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Research article

Effects of volatile fatty acids in biohydrogen effluent on biohythane production from palm oil mill effluent under thermophilic condition

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ABSTRACT

Background: Biohydrogen effluent contains a high concentration of volatile fatty acid (VFA) mainly as butyric, acetic, lactic and propionic acids. The presence of various VFAs (mixture VFAs) and their cooperative effects on two-stage biohythane production need to be further studied. The effect of VFA concentrations in biohydrogen effluent of palm oil mill effluent (POME) on methane yield in methane stage of biohythane production was investigated.
Results: The methane yield obtained in low VFA loading (0.9 and 1.8 g/L) was 15–20% times greater than that of high VFA loading (3.6 and 4.7 g/L). Butyric acid at high concentrations (8 g/L) has the individual significantly negative effect the methane production process ($P < 0.05$). Lactic, acetic and butyric acid mixed with propionic acid at a concentration higher than 0.5 g/L has an interaction significantly negative effect on the methanogenesis process ($P < 0.05$). Inhibition condition had a negative effect on both bacteria and archaea with inhibited on *Geobacillus* sp., *Thermoanaerobacterium thermosaccharolyticum*, *Methanoculleus thermophilus* and *Methanothermobacter delfuvii* resulting in low methane yield.
Conclusion: Preventing the high concentration of butyric acid, and propionic acid in the hydrogenic effluent could enhance methane production in two-stage anaerobic digestion for biohythane production.

1. Introduction

There are varieties of technologies for biological hydrogen production. Hydrogen production by microbial fermentation of organic waste is considered an environmentally friendly process with a high production rate from organic waste under realistic conditions approaching practical levels [1,2]. The microbial fermentation process can utilize organic materials such as cellulose and starch contained in common agricultural and food industry waste [3]. Some food industry waste products such as cheese whey, olive mill, oil palm mill and baker's yeast industry waste have been successfully used for hydrogen gas production at high production rates [3]. The hydrogen yield from organic waste such as apple processing wastewater, potato processing wastewater, food waste, starch processing wastewater and palm oil mill effluent have been previously measured at rates of 92, 128, 57, 92 and 115 mL H_2/gCOD, respectively [4,5,6,7]. Palm oil mill effluent (POME) is a suitable substrate for hydrogen production, and there are large amounts of POME generated in Thailand. It is estimated that around 94 million tons of POME is generated per year [8]. Successful hydrogen production from POME through fermentative processes was previously achieved under thermophilic conditions by O-Thong et al. [7]. Prasertsan et al. [9] obtained a continuous biohydrogen production rate (HPR) of 9.1 LH_2/L/d (16.9 mmol H_2/L/h) from POME. They used *Thermoanaerobacterium*-rich sludge under thermophilic conditions at optimum HRT values of 2 days, with an OLR of 60 g COD/L/d in an anaerobic sequencing reactor (ASBR).

However, the primary challenge for biohydrogen production is the low substrate conversion efficiency. It must be overcome before biohydrogen can become economically feasible. In a conventional microbial fermentation process, only about 7.5–15% of the energy contained in organic waste is converted to H_2. The rest of the energy is contained in volatile fatty acids (VFAs) [1,10]. However, 65% of the energy contained in the organic waste still remains in the liquid as VFAs mainly as butyric, acetic, lactic and propionic acids. Consequently, VFAs could be converted into a suitable product or energy carrier such as methane via methanogenesis by methanogens under anaerobic digestion [3,11]. The conversion of VFAs to CH_4 through anaerobic digestion (AD) is a faster and simpler than a conversion of VFAs to H_2 by photofermentation and a microbialelectrolysis process [1]. In addition, it has been shown to be an energy-efficient strategy

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for the production of a mixture of H_2 and CH_4, known as biohythane [12,13]. Biohythane can be used as a chemical, or as an energy carrier in gas combustion engines. Biohythane can be realized through a two-stage microbial fermentation [14]. In the first stage, the substrate is fermented to hydrogen and VFAs. VFAs are then converted to methane in the second stage [3,15]. The fermentation products from the hydrogen production process are very important for the entirety of the biohythane system performance because they can affect the loading, efficiency and running stability of the methanogenesis phase [16]. The effect of VFAs on methane production stage of two-stage thermophilic hydrogen fermentation and methanogenesis for biohythane production is still a lack of information.

The conversion rate from VFAs to acetic acid will affect the methanogenic archaea quantity, and subsequently, affect the degradation rate of acetic acid and methane yield. The accumulation of propionic acid typically results in the failure of methanogenesis. Siegert and Banks [17] found that VFA concentrations above 2 g/L led to inhibition of cellulose degradation, while VFA concentrations above 4 g/L caused only minimal inhibition of glucose degradation. Demirel and Yenigun [18] concluded that propionic acid would inhibit methanogenic archaea growth when its concentration was above 0.95 g/L. Researchers who have studied the inhibition of methanogenic archaea caused by a single VFA have found little information about the effects of mixed VFA on methanogenic archaea [19]. In two-stage thermophilic hydrogen fermentation and methanogenesis for biohythane, the presence of various VFAs (mixture VFAs) and their cooperative effects need to be further studied in order to better understand their impacts on methane production and methanogens community.

This work aimed to study the effects of a mixture of VFA concentrations contained in biohydrogen effluent on methane yield and microbial community for biohythane production via two-stage thermophilic hydrogen fermentation and methanogenesis.

2. Materials and methods

2.1. POME hydrogenic effluent and inoculum

The POME hydrogenic effluent was collected from a pilot-scale CSTR for hydrogen production under thermophilic conditions. POME hydrogenic effluent was analyzed for its physical–chemical parameters. The characteristics of the POME hydrogenic effluent employed are shown in Table 1. The POME hydrogenic effluent was stored in a sealed container and kept in a cold room at 4°C until it was used. The inoculum for the batch assays was collected from a pilot scale thermophilic biogas reactor (60°C) digesting POME. Thermophilic biogas inocula were placed in an incubator for 5 days until biogas production was ended in order to minimize the contribution of biogas from residual organic materials contained in the inoculum. The inoculum contained 8.1% total solids (TS), 6.2% volatile solids (VS), and 52.1 g/L volatile suspended solids (VSS).

Table 1
Characteristics of POME hydrogenic effluent.

Component	Concentration
Total solids (g/L)	68
Volatile solids (g/L)	62
Total nitrogen (g/L)	2.3
Lipid (g/L)	8.1
Total acids (g/L)	9.4
Acetic acid (g/L)	2.13
Butyric acid (g/L)	3.95
Lactic acid (g/L)	1.5
Propionic acid (g/L)	0.25
Ethanol (g/L)	0.99
Alkalinity ($gCaCO_3$/L)	1.3
pH	4.3

2.2. Biomethane potential of POME hydrogenic effluent

The biodegradability and biomethane potential of POME hydrogenic effluent were identified in batch assays under the thermophilic condition, as described previously by Angelidaki et al. [20]. The POME hydrogenic effluent was tested at different initial VS loading levels of 11.8, 17.7, 23.6 and 29.5 gVS/L, corresponding to initial volatile fatty acid loading of 0.9, 1.8, 3.6 and 4.7 g/L, respectively. Additionally, positive controls with 2 g/L of acetic acid and 2 g/L of butyric acid instead of samples were also included for testing the inoculum quality. Negative control with water instead of the sample was included for testing the substrate contamination in the inoculum. POME hydrogenic effluent was mixed with inoculums and adjusted pH to 7.0 by 1 M NaOH. Biogas production was determined through the use of the water replacement method [21]. Biogas composition in the headspace of the vials was monitored by GC-TCD. The gas produced by the negative control vials with inoculum only was subtracted from the actual gas produced through digestion of each treatment.

2.3. Experimental design

Response surface methodology (RSM) with central composite design (CCD) was employed to investigate the effect of lactic, acetic, butyric and propionic acid on methane production. The different concentrations of acetic, lactic, butyric and propionic acid were added into the POME hydrogenic effluent at the levels indicated according to Table 2. The amount of VFA addition into POME hydrogenic effluent depends on the concentration of VFA as a function of organic loading increase from a previous report by Prasertsan et al. [9]. The assay was conducted as batch cultivations in 500 mL serum bottles. In each bottle, 160 mL of inoculum and 40 mL of POME hydrogenic effluent supplemented with different concentrations of each VFA were added. All serum bottles were adjusted an initial pH to 7 with 1 M NaOH. Mixtures of inoculum and POME hydrogenic effluent were subsequently purged with N_2:CO_2 (80:20) to ensure anaerobic conditions. Afterward, the bottles were closed with butyl stoppers and placed in a 60°C incubator for 45 days. The experiments were run in triplicate. The evolved gas was collected every day with a gas-tight syringe (Hamilton, Reno, NV, USA), and the volume measured by a water displacement method using a graduated cylinder filled with acidic water in order to prevent dissolution of the gas components [21]. Methane production in the headspace of each vial was monitored by GC-TCD. The microbial community structure was determined by PCR-DGGE. Lactic acid (X_1), acetic acid (X_2), butyric acid (X_3) and propionic acid (X_4) were chosen as four independent factors in the experimental design (Table 2). The methane accumulation and methane yield were selected as the dependent output variables. The cumulative methane production curves can be identified over the time course of a batch experiment. A quadratic model can be used to evaluate the effects of factors [22]. The Design-Expert 7.0 version (Stat-Ease Inc., MN, USA) software was used for regression and graphical analysis of the experimental data obtained. The quality of the quadratic model was expressed by the coefficient of determination R^2 and its statistical significance was checked by t-test.

2.4. Microbial community analysis

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was used to study microbial community structure in the methane production. Total genomic DNA was extracted from sludge samples from each batch test by using the method described by Kongjan et al. [23]. First, Genomic DNA was used as a template for PCR reactions with a specific primer pair (Arch21f and Arch958r for archaea population and 1492r and 27f for bacteria population) [24,25]. DGGE analysis of the amplicons obtained from the second PCR was performed as previously described [26,27]. Most of the bands were

Table 2
A central composite experimental design with four independent variables and results of methane production and methane yield.

Run	Parameter (g/L)					Response	
	Lactic acid (A)	Acetic acid (B)	Butyric acid (C)	Propionic acid (D)	Methane ($mLCH_4$)	Methane yield ($mLCH_4/gVS$)	Biodegradability (%)
1	1.5	5.5	4.25	1.5	963	364	85
2	3	8	3	0	943	374	83
3	1.5	5.5	5.5	1.5	1080	389	93
4	1.5	5.5	5.5	1.5	1069	385	91
5	1.5	5.5	6.75	1.5	1019	339	75
6	1.5	4.25	5.5	1.5	1008	381	88
7	1.5	5.5	5.5	2.25	1008	349	78
8	0	8	3	3	1016	383	89
9	3	8	8	0	1038	302	65
10	0	3	8	0	1014	394	92
11	3	3	3	3	1008	413	99
12	3	3	8	3	1133	338	78
13	0.75	5.5	5.5	1.5	1056	392	93
14	1.5	5.5	5.5	1.5	1080	389	93
15	1.5	5.5	5.5	1.5	1069	385	91
16	1.5	6.75	5.5	1.5	1070	368	85
17	0	3	3	0	744	446	100
18	1.5	5.5	5.5	0.75	1016	381	88
19	0	8	8	3	1098	308	68
20	2.25	5.5	5.5	1.5	1104	387	92
21	1.5	5.5	5.5	1.5	1116	402	98
H_2 effluent	0	0	0	0	380	475	98

excised from the gel and re-amplified. After re-amplification, PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by database searches in Gene Bank using BLAST [28]. Similarity indices of the compared band profiles were calculated from the densitometric curves of the scanned DGGE band profiles by using the Dice product–moment correlation coefficient. The Dice correlation coefficient was directly applied to the array of densitometric values forming the fingerprint. The coefficient is robust and objective since whole curves are compared and subjective band scoring is omitted. Clustering of patterns was calculated using the unweighted-pair group method using arithmetic mean (UPGMA).

2.5. Analytical methods and calculation

The biogas composition was measured by a gas chromatograph (GC-8A Shimadzu) equipped with thermal conductivity detector (TCD) and fitted with a 2.0 m packed column (Shin-Carbon ST 100/120 Restek) [29]. Fermentation end products (volatile fatty acids and ethanol) in the supernatant were determined by gas chromatography (HP6850, Hewlett Packard) equipped with a flame ionization detector (FID) and Stabilwax-DA column (dimensions 30 m × 0.32 mm × 0.25 mm). Lactic acid was analyzed with a high-performance liquid chromatography (HPLC; Agilent 1200 series), equipped with an Aminex® HPX-87H ion exclusion column. The mass balance was made on a VS basis as described by Cullis et al. [30]. Chemical oxygen demand (COD), total solids (TS), volatile solids (VS), volatile suspended solids (VSS), pH, total nitrogen, and alkalinity were measured according to standard methods for the examination of water and wastewater [31]. The theoretical methane potential was calculated according to Bushwell's formula, which is derived by stoichiometric conversion of the compound of CH_4, CO_2, and NH_3 [32].

3. Results and discussion

3.1. Biomethane potential of POME hydrogenic effluent

The POME hydrogenic effluent was composed of 9.4 g/L of volatile fatty acids, 8.1 g/L of lipids, 68 g/L of total solids and low pH (4.3) (Table 1). The volatile fatty acids contained in the POME hydrogenic effluent were mainly composed of butyric and acetic acid. Butyric acid, acetic acid, propionic acid, lactic acid and ethanol contained in the POME hydrogenic effluent were 3.95, 2.13, 0.25, 1.5 and 0.99 g/L, respectively. The methane yield from the POME hydrogenic effluent at an initial volatile fatty acid loading of 0.9, 1.8, 3.6 and 4.7 g/L, respectively, was 510, 467, 428 and 401 mL CH_4/gVS (Fig. 1). Low methane yields at high volatile fatty acid loading indicated that a high

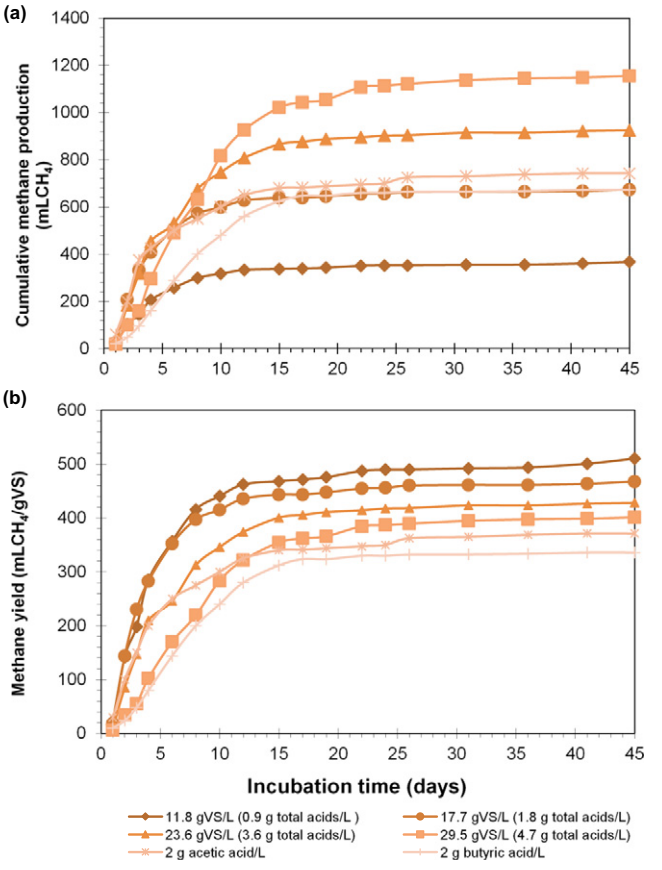

Fig. 1. Cumulative methane production (a) and methane yield (b) from POME hydrogenic effluent.

concentration of VFAs would affect the methane production process. Cumulative methane production at an initial volatile fatty acid loading of 0.9, 1.8, 3.6 and 4.7 g/L was 367, 673, 925 and 1155 mL CH_4. The cumulative methane production from acetic acid and butyric acid was 742 and 671 mL CH_4, corresponding to methane yields of 371 and 335 mL CH_4/gVS respectively, indicating a good quality of seed inocula. For VFA loading of 0.9 and 1.8 gVS/L, more than 90% of the methane production could be achieved within 6 days, indicating that they were very easily degradable. Methane production also reached a stationary phase within 6 days and resulted in methane yields of 510 and 467 mL CH_4/gVS, which was significantly higher than the yields of 428 and 410 mL CH_4/gVS (Fig. 1b) achieved at VFA loading of 3.6 and 4.7 g/L at the end of the 45 days of digestion. The methane yield obtained in low VFA loading (0.9 and 1.8 g/L) was 15–20% times greater than that of high VFA loading (3.6 and 4.7 g/L). VFAs are known as valuable substrates for methane production, but high concentrations of acetic acid, butyric acid and propionic acid cause inhibition of methanogenesis [33]. The POME hydrogenic effluent was easily degradable, and the methane development was fast at low VFA loading. On the contrary, the POME hydrogenic effluent at high VFA loading had poor biodegradability due to its high content of VFA. However, not all organic matter contained in the POME hydrogenic effluent could be completely degraded and converted into methane at high VS loading. As indicated by the biodegradability (Table 3), 76–89% of theoretical methane potential was achieved for POME at high VFA loading, while 97% of theoretical methane potential was achieved for low VFA loading.

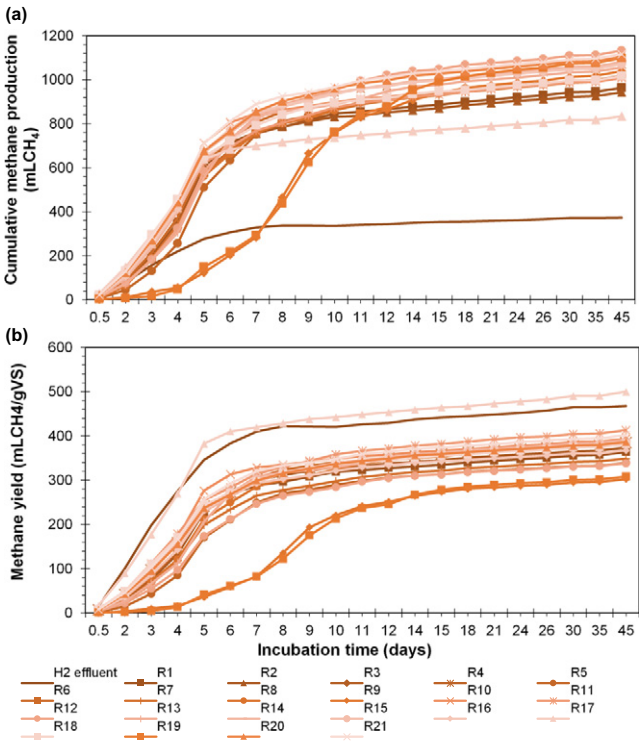

Fig. 2. Cumulative methane production (a) and methane yield (b) from POME hydrogenic effluent with additions of VFA at different concentrations.

3.2. Effect of VFAs on methane production

The degradation efficiency of lactic acid and VFAs was higher than 80% except for R5, R7, R9, R12 and R19, which had degradation efficiencies of lactic acid and VFAs of 75, 78, 65, 78 and 68%, respectively (Fig. 2). However, serious accumulation of butyric acid and propionic acid appeared in R9 and R19 (Fig. 3a). In these experiments, the highest concentrations of acetic acid and butyric acid reached 8 and 8 g/L, respectively, and resulted in the interruption of methane production (Fig. 2). Franke-Whittle et al. [34] also found that butyric and propionic acids at high concentration have inhibition effect on the methane production rate. The butyric acid concentration of 8 g/L mixed with other acids also showed significant inhibition of the bacteria and archaea communities by decreasing their number and diversity. These effects resulted in the accumulation of butyric and propionic acid, with consequently resulted in a low methane yield (302–308 ml CH_4/gVS) and low biodegradation efficiency (65–68%) (Table 3). Before being degraded to methane, all VFAs are first degraded to acetic acid and the conversion rates of VFAs to methane vary in the order of acetic acid > ethanol > butyric acid > propionic acid [35], resulting in an accumulation of butyric acid and propionic acid in the process. In addition, the propionic acid accumulation can inhibit the activity of methanogenic archaea and lead to cessation of fermentation [35]. Barredo and Evison [36] pointed that the methanogenic archaea quantity could decrease according to the propionic acid concentration increased. Wang et al. [35] found that when the pH was 7 and the propionic acid concentration was 5000 mg/L, the methane yield decreased to 22–38%, and indicated that the inhibition would be greatly strengthened when pH was decreased.

Acetic acid alone at the concentration of 3–8 g/L did not have an inhibition effect on the methanogenesis process. However, a mixture of acetic acid and propionic acid had significant interaction inhibition effects on methane production and biodegradation efficiency ($P < 0.05$) (Table 4). Butyric acid alone at the concentration of 8 g/L had a significant individual inhibition effect on the methanogenesis process ($P < 0.05$). The mixture of butyric acid and propionic acid had significant interaction inhibition effects on methane production and biodegradation efficiency, while more severe than butyric acid alone. The mixture of lactic and propionic acid had interaction negative effects on the methanogenesis process ($P < 0.05$). Lactic, acetic and butyric acid contained in POME hydrogen effluent had relation to high methane yield and methane production, while mixed with propionic acid has an adverse effect on methane yield. The accumulation of propionic acid also has inhibition effect on biohydrogen production [37]. Preventing propionic acid accumulation in the hydrogen fermentation process could enhance methane production in two-stage anaerobic digestion for biohythane production. An optimization analysis showed that lactic, acetic, butyric and propionic acid at concentrations of 2.88, 5.01, 0.44 and 5.55 g/L, respectively, led to the maximum accumulative methane of 1194 mL CH_4 and a methane

Table 3
Summary of biochemical methane potential methane yield and biodegradability of POME hydrogenic effluent.

Feedstock	Initial loading (gVS/L)	VFAs loading (g/L)	BMP yield (mLCH_4/gVS)	Theoretical yield (mLCH_4/gVS)	Biodegradability (%)
Control (acetic acid)	2	2	371	373	99
Control (butyric acid)	2	2	335	530	63
20% v/v POME hydrogenic effluent	11.8	0.9	510	522	97
30% v/v POME hydrogenic effluent	17.7	1.8	467	522	89
40% v/v POME hydrogenic effluent	23.6	3.6	428	522	82
50% v/v POME hydrogenic effluent	29.5	4.7	401	522	76

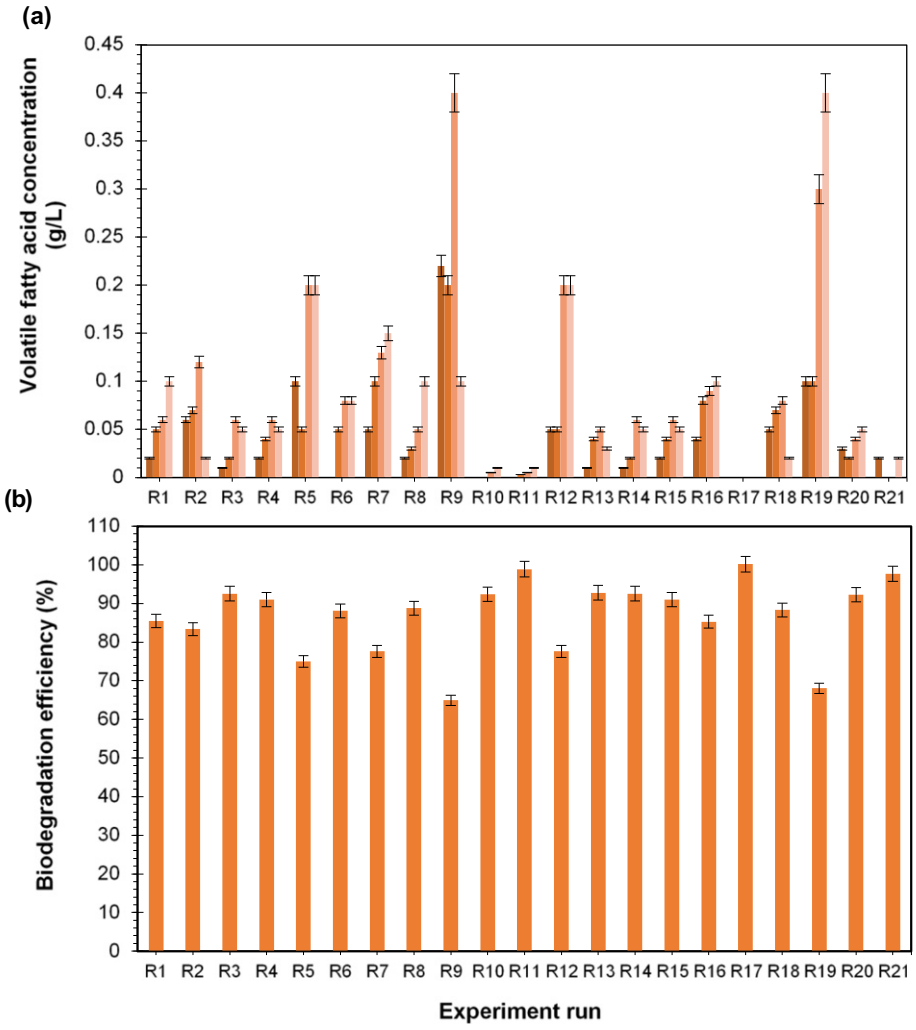

Fig. 3. Concentrations of lactic acid, acetic acid, butyric acid and propionic acid in fermentation liquid at the end of incubation (a) and biodegradation efficiency (b) from POME hydrogenic effluent with additions of VFA.

yield of 447 mL CH_4/gVS. The concentration of propionic acid was lower than 0.5 g/L and mixed others have no toxic effect on methane production process. Demirel and Yenigun [18] also found that propionic acid would inhibit the methanogenesis process when its concentration was above 951 mg/L. Considering VFA concentration in POME hydrogenic concentration range of 0.25–3.95 g/L (corresponding total VFA concentration of 9.4 g/L), an optimization lactic, acetic, propionic and butyric acid concentrations should be 2.88, 5.01, 0.44 and 5.55 g/L, respectively to avoid substrate inhibition in methanogenic process.

Table 4
Coefficients, t-statistics and significance probability of the effects of volatile fatty acids on methane accumulation, methane yield, and biodegradation efficiency.

Factor	Methane accumulation		Methane yield		Biodegradation efficiency	
	Coefficient estimate	Probability	Coefficient estimate	Probability	Coefficient estimate	Probability
Intercept	1053.5	–	378.4182	–	88.75592	–
A-Lactic acid	48	0.4138	-5	0.8238	-1	0.9072
B-Acetic acid	62	0.3001	-13	0.5674	-3	0.7279
C-Butyric acid	70.58824	0.0018*	-33.7059	0.0006*	-8.58824	0.0051*
D-Propionic acid	-8	0.8885	-32	0.1871	-10	0.2699
A^2	-72.5	0.2457	-22.75	0.3441	-9.25	0.3173
B^2	-16.5	0.2728	-2.5	0.6581	-1.25	0.5657
C^2	37.5	0.5305	15	0.5236	5	0.5770
D^2	-27.25	0.0933	-2.5	0.6581	-1.25	0.5657
AB	16.75	0.7763	8	0.7304	2	0.8214
AC	-19.75	0.1986	-3.25	0.5674	-2	0.3685
AD	184.1323	0.0201*	75.21232	0.0100*	26.82723	0.0010*
BC	20.13225	0.8389	15.21232	0.6973	2.827229	0.8495
BD	-171.868	0.0980*	-76.7877	0.0510*	-23.1728	0.0155*
CD	-87.8677	0.0898*	-22.7877	0.0533*	-11.1728	0.0463*

* Significant at 95% level ($P < 0.05$).

Mixtures of acetic with propionic acid, and butyric with propionic acid at high concentration (5–8 g/L) had significant negative interaction effects ($P < 0.05$) on the methanogenesis process. These findings indicate that acetic acid and butyric acid mixed with propionic acid can repress the microbial activity in the methanogenesis process, resulting in greatly decreased degradation rates of VFAs and consequent VFA accumulation. Among all the VFAs, methanogens have the lowest tolerance for inhibition by propionic acid. Complete inhibition triggered by propionate occurred at 5 g/L, which was two times lower than the inhibition concentration of acetate and butyrate. However, there was significant inhibition at higher acetate and butyrate concentrations of 2.4 g/L and 1.8 g/L, respectively [38].

3.3. Effect of VFAs on microbial community

As shown in the DGGE profile in Fig. 4, bacteria had similarities in numbers (band density) and diversity (number of the band) in most of the experiments except R1, R3, R4, R14, R15, R2, R9, and R19. The experiments R1, R3, R4, R14, R15, and R2 had small differences in microbial community, and methane accumulation, methane yield and biodegradation efficiency similar to other experiments. These phenomena in R1, R3, R4, R14, R15, and R2 indicate that, as the experiments proceeded, the species of bacteria gradually adapted to the new substrate. However, this did not happen in R9 and R19 due to high concentrations of acetic, butyric and propionic acid which inhibited bacteria. The DGGE profile showed bacteria composed of *Anaerobacter* sp., *Acetivibrio* sp., *Clostridium* sp., *Ruminococcus* sp., *Lactobacillus* sp., *Bacillus* sp., *Geobacillus* sp. and *Thermoanaerobacterium thermosaccharolyticum*. As shown in Fig. 4, the methane yields were lowest in experiments R9 and R19 with lacking *Geobacillus* sp. and *T. thermosaccharolyticum*. High loads of butyric acid led to inhibition of bacteria in methanogenesis process. *Thermoanaerobacterium* sp. and *Clostridium* sp. were the most abundant species throughout the variants, which dominated in normal conditions but disappeared at inhibited conditions. *Thermoanaerobacterium* species has been reported as a thermophile with optimal growth temperature at 60°C, which is able to convert carbohydrates to hydrogen with butyrate as the end soluble product and high concentration of end soluble product inhibited this microorganisms [39]. The butyric acid concentration of 8 g/L mixed with propionic acids also showed significant inhibition of the bacteria community by decreasing their number and diversity.

Archaea community analysis showed three significant bands in the DGGE-gels profile (Fig. 5). Three abundant archaeal species could be identified as *Methanoculleus thermophilus, Methanothermobacter delfuvii* and *Methanosarcina mazei*. Methanogenic archaea had similarities in numbers of archaea (band intensities) and species diversity (band appearance) in most experiments except R1, R6, R9, R11, R17, R19, and R20. The experiments R1, R6, R11, and R17 had small differences in microbial community, and the methane accumulation, methane yield, and biodegradation efficiency with most other experiments. These phenomena in R1, R6, R11, and R17 indicate that, as the experiments proceeded, the species of archaea gradually adapted to the new substrate, while the most abundant microorganisms were still present in low numbers in R9 and R19, which coincided with low methane production and low VFA biodegradation efficiency. The accumulated methane yields were lowest in experiments R9 and R19 containing high concentration of butyric acids. As shown in Fig. 5, it seems that *M. thermophilus* and *M. delfuvii* were influenced by high concentrations of butyric acid. High loads of butyric acid and acetic acid led to distinct methane formation for short time, whereas high concentrations of butyric acid and propionic acid caused a marked inhibition of methanogenesis. *M. thermophilus* was the most abundant species throughout the variants, which dominated in normal conditions but disappeared at inhibited conditions. These species seemed to play an important role in the thermophilic digestive performance since a distinct rise in methane production coincided with the change in band intensities and band appearance [12]. This observation was also noted by Chachkhiani et al. [40] that the most frequently detected in biogas production from anaerobic digestion of cattle manure was *M. thermophilus* and *Methanosarcina thermophila*. Both *Methanothermobacter* and *Methanoculleus* are hydrogenotrophic methanogens that could probably be a representative of the acetogenic community.

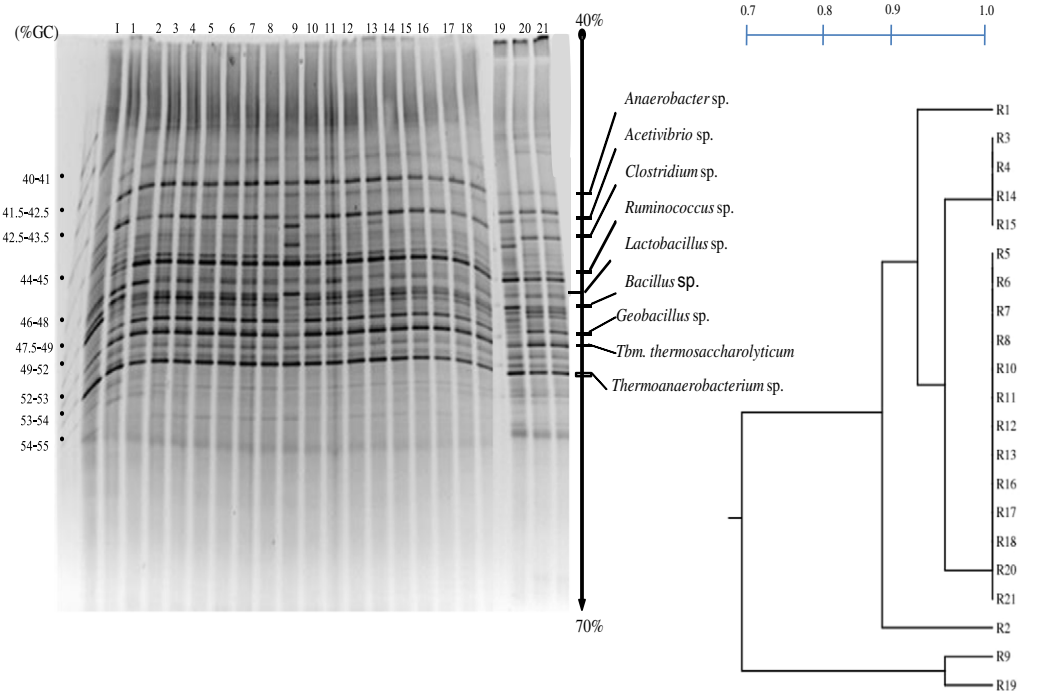

Fig. 4. DGGE profiles of 16S rRNA gene fragments of bacteria from methane production of POME hydrogenic effluent; with additions of different concentrations of lactic acid, acetic acid, butyric acid and propionic acid according to Table 2, and a corresponding similarity index (SI) dendrogram (UPGMA clustering).

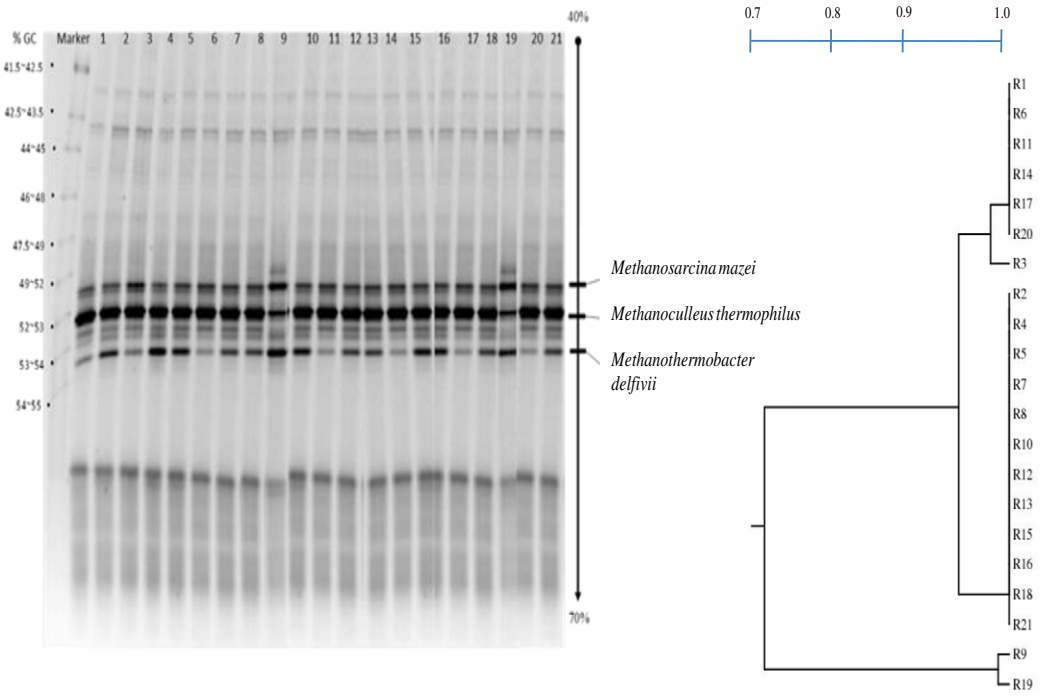

Fig. 5. DGGE profiles of 16S rRNA gene fragments of archaea from methane production of POME hydrogenic effluent; with additions of different concentrations of lactic acid, acetic acid, butyric acid and propionic acid according to Table 2, and a corresponding similarity index (SI) dendrogram (UPGMA clustering).

4. Conclusions

Butyric acid and propionic acid at high concentration (>8 g/L) in biohydrogen effluent have inhibition effect on the methane production process. Lactic, acetic and butyric acid at a concentration lower than 8 g/L has positive effects on the methanogenesis process, while propionic acid at a concentration of 3 g/L has negative effect on the methanogenesis process. Butyric acid and propionic acid at high concentration (>8 g/L) had a negative effect on both bacteria and archaea which inhibited on *Geobacillus* sp., *T. thermosaccharolyticum*, *M. thermophilus* and *M. delfuvii* resulting in low methane yield. Preventing the high concentration of butyric acid and propionic acid in the hydrogenic effluent could enhance methane production in two-stage anaerobic digestion for biohythane production.

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