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The Effect of Hydraulic Retention Time and Volatile Fatty Acids on Biohydrogen Production from Palm Oil Mill Effluent under Non-Sterile Condition.

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Abstract: The effect of hydraulic retention time and volatile fatty acids produced during fermentation were investigated on biohydrogen production from palm oil mill effluent in a 50 L bioreactor. The fermentation was done in three different hydraulic retention times; HRT 5, HRT 3 and HRT 2 days. Hydraulic retention time and volatile fatty acids concentration showed a vital role in response to the biohydrogen concentration, biohydrogen rate and biohydrogen yield. The maximum biohydrogen concentration was obtained at HRT 2 days with 30% hydrogen content in biogas. The biohydrogen yield and rate were 1054 NmL/L-POME and 44 NmL/h/L-POME, respectively. The lowest biohydrogen yield and rate were observed at HRT 5 days with 557 NmL/L-POME and 5 NmL/h/L-POME, respectively. Meanwhile, the accumulation of propionic acid concentration up to 7 g/L was suggested as a factor that reduced the biohydrogen production.

Key words: Biohydrogen, palm oil mill effluent, hydraulic retention times, volatile fatty acids.

INTRODUCTION

In the present world, energy is highly demanded by the industries, power plants, offices, households, as well as individual life. The demand of the energy is expending and lead to depletion of non-renewable energy such as coal, oil, gasoline and metal cores. Since this problem has overwhelmed all over the world, a lot of researches have been carried out to utilize biomass as alternative renewable resources (Lay *et al*., 1999, Levin *et al*., 2004, Prasertsan and Prasertsan, 1996, Vijayaraghavan *et al*., 2007). Biomass is known as by-products with no or low profit from agricultural crops or industrial processes. The production of biological hydrogen (biohydrogen) from biomass has gain wide attentions since it is one of the most reliable and sustainable energy for the future (Debabrata and Veziroglu, 2001, Levin *et al*., 2004). From the overview of environmental and engineering side, utilizing biomass wastewater or solid waste as a substrate for fermentation become an essential approach since it is capable for biohydrogen production in non-sterile conditions(Valdez-Vazquez *et al*., 2006)

In Malaysia, various type of biomass generated from palm oil mill processing, consist of empty fruit bunches, palm press fiber, palm kernel cake, palm kernel shell, sludge cake and palm oil mill effluent (POME) (Prasertsan and Prasertsan, 1996). POME is one of relatively potential as a substrate for generation of hydrogen, hence, the development of an improved fermentation process for this organic waste is needed (O-Thong *et al*., 2007, Vijayaraghavan and Ahmad, 2006, Atif *et al*., 2005). POME has been generated with an average values of 25 000 mg/L biochemical oxygen demand (BOD) and 50 000 mg/L chemical oxygen demand (COD), respectively (Yacob *et al*., 2005). Owning to its characteristic with high organic content, biohydrogen production could be achieved via dark fermentation as what have been shown in the previous study (Yusoff *et al*., 2009).

During anaerobic fermentation on carbohydrate-rich substrates, volatile fatty acids (VFAs), hydrogen (H_2), carbon dioxide (CO_2) and sometimes alcohols, are simultaneously produced as demonstrated in Fig 1

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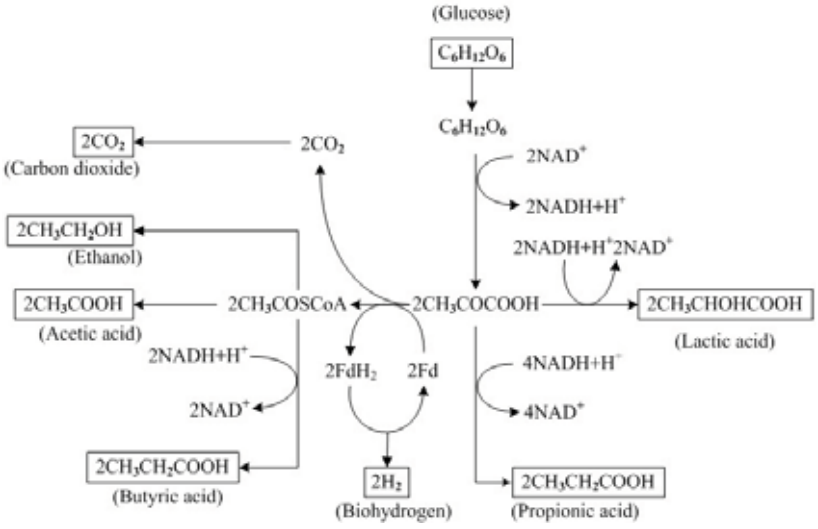

Fig. 1: Possible pathways of fermentative hydrogen evolution and other by-products during biohydrogen fermentation (Ren *et al*., 2006).

(Chen *et al*., 2006, Ren *et al*., 2006). VFAs are one of the by-products which might serve as limitation effect and could become a toxic to the fermentative bacteria depending on their concentration and as a result, the inhibition to the process would be occurred (Chen *et al*., 2006, Ren *et al*., 2006, Zhang *et al*., 2006, Van Ginkel *et al*., 2005). Apparently in anaerobic process, biohydrogen is produced during the exponential growth phase of clostridia (hydrogen producing bacteria). However during stationary phase, the reaction shifts from hydrogen production (gas phase) to solvent production phase. In the process, once hydrogen builds up, higher molecular weight acids such as butyric and propionic acids are accumulated in the system (Van Ginkel *et al*., 2005). The accumulation of soluble metabolites during biohydrogen production occurred due to enzyme synthesis, which is necessary for solvent production. Thus, the pH might drop to pH 3-4. If the pH is not controlled at the optimal range, it could inhibit the process because of microbial populations shift and some bacteria involved could not sustain its metabolic activity at pH values less than 5.0 and complete inhibition was reported in the pH range of 4.0-5.0 (Li and Chen, 2007, Venkata Mohan *et al*., 2007).

On the other hands, hydraulic retention time (HRT) become a vital parameters in controlling the anaerobic process. HRT also might play an important role in order to increase biohydrogen production as biomass maintained at certain density (Levin *et al*., 2004, Wu *et al*., 2008). In addition, the operation of continuous stirred tank reactor (CSTR) in the anaerobic fermentation at a high dilution rate will normally drive to the washout of biomass and tends to the operational instability and inefficient for biohydrogen production (Chen *et al*., 2006, Zhang *et al*., 2006). Therefore, HRT also impose a pivotal role to enhance cell retention under a high or low hydraulic loading rate. Since the system could retain high biomass content in different HRT, the biohydrogen production rate might be lifted to the optimum production at appropriate HRT.

In order to enhance the biohydrogen production in a large amount, the biohydrogen fermentation was carried out in 50 L continuous stirred tank reactor (CSTR). The fermentation was done in three different HRTs to evaluate their performance on biohydrogen production. In this study, the effects of HRTs and volatile fatty acids on the biohydrogen production rate, biohydrogen percentage and biomass concentration in the liquid effluent were evaluated during the course of biohydrogen fermentation from POME under non-sterile condition.

MATERIALS AND METHODS

Anaerobic Seed Sludge:

The anaerobic microflora used in this study was obtained from a settling tank of palm oil mill treatment plant at Serting Hilir Palm Oil Mill, Negeri Sembilan, Malaysia. The characteristics of sludge used were: volatile suspended solids (VSS), 15.1 g/L; total solids (TS), 45.9 g/L; pH 7.2; alkalinity, 1350 mg/ L as $CaCO_3$. Prior to heat-treatment, the sludge was settled down to get as much as solid which considered as biomass (Lin and Chang, 2004, Vijayaraghavan *et al*., 2007). Then, the sludge was heated at 80ºC for 20 min

to inactivate the hydrogentrophic methanogens and to harvest anaerobic spore-forming bacteria such as *Clostridium* sp (Lin and Chang, 2004, Yusoff *et al*., 2009). In order to provide an appropriate condition for biohydrogen producer, the seed sludge was undergo acclimatization phase with Reinforced Clostridium Medium (RCM) (Chong *et al*., 2009). The acclimatization phase was monitored based on gas production and biohydrogen concentration.

Substrate:

POME was taken from palm oil mill at Dengkil Selangor, Malaysia with a COD approximately 80 000-100 000 mg/L. The characteristic of POME used in this study is shown in Table 1.

Table 1: Characteristic of POME used in the biohydrogen fermentation.

Parameter	POME (mg/L)
Chemical Oxygen Demand (COD)	60,000 - 80,000
Biochemical Oxygen Demand (BOD_5)20°C, 5 days	20,000 - 35,000
Volatile Suspended Solid (VSS)	7,000 - 10,000
Total Solid (TS)	20,00 - 30,000
Oil & Grease	2,000 - 3,000
Lignin	1,000 - 1,500
Total Kjeldahl Nitrogen as (TKN)	200 - 450
Ammonium Nitrogen	100 - 200

Fermentation Operation:

Biohydrogen fermentation was carried out using 20 % (v/v) of acclimatized seed sludge with 20 000 mg/L VSS as inoculum. Slow feeding rate at 10 mL/min of POME with 50 000- 60 000 mg/L COD was used as substrate to supply carbon source. The fermentation time was started when the bioreactor was filling up with POME up to respective working volume. The operating hydraulic retention time (HRT) was set at 5, 3,and 2 days, respectively. At each HRT, the bioreactor was operated at 30 L working volume with pH controlled at 5.5, temperature monitored at 22-26ºC and mixing at 120 rpm. Nitrogen gas was purged to the medium for 30 min to provide anaerobic condition.

During the fermentation process, the development of gas production and its composition was monitored daily until the bioreactor naturally established a steady-state before shifted to another HRT. The steady-state term was considered when the product development (biohydrogen concentration) are stable with variation less than 15% (Mu *et al*., 2006, Shin *et al*., 2004, Yusoff *et al*., 2009). In the process, VFAs distribution, as well as solids concentration was monitored at 2 to 3 intervals day. The gas volumes were normalize (N) to standard temperature and pressure (STP) with temperature (0ºC) and pressure (760 mm Hg). (Kim *et al*., 2008).

Analytical Methods:

Total gas composition was determined by a gas chromatograph (Shimadzu Co., Kyoto GC- 8 A) equipped with a thermal conductivity detector (TCD) using a 1.83 m × 3.18 mm (inner diameter) stainless-steel column packed with Porapak-Q (80/100 mesh). The temperatures of column, injector and detector were kept at 50°C, 100°C and 100°C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 mL/min. Meanwhile, samples for organic acid analysis were centrifuged to remove suspended solid and supernatant was then filtered with 0.45µm pore size syringe filter before analyzed by HPLC (Shimadzu LC-10AS with UV-VIS detector SPD-10A) equipped with cation exchange resin column (300 x 7.8 mm Aminex HPX-87H column) and 4 mM of H_2SO_4 was used as mobile phase at a flow rate of 0.6 mL/min . TSS, VSS and COD were measured according to the standard methods (APHA, 1985).

RESULTS AND DISCUSSION

The Effect of HRT on Biohydrogen Production:

The effects of HRTs on the biohydrogen production rate, biogas composition and biomass concentration in the liquid effluent were investigated by many researchers and the experimental results showed that different HRTs influenced the biohydrogen production (Zhang *et al*., 2006, Wu *et al*., 2008). In this study, three different HRTs were evaluated on biohydrogen production from POME in the relation with VFAs.

Biohydrogen concentration increased gradually during the fermentation of biohydrogen from different HRT. The biogas produced in all set of experiments contained H_2, CO_2 and no methane gas was detected, suggesting that the facultative microbial flora as well as anaerobic microbe were competitive in the absence of methanogenic bacteria. The fastest steady state was observed at HRT 2 days. The biohydrogen concentration

attained was at the range of 27-30% after 14 days operation time, which is highest, compared to HRT 5 and 3 days. A faster steady state is important in order to cope with a bulk amount of waste substrate such as POME and becomes favorable to the industry due to energy requirement. On the other hands, with longer HRT, availability of CO_2 in the fermentation might be increased. Nath and Das, (2004) reported, by removing the CO_2 from the culture medium can increase the yield of biohydrogen. In this study, the biohydrogen concentration reduced at longer HRT operation due to mixing up with CO_2. The biohydrogen production seems diluted with CO_2. Regard to short HRT, the accumulation of CO_2 could be avoided and biohydrogen concentration could be increased.

The performance of biohydrogen in 50 L CSTR was monitored in term of biohydrogen yield, biohydrogen rate, CO_2/H_2 ratio as well as specific hydrogen production rate (SHPR). Table 2 summarizes the performance of biohydrogen production in different HRT. The highest yield obtained at HRT 2 days with 1054 NmL/L-POME as compared to the HRT 3 days (926 NmL/L-POME). The lowest yield was obtained at HRT 5 days, with 547 NmL/L-POME and the rate was 23 NmL/h/L-POME at steady state. The results obtained slightly higher as compared to Vijayaraghavan and Ahmad (2006). However, Atif *et al*., (2005) reported, that high yield of biohydrogen (4708 mL/L-POME) with concentration up to 60% in a batch fermentation under thermophilic conditions. Nazlina *et al*., (2009) also revealed that biohydrogen production in thermophilic condition gave higher yield as compared to the mesophilic condition. In this study, the operation temperature only achieved up to 26˚C, resulted in low biohydrogen concentration produced and affected on biohydrogen yield. Mu *et al*., (2006) and Yusoff *et al*., (2009) reported that temperature of 20 - 24°C is not the optimum temperature for biohydrogen fermentation and temperature was frequently a vital factor to the performance of biohydrogen fermentation.

Table 2: Performance of biohydrogen fermentation using POME in 50L CSTR at different HRT.

Time (Day)	CO_2 /H_2 ratio	Biohydrogen yield (NmL /L)	Biohydrogen production rate (NmL/h/l)	Specific biohydrogen generation rate (NmL/h/g VSS)	Accumulated Biohydrogen (NL)
1	10.1	80	3	1	0.5
4	6.1	201	8	2	4
8	5.7	290	12	3	10
12	8.1	238	10	2	17
16	4.6	433	18	4	25
20[a]	4.0	426	18	4	35
24[a]	4.3	460	19	4	47
27[a]	4.3	499	21	4	55
29[a]	3.8	547	23	5	62

a) Biogas profile during fermentation carried out at HRT 5 days.

Time (Day)	CO_2 /H_2 ratio	Biohydrogen yield (NmL /L)	Biohydrogen production rate (NmL/h/l)	Specific biohydrogen generation rate (NmL/h/g VSS)	Accumulated Biohydrogen (NL)
1	9.2	108	5	2	1
4	7.0	206	9	4	7
8	3.3	504	21	10	21
12	2.8	619	26	11	45
16	2.6	818	34	14	75
20[a]	3.3	819	34	13	106
24[a]	2.8	926	39	15	141

b) Biogas profile during fermentation carried out at HRT 3 days.

Time (Day)	CO_2 /H_2 ratio	Biohydrogen yield (NmL /L)	Biohydrogen production rate (NmL/h/l)	Specific biohydrogen generation rate (NmL/h/g VSS)	Accumulated Biohydrogen (NL)
1	19.0	53	2	2	1
3	9.0	136	6	4	5
6	5.7	302	13	8	17
9	4.0	474	20	15	35
12	3.2	711	30	22	62
15[a]	2.4	1026	43	33	103
18[a]	2.3	1054	44	35	151

c) Biogas profile during fermentation carried out at HRT 2 days.

([a]steady state operation. Nml, the volume was adjusted to standard temperature and pressure (STP); temperature of 273.15 K (0 C) and absolute pressure of 101.325 kPa (760 mmHg)).

Carbon dioxide was generated dominantly at initial fermentation for all experiments conducted. The CO_2/H_2 ratio at initial process was higher than the theoretical value which the ratio should be close between 0.5 – 1(Wang *et al*., 2007). For the experiment at HRT 5 days, the ratio was started with 10.1 and gradually

decreased down to 3.8. The decrease in the ratio indicated that the biohydrogen concentration gradually increased in the biogas produced. As the case of HRT 3 days, the CO_2 was dominant at the initial stage and gave the higher ratio. However for the HRT 2 days, the ratio indicated slightly divert from the other experiments. At initial fermentation, CO_2 still remain as a major gas in biogas produced. Later, the ratio was reduced at day 3 about 2 folds and this demonstrated that hydrogen producing bacteria take an adaptation very fast and became dominant in the reaction process due to short HRT. As a result, the biohydrogen content increased and decreased the ratio to 2.3. In the other aspect, the higher ratio at initial process due to high volume of POME contained soluble O_2 introduced in the system (oxidation-reduction potential, ORP 70 – 200 mV). As described by Chynweth and Isaacson, (1987) an obligate anaerobes are performed in a highly reduced environment and having a highly negative potential and widely recognized in anaerobic digestion. It was suggested that raw POME should undergo another pre-treatment prior to utilize in order to reduce soluble O_2 for improve result in term of yield and operation time. From the observation, it is assumed that the initial stage of the fermentation was dominated with facultative bacteria, which utilizing soluble O_2 in the POME and produced a lot of CO_2 as biogas. Eq 1.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (1)$$

All experiments shown the change in biohydrogen rate at steady state. The rate was gradually increased from the day 1 and become stable at the steady state with 23, 39 and 44 N mL/h/L-POME at HRT 5, 3, and 2 days, respectively. The rate obtained was in agreement to Lee *et al*., (2006). The decrease in HRT would increase the organic loading rate and vice versa. Therefore, with the decrease of HRT, more substrate is required to feed into the system and consequently, biohydrogen production rate should be increased. On general basis, biohydrogen production rate might be lifted up since the hydrogen producing bacteria population maintained in the process with VSS as indicator (Lee *et al*., 2006). The biohydrogen production rate obtained in this study was higher compared to Wang *et al*., (2007).

Meanwhile for SPHR, the HRT 2 days gave the maximum value (35 NmL/h/gVSS) followed by HRT 3 days and 5 days with 15 NmL/h/gVSS and 5 NmL/h/gVSS, respectively. Conceding on the data obtained, although the VSS kept maintained at 20–28 g/L, but the SPHR for each HRTs shown a different trend. For the operation at short HRT, the SPHR is higher as compared to the high HRT operation. Hence it was compromise that the HRT was the one of the accountable parameter for biohydrogen fermentation. Therefore, in order to build up the production of biohydrogen in a bulk amount, HRT should be one of the crucial parameter to be considered. HRT is also important to control the biohydrogen concentration in biogas produced since the reaction is very fast. The biohydrogen produced is needed to capture as soon it was released. From the anaerobic degradation pathway, biohydrogen can be harvested during second and third stage of anaerobic digestion which is acidogenesis and acetogenesis process (Chong *et al*., 2009).

In this study, the HRT revealed a significant and vital parameter for the biohydrogen production from POME in a 50 L CSTR. The short HRT gave a higher yield as well as the production rate for biohydrogen production since the operation was maintained in the optimal condition such as pH, temperature and biomass concentration (Hawkes *et al*., 2002, Shin *et al*., 2004).

The Effect of VFAs on Biohydrogen Production:

During the biohydrogen fermentation from glucose, the substrate was converted to VFAs, H_2, alcohols, CO_2 and biomass. As well as degradation of organic material like POME in biohydrogen fermentation, it always accompanied with the production of soluble metabolites in aqueous phase through anaerobic metabolic pathway (Ren *et al*., 2006, Wang *et al*., 2007, Zhang *et al*., 2006, Zheng and Yu, 2005).

In biohydrogen fermentation, pH is one of the greatly influenced factors in order to give an optimum condition (Khanal *et al*., 2004, Mu *et al*., 2006). The pH value was found associated with the effect of VFAs which arise as soluble metabolites. In this operation, VFAs produced were monitored to investigate the effect and interrelation to the yield of biohydrogen. According to Zheng and Yu (2005) an excess production of VFAs might result in an inhibitory effect on the biohydrogen fermentation. Hence, undissociated forms of VFAs accumulated inside the culture are freely able to permeate inside the plasma membrane, the action might interrupt the cell metabolisms consequently disturb cell activity as well as cell growth (Zhang *et al*., 2006). The major VFAs detected in the process were acetic acid (HAc), propionic acid (HPr) and butyric acid (HBr). The typical VFAs production profile in this study is summarized in Table 3. According to the stoichiometric correlations, composition of VFAs was often closely related with the yield and performance of the biohydrogen production in the fermentation. Theoretically, 2 mol of HAc or 1 mol of HBr as end product is accompanied

with 4 mol or 2 mol of H_2, respectively (Eq 2 and 3) (Wu *et al*., 2008).

Table 3: Distribution of soluble metabolites during fermentative biohydrogen using POME in 50 L continuous stirred tank reactor at different HRT.

Time (day)	Hac (mg/L)	Hbu (mg/L)	Hpr (mg/L)	Ratio Hbu/HAc
4	0	471	2987	0
8	7372	471	4576	0.2
12	3257	1404	7366	0.6
16	0	1956	3191	0
20	0	1708	2920	0
24	0	1788	7657	0
26	0	1359	7561	0
29	0	3756	6846	0
a) VFAs profile during fermentation carried out at HRT 5 days.				
Time(day)	HAc(mg/L)	HBu(mg/L)	HPr(mg/L)	Ratio Hbu/HAc
2	2768	2162	345	0.8
4	2833	0	334	0
8	6035	0	512	0
12	5768	0	2242	0
16	7351	8069	2120	1.1
20	8507	5919	6871	0.7
24	9160	4381	6242	0.5
b) VFAs profile during fermentation carried out at HRT 3 days.				
Time(day)	HAc(mg/L)	HBu(mg/L)	HPr(mg/L)	Ratio Hbu/HAc
1	674	3567	0	5.3
2	1179	2764	0	2.3
4	3896	2952	0	0.8
6	2405	2149	0	0.9
8	2495	2285	0	0.9
12	1904	2477	487	1.3
16	2834	3501	369	1.2
18	3118	3715	423	1.2

c) VFAs profile during fermentation carried out at HRT 2 days.

During fermentation at HRT 5 days (Table 3a), HPr and HBr showed dominant in the whole fermentation. The result shown the yield obtained was not maximum since the HAc produced at initial stage of fermentation and then depleted with the increased of HPr. The high concentration of HPr was not only resulted a lower yield of biohydrogen but also affect biohydrogen concentration since the HPr concentration started to accumulate up to 7 g/L at day 24. The biohydrogen concentration in biogas produced was reduced about 10%. During the fermentation, HPr was remained higher as dominant VFA in the system compared to Vijayakrishnan and Ahmad (2006) (1.2-1.5g/L) and Kim *et al*., (2008) reported, about 2000 mg/L.

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

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + \mathbf{2H_2} + 2CO_2 \quad (3)$$

$$C_6H_{12}O_6 + \mathbf{2H_2} \rightarrow CH_3CH_2COOH + 2H_2O \quad (4)$$

In comparison with others HRT, a different patent revealed from the HRT 3 and 2 days, (Table 3b and Table 3c). Once the HRT reduced, HAc started to accumulate. Unfortunately for HRT 3 days, HPr gradually increased in the fermentation, simultaneously biohydrogen concentration started to decrease and remain constant since HPr maintain at concentration at 5 – 8 g/L. From the observation, the performance of biohydrogen fermentation was interrupted due to the accumulation of HPr. Regard to Eq 4, 1 mol of HPr produced might not give any H_2 production but consumed by the bacteria involved (Ren *et al*., 2006). In the HRT 2 days, a significant improvement was observed. The HPr was not detected at initial stage of fermentation and only about 300 – 400 mg/L was observed during steady state. The pattern of the VFAs accumulation seems followed the optimal pathway with HAc tends to accumulate and give a higher yield in biohydrogen production (Eq 2). The HAc detected around 1-5g/L along the fermentation process as well as HBr kept maintained at 1-3g/L. Zheng *et al*., (2005) reported that, high concentration HBr might inhibited biohydrogen production. In this study, the results not shown any inhibition effect from HBr accumulation since the concentration kept maintain at low concentration (3g/L) and it was operated in the continuous operation with short HRT compared to batch fermentation operation.

In biohydrogen fermentation, HBu/HAc ratio has been used as an indicator for the present of biohydrogen in an acidogenesis phase (Chen *et al*., 2001, Debabrata and Veziroglu, 2001, Cheng *et al*., 2008). The ratio has been frequently used as a monitoring parameter for biohydrogen yield. A few reports showed the optimal HAc/HBr ratio for biohydrogen fermentation varies with the differences in the substrate and cultures used. For instance, the optimal HBu/HAc ratio for biohydrogen production using glucose or sucrose as a substrate was 5.0 (Chen *et al*., 2001) and Kim *et al* (Kim *et al*., 2008) obtained the ratio about 4.0, meanwhile Cheng *et al*., (2008) reported the optimum ratio obtained was 1.79 using starch as a carbon sole. However, on the ratio alone, was not a significant indication to justify the performance of biohydrogen fermentation, however, some other factors should also be considered such hydrogen yield, hydrogen production rate and hydrogen content (Kim *et al*., 2006, Wang *et al*., 2007).

According to Khanal (2004), that an increase in the HAc/HBr ratio should be accompanied by an increased production of biohydrogen yield and vice-versa. In this study at HRT 5 days, no HAc was detected and the fermentation pathway was favored to follow Eq 3 and 4 where the propionic acid bacteria and butyric acid bacteria was dominated (Kim *et al*., 2008). For the HRT 3 days, the HBr/HAc ratio was improved since the ratio remain constant at 0.5 – 1.1. However HPr accumulated at the concentration up to 6g/L at steady state. Since the HPr accumulated, the biogas concentration also reduced about 8% in the biogas content. Despite the ratio was quite far from the optimal, but the HAc started to accumulate and would contribute in the bohydrogen production instead of HPr.

Exclusively at HRT 2 days, the HBr/HAc ratio was 5.3 at the initial stage and gradually decreased to ratio 1.2 and attained to avoid HPr accumulation in the soluble metabolites. According to the results obtained, with decrease of HRT (2 days), the biohydrogen concentration significantly increased to 30% in the biogas content. The HAc and HBr produced in soluble metabolites gradually increased in initial stage and finally almost constant in the steady state. From the observation, with decrease the HRT, it might change the bacteria communities involved in the process since HAc and HBr were dominant in soluble metabolites and affect on biohydrogen yield. On the other hands, beside the HRT itself, the optimal ratio of HBr/HAc also could be controlled by environmental conditions such as pH, mixing intensity, organic loading rate and nutrients supplementation (Han and Shin, 2004, Khanal *et al*., 2004).

Conclusions:

Production of biohydrogen in 50 L CSTR was successfully operated in different HRT (5, 3 and 2 days). HRT 2 days gave an optimal operation with maximum biohydrogen yield and biohydrogen rate of 1054 NmL/L-POME and 44 NmL/h/L-POME, respectively with maximum biohydrogen concentration 30%. Meanwhile the VFAs as soluble metabolites affected the biohydorgen fermentation, especially HPr. HPr interrupted the efficiency of the fermentation process and consequently reduced the amount of biohydrogen production by 8-10%. HRT and VFAs showed the vital parameters that effect the biohydrogen production and should be considered in biohydrogen fermentation.

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