African Journal of Biotechnology Vol. 11(36), pp. 8872-8879, 3 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2860
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Utilization of hydrogen gas production for electricity generation in fuel cell by *Enterobacter aerogenes* ADH 43 with many kinds of carbon sources in batch stirred tank reactor

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Accepted 27 January, 2012

Enterobacter aerogenes **ADH-43 is a hydrogen gas (H_2) producing mutant bacterium and a facultative anaerobic microbe. This double mutant was obtained by classical mutagenetically treated in order to enhance H_2 production. In addition, this mutant has ability to degrade molasses from sugar factory as well as other carbon sources. The main goals of this research were to use *E. aerogenes* ADH-43 for fermentation in order to decide the best carbon sources and optimum concentration from molasses, glucose, cassava sugar, glycerol and biodiesel waste media in vial bottle. Moreover, to stabilize H_2 production, it was operated at 37°C and an initial pH 6.8. Performing the research in batch and fed-batch culture, utilizing it by converting to electricity using fuel cells in 50 ml vial bottle, 2% total sugar concentration of sugar cane molases was found to be the highest H_2 production (9.38 L H_2/L medium) during 24 h fermentation. It was also observed that in batch culture at 12 h fermentation, the volume, flow rate maximum, and the yield of H_2 production were 1.6 L H_2/L sugar molases, 73.4 ml H_2/min, and 2.99 mol H_2/mol sugar molases, respectively. Both sugar was consumed perfectly, colony count was 1.7 10^7 cfu/ml, pH was nearly constant at 6.0, and finally the H_2 was drifted to fuel cell to generate electrical power until 4 V 0.25 A. Moreover, the volume, flow rate maximum, and the yield of H_2 production were 2.12 L H_2/L sugar molases, 107.7 ml H_2/min, and 3.84 mol H_2/mol sugar molases, respectively when the fed-batch culture was performed. This means nearly 1.3- fold yield of H_2 as compared to batch culture was obtained by adding 0.5 L of 2% sugar cane molasses at 5 and 12 h of fermentation in order to reach 7.0 V, 5.3 W and 0.38 A by a fuel cell.**

Key words: *Enterobacter aerogenes* ADH-43, H_2 production, molasses and fuel cell.

INTRODUCTION

Recently, one of the most important clean fuel materials is expected to be hydrogen gas (H_2) (Das et al., 2008), which is also widely recognized as a clean and efficient energy resource of the future with a high energy content of 122 KJ g^{-1}. Approximately 95% of commercially produced H_2 comes from carbon-containing raw materials, primarily of fossil origin (Claassen et al., 1999). 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 (Lee et al., 2000). Furthermore, fermentative organisms have high growth rates, and H_2 evolution takes place under anaerobic conditions during sugar fermentation by a variety of facultative anaerobic

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bacteria, such as *Escherichia coli*, *Enterobacter aerogenes*, and strict anaerobes, e.g. Firmicutes. Fermentative bacteria have very high rates of H_2 evolution compared with other biological hydrogen production processes and recent research has led to many improvements in bioprocess parameters leading to impressive gains in volumetric production rates. However, substrate conversion yields are low, both due to competing reactions, such as H_2 consumption or reductant diversion to other products, and to theoretical limits of the natural metabolic pathways (Hallenbeck, 2009).

The potential of these various pure cultures had been exploited to produce H_2 using a variety of substrates, in batch culture the highest H_2 yield production was achieved 3.31 mol H_2/mol glucose by *Enterobacter cloacae* DM 11 (Nath et al., 2004), 0.73 mol H_2/mol xylose and 6.0 mol H_2/mol sucrose by *E. cloacae* II-BT-08 (Kumar and Das, 2000), 9.95 mmol H_2/COD in starch by *Clostridium acetobutylicum* CGS 2 (Chen et al., 2001), 2.2 mol H_2/mol chitinous waste by *Clostridium puraputripicum* M-21 (Evvyernie et al., 2001) 2.3 mol H_2/mol glucose in cellulosic biomass by *Clostridium thermocellum* 27405 (Levin et al., 2006). In continuous culture with a packed-bed reactor using porous ceramics as a support material to fix cells in reactors, the maximum H_2 production from biodiesel wastes reached 63 mmol/ L·h (Ito et al., 2005). Moreover, the highest H_2 production was 3.5 mol H_2/mol sugar in molasses by *E. aerogenes* E-82005 (Tanisho and Ishiwata, 1995) and 3.0 mol H_2/ mol lactose by *Clostridium thermolaticum* (Collet et al., 2004)

Dark fermentation is a promising method of bio-hydrogen production due to its high rate of hydrogen evolution from a diverse range of substrates, and its lack of a requirement for a direct input of solar energy (Hallenbeck, 2009). A variety of cheap agricultural residues, or even wastes otherwise requiring treatment are readily available feedstocks for H_2 production by dark fermentation (Kaparaju et al., 2009). Any other glucose sources (pine apple cannery waste, sweet sorghum, molases sugar cane, etc), starchy material sources (sago, rice husk,etc) and glycerol sources (biodiesel waste, palm oil waste, etc) can be converted into H_2 by three fermentation types, namely butyric acid type (Lay, 2000), propionic acid type (Cohen et al., 1984) and ethanol type fermentation (Ren and Huang, 1997). Bio-mass fermentation for bio-H_2 production offer significant advantages over costly chemical processes due to operation under mild conditions (30 to 35°C, 1 atm). H_2 gas may be produced by a number of processes, including electrolysis of water, hermocatalytic reformation of H_2-rich organic compounds, and biological processes. Currently, H_2 is produced almost exclusively, by electrolysis of water or by steam reformation of methane. Biological production of H_2 using microorganisms is an exciting new area of technology development that offers the potential production of usable H_2 from a variety of renewable resources (Nishio and Nakashimada, 2004). Biological systems provide a wide range of approaches to generate H_2, and include direct biophotolysis, indirect bio-photolysis, photo and dark-fermentation (Rachman et al., 1998).

A fuel cell (FC) combines H_2 and O_2 to produce electricity, heat, and water. FCs are often compared to batteries; both convert the energy produced by a chemical reaction into usable electric power. However, the FC will produce electricity as long as fuel is supplied, never losing its charge. Fuel cells are a promising technology for use as a source of heat and electricity for buildings, and as an electrical power source for electric motors propelling vehicles. Fuel cells operate best on pure H_2, although fuels like natural gas, methanol or even gasoline can be reformed to produce the H_2 required for FC. Some FC even can be fueled with methanol without using a reformer (Lamine and Dicks, 2000). The main goal of this experiment was to compare the C6 and C3 (glucose and glycerol) as carbon sources for maximum H_2 production and to decide the the optimum concentration of the selected substrates in 50 ml vial bottle, and also to convert the best substrates using the potential strain of *E. aerogenes* ADH-43 to produced H_2 in batch, and fed-batch system in 4 L of batch stirred tank reactor (BSTR). The next idea was how to utilize this H_2 by converting it to electricity by using FC.

MATERIALS AND METHODS

Microorganism and culture condition

H_2 producing bacteria of *E. aerogenes* ADH-43, a double mutant, was obtained by treatment of mutant *E. aerogenes* AY-2 (Rachman et al., 1997) using mutagen ethyl methane sulfonate (EMS). The bacterial strain was stored in glycerol broth at −80°C, it was grown on complex medium and cultured anaerobically at 37°C for 24 h. The synthetic medium used contained (per liter) 7.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, 0.021 g $CaCl_2.2H_2O$, 0.029 g $Co(NO_3)_2.6H_2O$, 0.039 g $Fe(NH_4)_2SO_4.6H_2O$, 0.172 mg Na_2SeO_3, 0.02 mg $NiCl_2$, 0.5 g $MnCl_2.4H_2O$, 0.1 g H_3BO_3, 0.01 g $AlK(SO_4)_2.12H_2O$, 0.001 g $CuCl_2.2H_2O$, 0.5 g $Na_2EDTA.2H_2O$, and 2.0 mg nicotinic acid (Rachman et al., 1997). A complex medium was prepared by adding 2% reducing sugar of molasses to synthetic medium.

A modified hungate technique in combination with the serum bottle technique (Miller and Wolin, 1974) was used to culture the bacterium anaerobically. The medium without molasses and phosphate buffer was boiled for 20 min, cooled on ice with continous bubbling of N_2 gas, dispered into serum bottles sealed with black butyl rubber stoppers and then sterilized (18 min at 121°C). Molasses and phosphate buffer autoclaved separately were injected into serum bottle. After the innoculation 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 (Nakashimada et al., 2002)

Analyses

The number of bacterial colonies was measured by the method of total plate count (TPC). Gas volume measurements were made using respirometer connected with the holes on the top of the

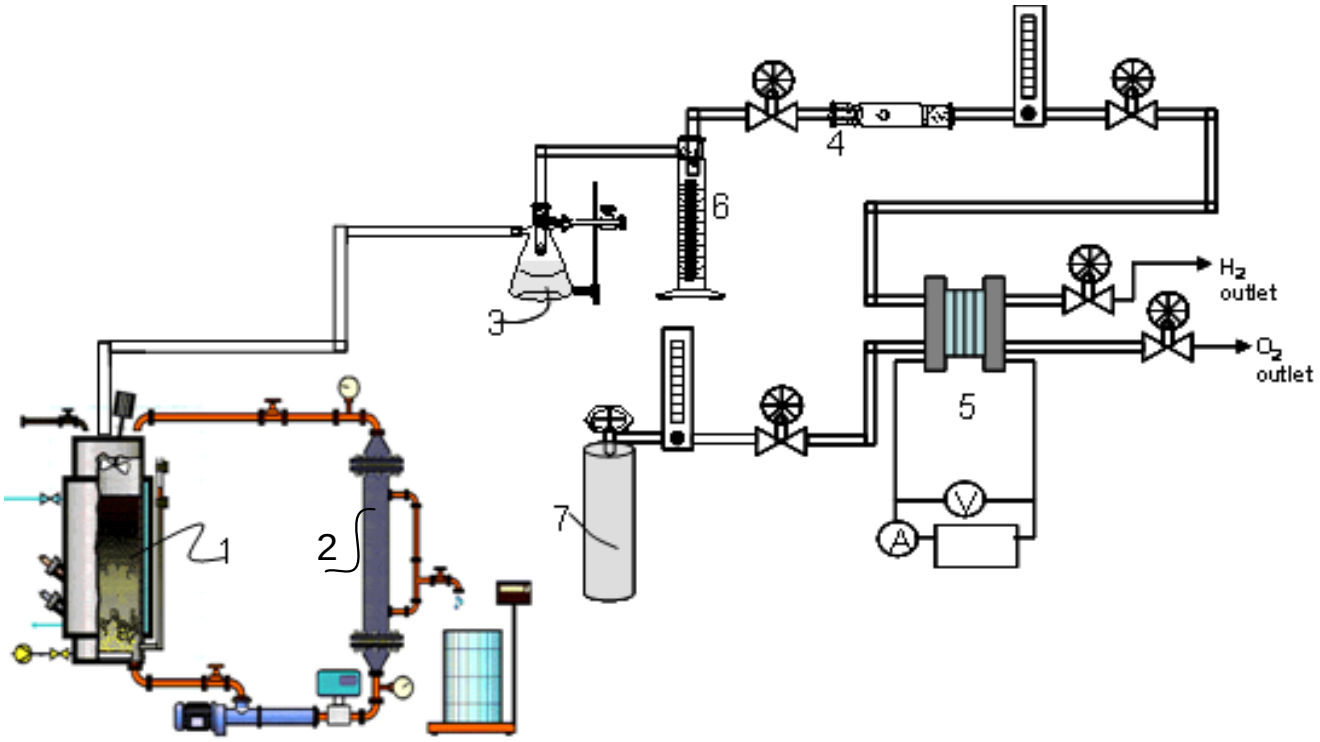

Figure 1. Schematic process of H_2 production in BSTR and FBSTR for electricity. 1, 4 L working volume of BSTR; 2, ceramic membrane; 3, replacement NaCl measuring cylinder; 4, pressure regulator; 5, FC; 6, NaCl saturated cylinder; 7, O_2 tube. BSTR, Batch stirred tank reactor; FBSTR, fed batch stirred tank reactor; FC, fuel cells.

fermentor. CO_2 and H_2 gases formed flow through the hose into the Erlenmeyer containing a solution of Ca (OH) $_2$. CO_2 gas and react with Ca (OH) $_2$ to form $CaCO_3$, while the H_2 will get into the respirometer tube containing a saturated NaCl. The reaction was as follows:

$$CO_2\ (g) + Ca\ (OH)_2\ (l) \rightarrow CaCO_{3\ (s)} + H_2\ (g)$$

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 measured volume of H_2 is calculated based on the difference in volume that occurs due to gas pressure cylinder H_2 between large and small cylinders. The concentration of CO_2 and H_2 were determined by gas chromatography (GC 8A, Shimadzu Kyoto) with a thermal conductivity detector, while measurement of the concentration of glycerol, lactic acid, ethanol, acetate and 2,3-butandiol was by HPLC. A total of 1 ml of sample is introduced into micro centrifuge sterile tube and then centrifuged at 5000 rpm for 15 min. The supernatant was transferred to another tube micro centrifuge tube and then filtered using 0.2 µM micro filter. Standard solution of 100 mM lactic acid was injected into the HPLC and after injection of the centrifugation supernatant it was injected into the HPLC (Rachman et al., 1997). Total sugar (TS) and reducing sugars (RS) were measured by phenol sulfuric method (Dubois et al., 1956).

Stirred tank reactor and fuel cell installation

As shown in Figure 1, 4 L working volume of 7 L of total volume of BSTR was used and the speed of agitation was nearky constant at 50 rpm, while the fed batch stirred tank reactor (FBSTR) condition was treated by adding 0.5 L 2% each fresh molasses at 5 and 12 h of fermentation time by peristaltic pump in 3 L fermentation volume of the initial batch culture. The ceramic membrane with 0.2 m pore size was also set together for recycling of retentate cell into reactor and separating of permeate supernatant such as acids and alcohols when the batch and fed-batch culture were carried out. Evolved gas was discharged from the top and effluent liquid were discharged from the bottom of reactor. A quasi-steady state was confirmed on the basis of a constant H_2 evolution rate and the amount of evolved electricity. The gases which contains 60% of H_2 and 40% of CO_2, N_2 and H_2O were analyzed using gas chromatography purification unit consisting of absorber filled with zeolites, silica and calsium hidroxide in order to decrease H_2O and CO_2 contained.

After undergoing the purification system, 99% purity of H_2 was achieved. High humidity of H_2 is needed for FC performance, but water flooding could be affected. Thus, water management inside fuel cell stack should be controlled not too high as FC requirement condition (Stephen and Kathya, 2005). Four standard cell of FC was used from Electrochem Co. which consisted of four membrane electrode assembly (MEA).

RESULTS AND DISCUSSION

Selected of carbon sources in vial bottle

Figure 1 shows that substrates in the form of cassava, sugar cane, molasses, and glucose can produce H_2 higher than the glycerol and biodiesel waste. This is because cassava, sugar cane, molasses and glucose-containing carbon sources derived from simple sugars, especially glucose (1 mol of glucose contains C 6 atoms),

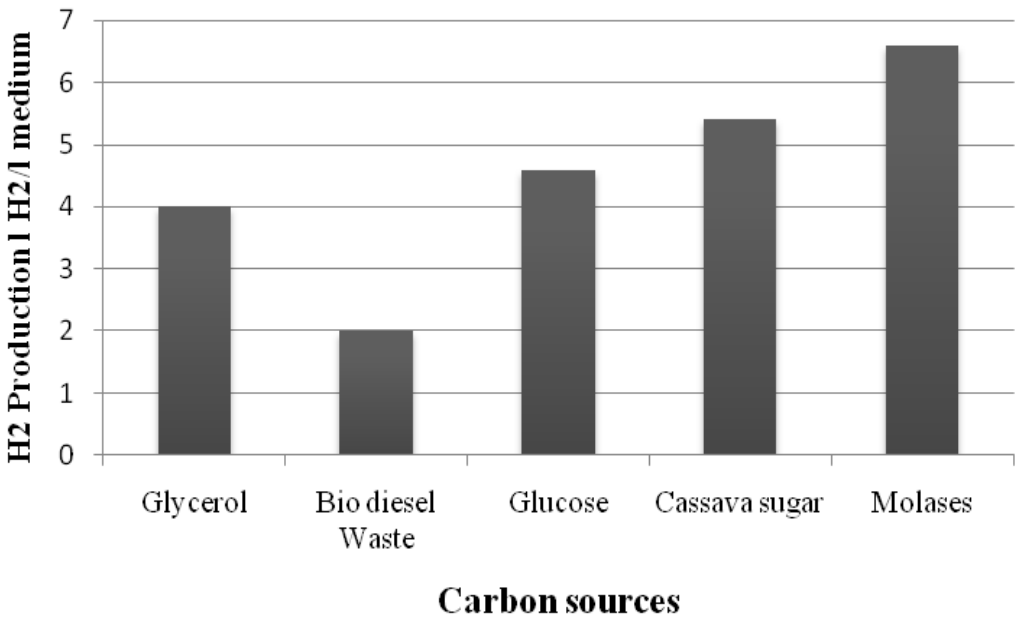

Figure 2. Influence graph types 1% substrates of 50 mL working volume in the vial bottle by *E. aerogenes* ADH 43 on the production of bio-H_2 for 24 h of fermentation.

form 4 mol H_2 / mol of substrate theoretically when acetic acid is produced (Gottschalk, 1986). On the opposite, microbial conversion of glycerol and biodiesel waste containing C 3 have been investigated with particular focus on 1,3-propanediol for polyester industries and it was found that maximum rate of H_2 production was 80 mmol/L·h and 30 mmol/L·h for pure glycerol and biodiesel waste, respectively (Ito et al., 2004). Among several carbon sources with concentration of 1% sugar for each substrate, sugarcane molasses produced the highest amount of H_2, ranging from 6.37 to 6.61 (L H_2 / L molasses), while the waste biodiesel produced the lowest amount of H_2 which ranged from 1.89 to 2.04 (L H_2/ L biodiesel waste). This type of molasses is a byproduct of the manufacture of crystalline sugar from sugar cane juice. It has not crystallized and still has fairly high sugar content, especially sucrose as a disaccharide sugar. During the non-reduction, it splits into reducing sugars (glucose and fructose) that are then ready for use by bacteria as a source of carbon or energy sources for growth and multiplication of cells, to produce compounds of metabolites including H_2.

In addition to very high sugar content, molasses also contain nitrogen (N) as compared with other substrates. Elemental nitrogen is very influential on the growth and development as the formation of bacterial cell walls. According to Özgür et al., (2003), nutrients for the microbes to function as an energy source for growth and cell shape products metabolite biosynthesis. Nutrients needed by the microbes include water, carbon source, nitrogen source, vitamins and minerals. While on cassava sugar substrate, glucose, waste biodiesel, and glucose did not contain elements of N, so that the elemental N is available only in complex media and tripton of yeast extract. biodiesel waste results in the lowest H_2 because these wastes contain crude glycerol only 10% of byproduct biodiesel production process by converting triglyceride oil into methyl or ethyl esters by trans-esterification, that is by reacting the alcohol with the oil to produce a three-chain esters of glycerin. So the crude glycerol is still mixed with the rest of the trans-esterification process, among others, residual catalyst, methanol and soap. In addition to methanol and soap, crude glycerol also contain various elements such as calcium, magnesium, phosphorus, or sulfur and the content of impurities which can inhibit the growth of cell (Abbad et al., 1996). While for the glycerol substrate, the volume of H_2 produced is higher than biodiesel waste because glycerol is a pure substrate, making them easier to be utilized by the bacterial metabolisms (Ito et al., 2004).

The inhibition of molasses in 50 ml vial bottle

The influence of 54% dextrose equivalent of used molasses was carried out in 50 ml vial bottle. Fermentation was performed for 24 h from 1 to 5% of molasses concentration by diluting the pure molases to an expected concentration. We found in Figure 2 that the best sugarcane molases was 2% of RS for achieving maximum H_2 production (9.38 L H_2/L molasses). In comparison, diluted molasses containing about 2% sugars were fed at a rate of approximately 80 ml/h and

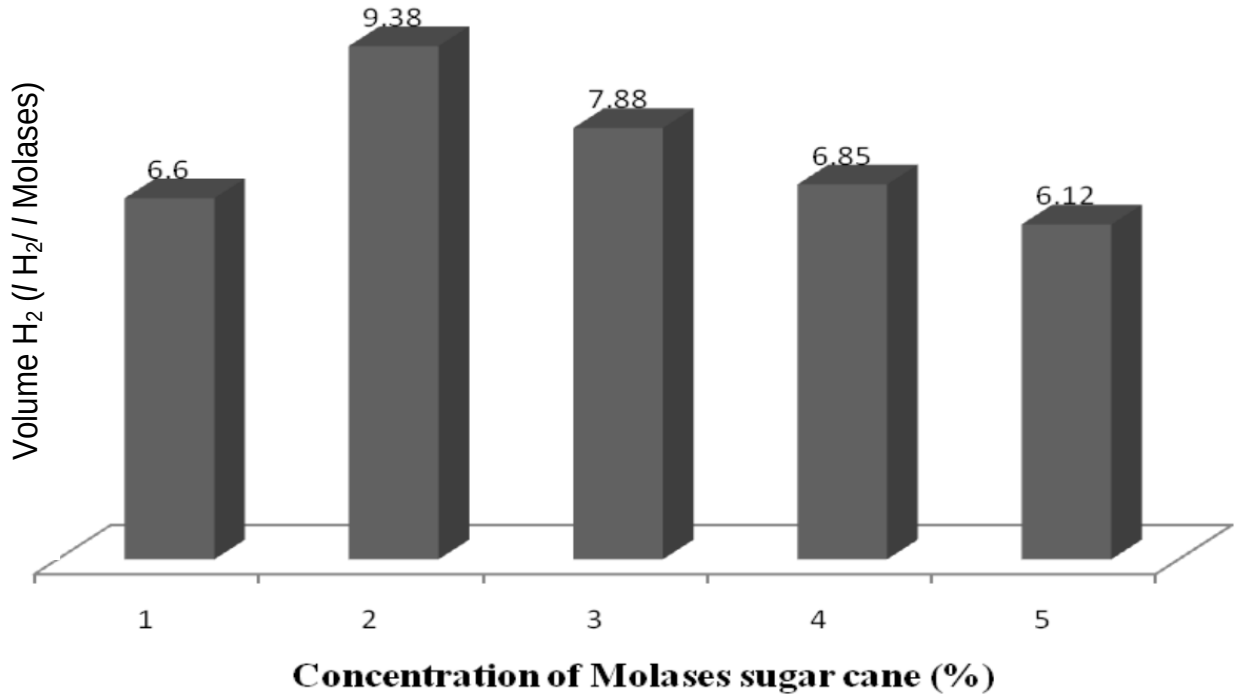

Figure 3. Molasses inhibition on bio-H_2 production by *E. aerogenes* ADH-43 in 50 mL of vial bottle for 24 h fermentation of molasses.

the maximum and the available rate of H_2 production at 38°C in a 300 ml fermenter with 250 ml molasses fluid were 36 mmol-H_2/(L-culture· h) (Tanisho, 1994) figure 3. The increasing concentration of molasses RS from 3 to 5% indicated substrate inhibition (Wang and Jin, 2009) and H_2 production decreased to 6.5 L H_2/L molasses. Hence, we decided to use 2% of RS molasses or nearly 4% base of TS in molases for the batch and fed-batch culture.

The batch analyses for bio-H_2 production with 2% of molases as a susbtrate

The relationship between BSTR fermentation system with the energy generated by the FC is described in Figures 4 and 5. Both equipment was synergtically performed on 2% of molases concentration. The fermentation was carried out at 6.8, 37°C, and 20 h of pH, temperature, and fermentation time, respectively.

As shown in Figure 4, *E. aerogenes* ADH43 consumed a carbon source for cell growth up to 2 h and then began to metabolize the carbon source as a gaseous H_2 in the early hours up to 2 to 3 h to when the cells where in early log phase. It was indicated that the dark-fermentation is faster than photo-fermentation (Özgür et al., 2003). Flow rate and volume of H_2 production showed the same pattern of the curve increased from 3 to 12 h of fermentation with a maximum flow rate of 73.8 m/ H_2/min and total volume of and 1.6 L H_2/L sugar molasses at 12 h fermentation. The RS and TS decreased sharply by the increase of fermentation time and both sugars were consumed perfectly after 20 h of fermentation. Colony count was 7.7 10^7 cfu/ml, pH was nearly constant at 6.0, and finally the H_2 was drifted to fuel cell to generate electrical power until 4 V 0.25 A. When the cells were in a phase of death, H_2 productivity decreased so that the voltage generated also decreased. This is because the RS and TS in molasses have declined and are not quite used to the survival of cells, so that the cells undergo death during the 22 h fermentation with BSTR. The obtained volume of H_2 was 9.13 L H_2 / L sugar molasses and the yield of H_2 was 2.99 mol H_2 / mol sugar molasses during 20 h fermentation. The resulting H_2 directly connected to the FC in the FC electrochemical reaction occurs, which react with H_2 and O_2 to generate energy in the form of electricity and the voltage of 4 V of electricity was generated, with efficiency substrate ranges of 90% (Stephen and Kathya, 2005).

The fed-batch fermentation of *E. aerogenes* in 4 L volume of reactor

To investigate the influence of application the fed-batch system for H_2 production, an electrical power formation is illustrated in Figure 6. The experiment was carried out twice and *E. aerogenes* ADH-43 was incubated similar to 24 h for batch system. At first, the working volume was 3

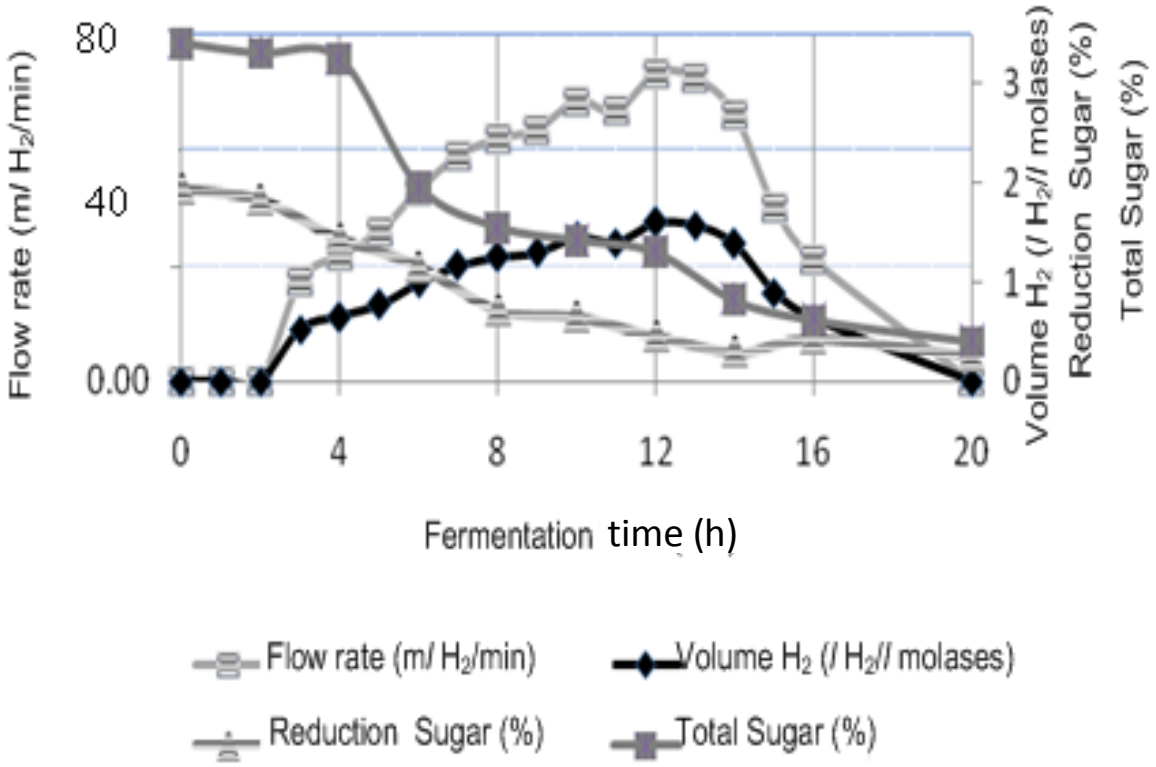

Figure 4. The relationship between duration of 2% molasses fermentation time with the production of H_2 and the sugar consumed by *E. aerogenes* ADH-43 in 4 L of BSTR. BSTR, Batch stirred tank reactor.

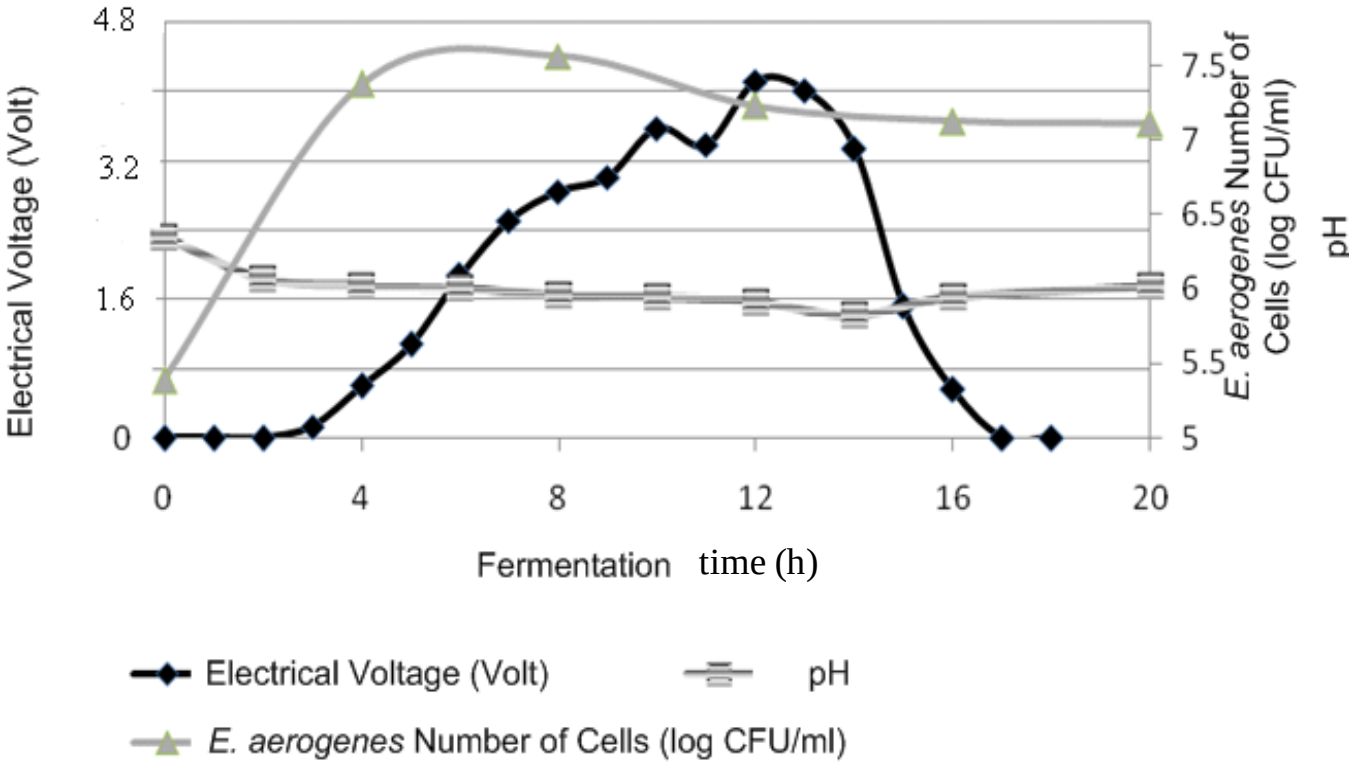

Figure 5. The relationship between duration of 2% molasses fermentation time with the production of electrical voltage, the growth of cells and pH by *E. aerogenes* ADH-43 in 4 L of BSTR. BSTR, Batch stirred tank reactor.

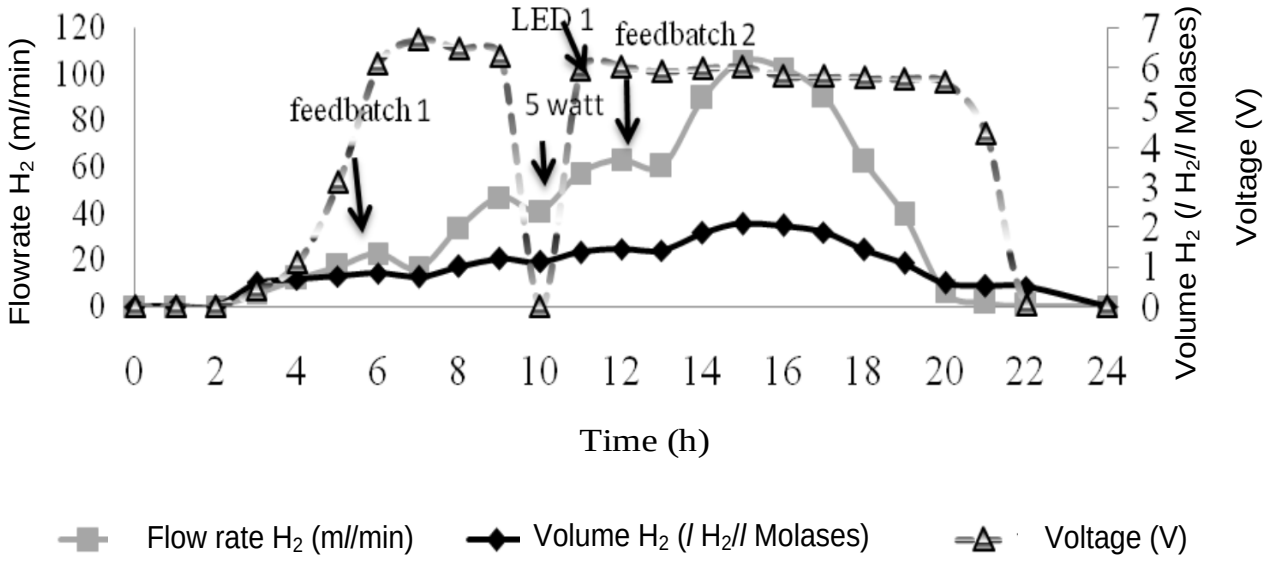

Figure 6. H_2 production curve and the electrical energy generated by the fermentation of *E. aerogenes* ADH-43, 0.5 L , 2% molasses fresh medium was added at 5 and 12 h of fermentation in order to have 4 *l* volume of FBSTR. BSTR, Batch stirred tank reactor.

L for the initial fermentation in BSTR. Each 0.5 L of fresh molasses medium at 5 and 12 h fermentation was added in order to achieve 4 L working volume. At the beginning, the TS was 2%, but decreased together with RS by fermentation time. The flow rate and volume maximum of H_2 in FBSTR were 107.7 ml/min and 2.12 L H_2/L sugar molasses at 15 h of fermentation. The total volume of H_2 production was 13.61 L H_2/L sugar cane molasses when the fed-batch culture was performed. This indicates nearly 1.5-fold compare to batch culture by adding 0.5 L, 2% of sugar cane molasses at 5 and 12 h of fermentation in order to reach 7.0 V, 5.3 W, and 0.38 A by a fuel cell. When 5 W of LED lamp was connected into FC at 10 h of fermentation, the voltage decreased to 0 V and increased again to gain the steady state phase from 12 to 20 h of fermentation at 7.0 V as maximum voltage for seven stack of FC.

Biofuels can partially overcome the disadvantages posed by impeding climate change and deteriorating fossil fuel reserves. Biohydrogen can be viewed as an ideal biofuel, although its production at present is problematic with large number of technical barriers that have resolved. H_2 gas production through biological processes, however, represents an exciting new avenue of technology development for bioenergy generation. On the hand, the lower yield obtained with fermentation with respect to other methods of H_2 production continues to be the principal issue to be addressed. In the present study, several main factors influencing fermentative H_2 production and attempts to improve the H_2 production has been introduced and discussed. Significant improvement can be made through rapid gas removal and separation, hybrid system, reverse micelles and by metabolic engineering. Moreover, identifying novel hydrogenases and metabolic pathways through genetic engineering, high-throughput genomic sequencing, environmental genomics and/or metagenomic technologies, hybrid system using photosythetic and fermentative bacteria may assist to make biological H_2 production more economical, practical and commercially feasible (Nath et al., 2004). Instead of molasses or any sugar as a substrate for H_2 fermentation production (Tanisho and Ishiwata, 1994), glycerol and biodiesel waste are also to be considered for obtaining an ethanol yield of 0.85 mol/mol-glycerol by *E. aerogenes* HU-101 0 (Ito et al., 2005) since 90 g/L raw glycerol derived from biodiesel wastes was completely consumed and 47.1 g/L 1.3-propanediol was produced by *Clostridium butyricum* F2b (Papanikoloau et al., 2004). Moreover demand for renewable energy is still an open question on the biohydrogen, since fermentation is a technology for converting low economic materials to high value matters.

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