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In-Situ Continuous Production of Hydrogen Gas from Molasses Using Mutated *Enterobacter aerogenes* ADH43 for Fuel Cell Application

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Abstract: The performance of the continuous stirred tank reactor (CSTR) for the in-situ production of hydrogen gas (H_2) integrated with a polymer electrolyte membrane fuel cell (PEMFC) was investigated. Facultative anaerobe fermentation of *Enterobacter aerogenes* ADH-43 was conducted into CSTR 50 rpm of agitation speed, 37°C of temperature, 6.3 of pH and 0.15; 0.3; 0.45; 0.60 hG^{1} of dilution rate. Bio-H_2 produced was assessed after inserting it into a fuel cell to generate electricity and measuring voltages. The system was integrated with a ceramic membrane having 0.2 μm pore size for recycling the retentive cell into reactor and separating the permeate supernatant during the fermentation. The obtained H_2 was purified from CO_2 by absorption in $Ca(OH)_2$ solution prior to feed to PEMFC. The CSTR was initially operated on batch basis to increase the bacterial cell density to ensure the production of sufficient H_2 and develop a feeding culture strategy for continues operation mode. The result showed that the highest H_2 production achieved at continuous system resulted in 0.30 hG^{1} of optimum dilution rate. The maximum H_2 volume of 9.76 *l* H_2/*l* sugar, the yield of 1.84mol H_2/mol sugar and the flow rate of 115 m*l* H_2/min were obtained. Furthermore, colony count of 9.81 log cfu/m*l*, pH of 5.73, maximum electrical current of 0.38 Ampere, electrical power of 2.20 Watt and electrical voltage of 5.75 volt after given resistance using LED of 25 ohm was also obtained.

Key words: H_2 production % *E. aerogenes* % Molasses % CSTR % Ceramic membrane % PEMFC

INTRODUCTION

The future form of energy might be H_2, which is a highly attractive clean energy carrier especially when mentioned as an everlasting energy source. Another advantage would be having water as a by-product from hydrogen combustion to get the energy thus reducing the pressing greenhouse issues raised from fossil fuel usage [1]. The insecurity in fossil energy prices and sources further heightens the demand for an alternative, sustainable and clean energy carrier. Furthermore, all renewable energy sources is ultimately based on solar energy that is made available to us through photovoltaic cells, wind energy or stored as chemical energy in biomass [2]. The latter will play a major role as a feedstock for sustainable production of electricity as well as gaseous and liquid biofuels. A distinction can be made between the use of dry biomass such as wood and the use of wet biomass sources such as the organic fraction of domestic waste, agro-industrial wastes and slurries and wastewater [3, 4].

Currently South East Asian countries such as Malaysia, Indonesia, Thailand, etc. produces substantial amount of biomass in the form of agricultural wastes [5]. Rather than the current practice of disposing these bio waste via open burning that can aggravate the environmental issues, these biomass can be converted into H_2. However, the public is still very unfamiliar with the idea of H_2 as an energy source [6]. Moreover, any production route emitting carbon dioxide that starts from biomass ensures zero net carbon emission as the carbon dioxide released is taken up by the plants to perform photosynthesis, the idea of using hydrogen to produce energy is indeed attractive [7].

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The difficulty of storing and transporting bio- H_2 limits its commercial application. This problem can be solved by combining the bio- H_2 -producing system with fuel cell system for electricity generation [8, 9]. The possibility of converting H_2 into electricity via fuel cells makes the application of H_2 energy very promising [10]. However, there is little information in the literature referred to direct fuel cell electricity generation from bio- H_2 In this study, the bio- H_2 produced inserted directly to a home-made proton-exchange-membrane fuel cell (PEMFC) for electricity generation.

Potential of various pure cultures had been exploited to produce H_2 using a variety of substrates. Fermentation of bacterium based on the used substrates in batch culture leads to the highest production yield of H_2 as follows: 3.31 mol H_2 / mol glucose by *E. cloacae* DM 11 [11], 0.73 mol H_2/ mol xylose in batch culture, 6.0 mol H_2/ mol sucrose by *E. cloacae* II-BT-08 [12], 9.95 mmol H_2/ COD in starch by *C. acetobutylicum* CGS 2 [13], 2.2 mol H_2/ mol chitinous waste by *C. puraputripicum* M-21 [14], 2.3 mol H_2/ mol glucose in cellulosic biomass by *C. thermocellum* 27405 [15] and 1.12 mol H_2/ mol glycerol in biodiesel waste by *E. aerogenes HU*-101 [16]. On the other hand, maximum H_2 production in continuous culture was 3.5 mol H_2/mol sugar in molasses by *E. aerogenes* E-82005 [17] and 3.0 mol H_2/mol lactose by *C. thermolaticum* [18].

Members of *Clostridium* and *Enterobacter* were most widely used as inoculums for fermentative H_2 production. Species of *Clostridium* are Gram negative, rod shaped strict anaerobes and endospore formers whereas *Enterobacter* are Gram negative and rod shaped facultative anaerobes [19]. In most of studies, pure cultures of bacteria were used for fermentative H_2 production. These experiments were conducted in batch and glucose was used as substrate, however H_2 production from organic wastes is more desirable as it is feasible process for industrialization to realize the goal of waste reduction and energy production. Thus, fermentative H_2 production by pure cultures using organic wastes is widely recommended [20].

In our previous study, the vial bottles fermentation of *E. aerogenes* ADH43, sugarcane molasses used as carbon sources. It was shown that by feeding 0.5 *l* 4 % (v/v) of sugar cane molasses into 3 *l* of working volume of batch reactor at 5 and 12 hrs fermentation, the flow rate and yield of H_2 production increased nearly 1, 3-fold and 1.5-fold compared to batch reactor, respectively. It also indicated, that a maximum current of 0.40, electrical power of 2.42 Watt and a maximum electrical voltage of 6.03 volt after given a resistance using a LED of 15 ohm (Data are not reported).

The main objective of present work was to investigate the effect of dilution rate to biohydrogen production in continuous stirred tank reactor (CSTR) system. The produced biohydrogen was directly inserted into a fuel cell to generate electricity.

MATERIALS AND METHODS

Microorganism and Culture Condition: H_2 producing bacteria of *E. aerogenes* ADH-43 was obtained by classical mutagenesis working and was maintained at -80°C with 15 % glycerol. A synthetic medium used in this contained (per liter) was included as follows: 7.0g K_2HPO_4, 1.0 g $(NH_4)_2$ SO_4, 0.25g $MgSO_4.7H_2O$, 0.021g $CaCl_2.2H_2O$, 0.029g $Co(NO_3)_2.6H_2O$, 0.039g $Fe(NH_4)_2$ $SO_4.6H_2O$, 0.172mg Na_2Se0_3, 0.02 mg $NiCI_2$, 0.5g $MnCl_2.4H_2O$, 0.1g H_3BO_3, 0.01g $AlK(SO_4)_2.12$ H_2O, 0.001g $CuCl_2$. $2H_2O$, 0.5g $Na_2EDTA.2H_2O$ and 2.0mg nikotenic acid [21]. A complex medium was prepared by adding 2% of reducing sugar of molasses or equal to 4% of total sugar to synthetic medium.

A modified hungate technique combined with serum bottle technique [22] was used to culture the bacterium anaerobically. The medium without molasses and phosphate buffer boiled for 20 minutes, cooled on ice with continuous bubbling of N_2 gas, dispersed into serum bottles sealed with black butyl rubber stoppers and then sterilized (18 min, 121°C). Molasses and phosphate buffer autoclaved separately and injected into serum bottle. After the inoculation of 10 % of seed culture into serum bottles and adjustment of the pH to 6.8, the bottles were incubated at 37°C with 50 rpm of agitation [23]. Seed culture was obtained by 40 ml of pre-culture of *E. aerogenes* ADH43 (OD = ± 0.82) before the end of logarithmic growth phase was inoculated into 400 ml complex medium supplemented with 2 g/*l* total sugar of cane molasses and then incubated at 37°C, 120 rpm, 8 hours of temperature, agitation time and fermentation time.

Analysis: The number of bacterial colonies was measured by the method of total plate count (TPC). Gas volume measurements made using respirometer connected with the holes on the top of the fermentor. Gas of CO_2 and H_2 were formed and flowed through the hose into the Erlenmeyer containing a solution of $Ca(OH)_2$. Gas of CO_2 reacted with $Ca(OH)_2$ to form $CaCO_3$, while H_2 got into the respirometer containing a saturated NaCl as follows:

$$CO_2(g) + Ca(OH)_2(l) \rightarrow CaCO_3 + H_2(g) \quad (1)$$

The amount of H_2 produced is shown by the difference in volume between the cylinders in a NaCl solution (small cylinder) with the outer cylinder (large cylinder) on the respirometer. A volume of H_2 is calculated based on the difference in volume that occurs due to gas pressure cylinder H_2 between the large and small cylinders. The concentration of CO_2 and H_2 were determined by gas chromatography (GC 8A, Shimadzu Kyoto) equipped with a thermal conductivity detector [24]. Total sugar (TS) and reducing sugars (RS) were measured by phenol sulfuric method [25].

Stirred Tank Reactor and Fuel Cell Installation: Amount of 7*l* stirred tank reactor with a working volume of 4*l* was used for the continuous culture. A volume of 400 m*l* seed culture was inoculated into 3.6 *l* fermentation media containing sterile complex medium supplemented with 4 g/*l* total sugar of cane molasses. The ceramic membrane with 0.2 µ pore size was also set together for recycling of retentive cell into reactor and separating of permeate supernatant such as acids and alcohols when the batch and continuous culture were carried out. The cells were cultivated anaerobically by replacing the gas phase with N_2 gas at the start of the culture and cells were cultivated anaerobically. The initial pH was adjusted to 6.8. The temperature and agitation rate were maintained 37°C and 40 rpm, respectively. After 6 h of incubation in batch culture, continuous cultivation was initiated by feeding sterilized fresh medium. The flow rate was sharply increased in order to have the dilution rate of 0.15; 0.30; 0.45; 0.60 h^{-1} (in sequence) with a peristaltic pump under anaerobic conditions. Evolved gas and effluent liquid were discharged from the top of the reactor. A quasi-steady state was confirmed based on a constant H_2 evolution rate and the amount of evolved electricity. The gases which contained 60% of H_2 and 40 % of CO_2, N_2 and H_2O were analyzed using Gas Chromatography. The purification unit consists of absorber filled with zeolites, silica and calcium hydroxide in order to decrease H_2O and CO_2 contents. After passing through purification system, 99 % purity of H_2 was achieved. While the flow rate reached in steady state, the next dilution rate was selected. The total running period for the culture was 2 days. The changing of dilution rate was performed by peristaltic pump. Flow rate of pump can be estimated from the dilution rate with the Eq. (2):

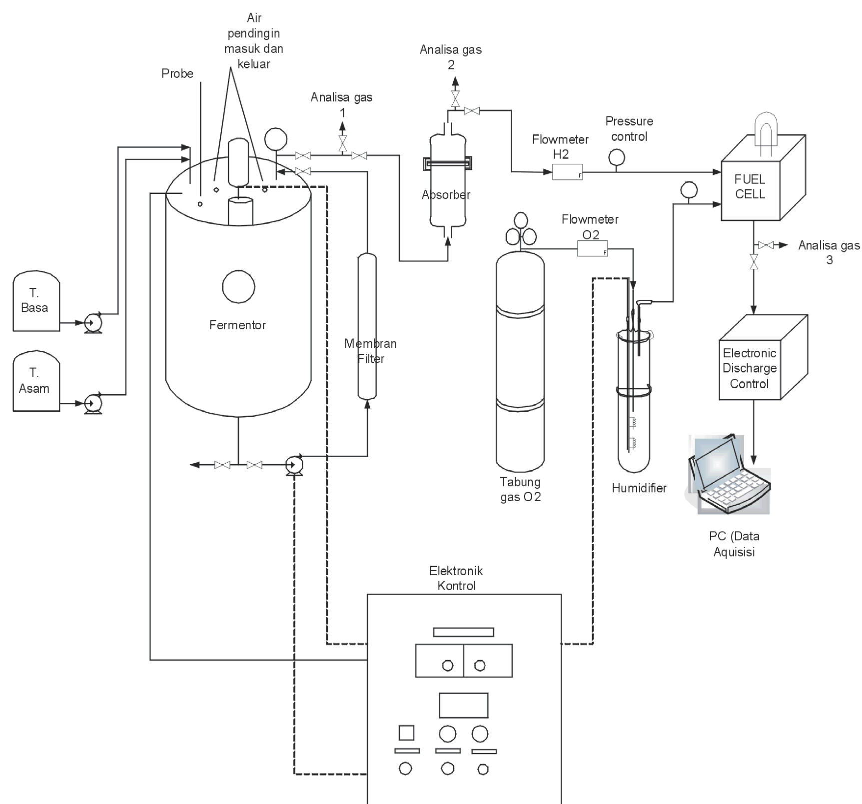

Fig. 1: Schematic diagram for biohydrogen production process and its application in PEM fuel cell stack

$D(h^{-1})$ = Flow rate of pump *(l/h)* / Working volume (*l*) (2)

Measurements were performed on optical density, viable cell count, H_2 production, pH, sugar reduction, total sugar. The H_2 produced was introduced into PEMFC, voltage was assessed after given resistance using LED of 25 ohm. The described experiment was reproduced for three times.

FC performance needs a High humidity of H_2 but water flooding may affect. Thus, water management inside fuel cell stack should be controlled not too high as FC requirement condition [26]. A 4 standard cell of FC was used from Electrochem Co., which is consist of 4 Membrane Electrode Assembly (MEA). The schematic diagram for biohydrogen production and its application in PEM fuel cell stack is shown in Fig. 1.

RESULTS AND DISCUSSION

Fermentation Performance in Batch Culture System: Table 1 shows the growth curve of *Enterobacter aerogenes* ADH-43 using a batch culture system at the reactor volume, 4 *l*, 37°C, 6.8 of pH and 2 % of molasses concentration. As well as gram-negative is growing faster than Photosynthetic microorganisms, Phase lag was only about 2 hours and H_2 being produced during the early log phase is approximately 2 hours. Microorganisms enhanced growth along with rising H_2 production and the sugar consumed up to 6 hours of fermentation time. The pH values of 6.1-6.3 were ideal for the production of H_2 [19]. Fresh 2 % molasses was pumped towards the end of the log phase of microbial growth, or about 6 hours into the fermentation time, at the time stability of the H_2 production of about 42 m*l* H_2/min.

Effect of Dilution Rate to Total Colony, pH, Total Sugar and Reduction Sugar: In this study, after 6 h of incubation in batch culture system, continuous cultivation was initiated by feeding sterilized sugar cane fresh medium at the dilution rate of 0.15; 0.30; 0.45; 0.60 h^{-1} (in sequence). In Fig. 2, the effect of dilution rate on total colony of *E. aerogenes* ADH 43, pH, total sugar and reduction sugar during fermentation in CSTR system

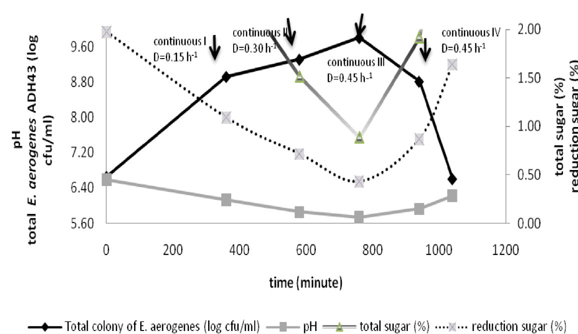

Fig. 2: Changes in Total Colony of *E. aerogenes* ADH43, pH, Total Sugar and Reduction Sugar in CSTR with Varying Dilution Rate

were described based on the fermentation time. Flow rate pump changed at minute 400, 600, 800 and 1000 to achieve dilution rate of 0.15, 0.30, 0.45 and 0.60 h^{-1}, respectively. In pattern 1, with rising of D from 0.15 to 0.30 and finally to 0.45 h^{-1}, the number of colonies increased. It also showed the reduction for sugar and even when D is 0.45 h^{-1}, the reduction of sugar is close to zero. It indicated that the sugar perfectly consumed. With an increase in D, the pH showed an opposite changes from 6.13 to 5.73 associated with increased formation of acetic acid and lactic acid as well as an increased number of colonies from 8.93 to 9.60log cfu/m*l*. It showed that sugar from cane molasses was utilized for cell growth and produce H_2, organic acids and alcohol. *E. aerogenes* produces 2,3-butanediol (BD), ethanol and organic acids (lactate, acetate and formate) besides H_2 [16, 17] Organic acids produced by *E.* aerogenes decrease pH of fermentation medium.

For the second pattern in which dilution rate was increased from 0.45 to 0.60 h^{-1}, the amount of sugar consumed was decreased with increasing total sugar and reducing sugar in effluent. It is also associated with rising pH to be 5.93 and 8.82 log cfu/m*l* fall in the number of colonies. In the last pattern to retain D at 0.60 h^{-1}, the number of colonies dropped very drastically and the effluent pH rose with increased RS and TS. A decrease in total colony leads to washout at higher dilution rates. According to this figure, in the CSTR, operation at a high dilution rate or short residence time can lead to washout.

Table 1: Fermentation growth and production during batch culture of *E. aerogenes* ADH-43

Time (hr)	OD (log CFU/m*l*)	pH	RS (%)	TS (%)	Flow rate H_2 (m*l* H_2/min)
0	5.45	6.5	2.0	3.0	0
2	6.5	6.3	1.8	2.9	0
4	7.5.	6.2	1.5	2.8	26
6	7/.6	6.2	1.0	2.0	42

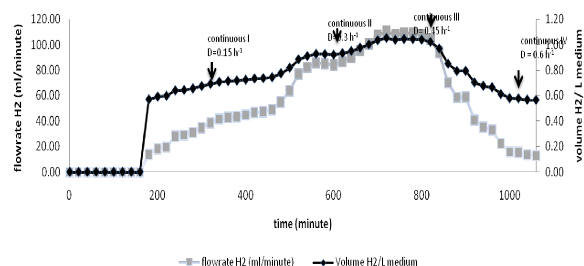

Fig. 3: Changes in H_2 flow rate and H_2 volumetric in CSTR with varying dilution rate

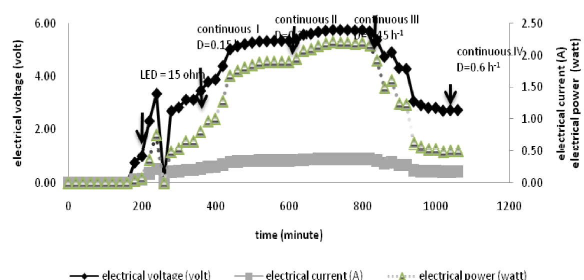

Fig. 4: Changes in electrical current, voltage and power in CSTR with varying D

Effect of Dilution Rate in H_2 Production: Fig. 3 described changes in H_2 production by *E. aerogenes* ADH 43 in continuous culture with varying dilution rate. This figure, as well as Fig. 1 for pattern 1, with the increase in D from 0.15 to 0.30 and then finally to 0.45 hG¹, flow rate of H_2 formation was increased nearly linear from 70 to 110 m*l*/min and also identical for the volume of H_2 production. Conversely, an increase in D up to 0.60 hG¹ and maintained it in such away, the washout reached only 10 m*l*/min, even if production of H_2 continued no longer. Therefore, these disadvantages may cause operational instability and limit H_2 production. H_2 production in a CSTR operation has some drawbacks. In general, a CSTR system is very sensitive to environmental changes (i.e., pH, HRT) [10].

The highest H_2 production was obtained at dilution rate 0.45 hG¹, resulted a maximum H_2 volume of 9.76 *l* H_2/*l* sugar (1.84 mol H_2/mol sugar), maximum flow rate of 114.66 m*l* H_2/minute.

Electricity Generation via PEMFC: In this study, the CSTR H_2-producing system was combined with PEMFC to generate electricity. Levin *et al.* [15] pointed out that choosing a PEMFC system to produce electricity from biohydrogen is based on the idea that the best use of biohydrogen systems might be as a means of delivering small, distributed power systems to communities. They also estimated that if a continuous biohydrogen system can deliver enough H_2 to power a PEMFC for 24 h for at least one year; then, the biohydrogen system could have a truly useful and potentially commercial application. In our previous study, biohydrogen production using the fed-batch system was inserted into PEMFC to generate electricity. It resulted in a total H_2 volume of 13.61 *l* H_2/*l* sugar, total yield H_2 of 3.84 mol H_2/ mol sugar, flow rate of 39.73 m*l* H_2/minute and a maximum current of 0.40, electrical power of 2.42 Watt and a maximum electrical voltage of 6.03 volt after given a resistance using a LED of 25 ohm. Dark fermentation is known to be the most promising way of mass production of H_2, thereby feasible for continuous electricity generation via PEMFC [26].

In this CSTR system, H_2 from respirometer was fed to the PEMFC device to generate electricity that turned on the light on the LED 25 ohm. The highest electricity generation was obtained at dilution rate 0.30/h, resulted a maximum electrical current of 0.38A, electrical power of 2.20 Watt and electrical voltage of 5.75 volt after given resistance using LED of 25 ohm. It could be the highest H_2 produced at dilution rate 0.30; so, the higher H_2 inserted into fuel cell, the higher electricity generated.

CONCLUSION

In this paper, we investigated the effect of dilution rate to biohydrogen production in continuous stirred tank reactor (CSTR) system. The produced biohydrogen was directly inserted into a fuel cell to generate electricity. The maximum H_2 volume of 9.76 *l* H_2/total sugar, the yield of 1.84mol H_2/mol total sugar and the flow rate of 114.66 ml H_2/minute were obtained at dilution rate 0.30hG¹. Furthermore, colony count of 9.81 log cfu/m*l*, pH of 5.73, maximum electrical current of 0.38 A, electrical power of 2.20 Watt and electrical voltage of 5.75 volt after given resistance using LED of 25 ohm were also achieved.

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