high concentration from Day 4 until the end of fermentation. This sharp fall in pH led to a drastic decrease in cell concentration on Day 4.

A dramatic decrease in pH has been found to affect cell stability and result in cell deactivation and low product formation (Mohammadi et al., 2012). During the cell deactivation period, as indicated by the steady growth of cells at a low level, there was a metabolic shift from acidogenesis to solventogenesis as the cells counteracted against the drastic pH changes by producing ethanol (Kundiyana et al., 2011; Mohammadi et al., 2012). In this study, the decrease in fermentation pH indicated the same phenomenon, as ethanol started to be promoted (0.13 g/L) and was then continuously produced as the act of acetate consumption by the cells with the highest concentration of 0.35 g/L was achieved at the end of fermentation. A gradual decrease in acetate concentration was followed by an increase in cell concentration after Day 6. However, acetate still appeared as the dominant product, which may have been the result of low availability of reducing equivalents from CO or H_2 oxidation as the fermentation was not conducted in a high-pressure system.

High availability of reducing equivalents from CO oxidation is often achieved by using a high-pressure gas fermentation system. In a study applying high partial pressure of CO ($P_{CO} \geq 1.35$ atm) with a P_{CO}/P_{CO2} ratio of 4.0, acetic acid was observed to be consumed when the cell growth started to level off (Hurst & Lewis, 2010). Another result using the newly built high-pressure gas fermentation system also showed a significant effect of the increased pressure of mixed gas (H_2/CO_2) on the formation of *C. ljungdahlii* products (Stoll et al., 2018). In the fermentation with an atmospheric condition applied, only a small amount of ethanol (C_{max} 13 mmol/L) with acetate dominating as the main product was observed. However, by increasing the pressure up to 7 bar, the acetate concentration decreased to 85%.

3.2. Syngas Fermentation Performance in HFM-supported Bioreactor

By using the same working volume and applied condition, fermentation was carried out with the application of HFM as the gas–liquid contactor to evaluate the performance of the membrane in enhancing the CO mass transfer. HFM was connected to the STR bioreactor, which acted as the reservoir (Figure 1). As shown in Figure 3a, there was rapid cell growth after the membrane was connected to the bioreactor at the end of Day 2 until peak cell density was reached on Day 6 (0.65 g/l).



(a) (b)

Figure 3 Syngas fermentation performance in HFM-supported bioreactor system (a) medium pH and cell concentration, (b) ethanol and acetate concentration

This was also followed by a drop in the pH of the medium to its lowest point of 5.03 as a result of acetate accumulation, which also led to a halting of cell growth. In this system, however, a gradual decrease of pH was observed, which is believed to have been influenced by a higher titer of ethanol that was produced earlier in its promotion compared to the STR system. On Day 4, ethanol started to be produced (0.56 g/L) at a rate exponential to that of the cell growth, and it continued to increase following the acetate consumption that began on Day 8 (mixed growth-associated product). The consumption of acetate by cells was also followed by a rise in the medium pH.

A comparison of the data for the syngas fermentation performance in STR with and without HFM support is shown in Table 1. It is believed that effective CO mass transfer also occurred along the microporous HFM surface to which the cell was attached so that the performance of the overall system was improved. Keryanti et al. (2019) proved that the CO volumetric mass transfer coefficient (k_La/V_L) of an STR with external-module HFM of the type used in this study was higher (300.5 h^{-1}) than for an STR without HFM (90 h^{-1}) at the specific CO flow rate of 1.05 vvm. However, the carbon conversions, as the ratio of CO utilization per CO supplied, have similar values for both reactor systems (Table 1). The results indicate that CO supply may have exceeded the maximum cell capacity. Therefore, in that condition, the fermentation performance was no longer limited by mass transfer but rather by growth kinetics. Shen et al. (2014) reported a similar result as they found that carbon utilization fell sharply when the gas flow rate was further increased to 300–500 mL/min.

Based on the similar CO utilization values, it can be deduced that the production of ethanol in the bioreactor with HFM support was three times higher than without HFM, using the same flow rate of mixed gaseous substrate. As the mixed gas used in this study also contained H_2, which can act as an electron donor from H_2 oxidation, it is also possible that CO_2 was consumed by the cells as a carbon source. As suggested in a previous study, it is possible for the high availability of electrons from CO and H_2 oxidation to induce ethanol production as a mixed growth-associated product, which was also observed in our study (Hurst & Lewis, 2010).

Table 1 Comparison of syngas fermentation performance

Fermentation parameter	STR without HFM	STR with HFM	Batch bottle Fermentation[a]
CO utilization (mol)	0.63	0.48	ND[d]
Maximum ethanol concentration (g/L)	0.35	1.09	0.21
Ethanol yield from utilized CO (mol/mol)[b]	0.05	0.22	ND[d]
Carbon conversion (%)	15.6	14	ND[d]
Maximum cell concentration (g/L)	0.47	0.65	0.13
Specific growth rate (h^{-1})	0.024	0.012[c]	ND[d]
EtOH/Acetate (mol/mol)	0.29	1.43	0.95

[a]18 days' fermentation in 100 % CO supply (Anggraini et al., 2018)
[b]values were calculated at 7 days' fermentation
[c]lower specific growth rate in HFM-supported bioreactor due to biofilm formation on the membrane surface from bioreactor-to-membrane shells liquid recirculation
[d] not determined

Ethanol as a mixed growth-associated product can also be observed in a system that applies a high total pressure of mixed gas in the reactor headspace (Younesi et al., 2005). The high initial total pressure (1.6 atm and 1.8 atm) applied in the system yielded results for early ethanol production in the exponential phase after 48 h of inoculation, with 100% CO consumption achieved after 60 h (Younesi et al., 2005).

Theoretically, a greater H_2:CO molar ratio promotes more efficient incorporation of carbon from CO to produce ethanol (Wilkins & Atiyeh, 2011). With the mixed gas lacking H_2, the carbon mole conversion from CO to ethanol was only 33%, while at an H_2:CO molar ratio of 2.0, it could reach 100% (Lee, 2010). In this study, the carbon mole conversion from CO to ethanol with HFM-supported bioreactor system could achieve 44% with a molar H_2:CO ratio in the mixed gaseous substrate of 0.6. Effective mass transfer in an HFM-supported bioreactor can provide high availability of H_2 as an additional electron source from its oxidation; thus, more CO will be converted to ethanol instead of CO_2 (Wilkins & Atiyeh, 2011).

3.3. Comparison with Other Studies

A comparison between the HFM-supported bioreactor and other fermentation systems examined in our study revealed the potential of the membrane to provide high CO mass transfer efficiency. By employing the same strain of *C. ljungdahlii* as a previous study on serum bottle fermentation, Anggraini et al. (2018) observed a lower ethanol titer despite the presence of a high ethanol-to-acetate ratio, which corresponded to the low density of cells. This may have been caused by a limited CO gas supply (Table 1). The continuous gas supply applied in this STR system may have induced earlier ethanol production, starting on Day 3, in comparison to the bottle serum study in which ethanol was promoted after 7 days of fermentation.

Of the three fermentation systems studied, the application of membrane as a gas–liquid contactor significantly improved the production of ethanol (Table 1). Compared to the STR system without HFM, the ethanol-to-acetate molar ratio increased, with the highest value of 1.43 achieved on Day 10. Another study using *C. carbodixovorans* P7 with HFM as the gas–liquid contactor also demonstrated a high ethanol titer, 23.93 g/l, and a high ethanol-to-acetate molar ratio, 4.79 (Shen et al., 2014). In Shen's study, however, the membrane had a larger active surface area (1.4 m^2 with k_La 1096.2 h^{-1}) compared to our study (0.46 m^2 with k_La 300.5 h^{-1}) and also featured fructose as an additional carbon source to CO. A larger membrane active surface area can increase the contact of the gas–liquid phase. The microbubble syngas that flows through the lumen of the membrane diffuses through the microporous membrane, as shown by higher retention time. Therefore, a larger membrane active surface area is able to produce an optimal flow rate of syngas supply and medium recirculation.

It is also possible to improve the ethanol-to-acetate ratio by maintaining the pH value within the acidic range at the point when the cells start to produce ethanol (i.e., the solventogenic phase) (Lee et al., 2012). In a study using the *C. ljungdahlii* strain, which was also carried out using HFM as the gas–liquid contactor, the molar ratio of ethanol to acetate reached 2.6 with the pH regulated at 5.0 and a higher syngas flow rate (100 mL/min).

Future research should be oriented more toward modifying either the medium composition or operating condition to increase biomass density and optimize the growth kinetics, with the aim of determining the optimal flow rate of syngas supply and medium recirculation.

4. CONCLUSION

The design of HFM as a gas–liquid contactor and biofilm platform has demonstrated a significant improvement in the amount of ethanol production with the achievement of a high ethanol-to-acetate molar ratio (1.43) and a threefold-higher maximum ethanol concentration (1.09 g/L) than that produced in an STR without HFM support (0.35 g/L). The application of HFM to bioethanol production via syngas fermentation is an innovative approach that offers a reduction of the CO mass transfer barrier observed in other conventional reactor designs.

5. ACKNOWLEDGEMENT

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