CHAPTER III

Two stages hydrogen and methane production by using thermophilic dark co-fermentation of skim latex serum (SLS) and palm oil mill effluent (POME): Optimization of mixing ratio and nutrients

3.1 Abstract

A batch experiment was conducted to determine the optimal mixing ratio of skim latex serum (SLS) to palm oil mill effluent (POME), and further buffer and nutrients optimization in the substrate which consisting of the optimal mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L, respectively for biohydrogen production in the first phase under thermophilic temperature (55°C) by using thermophilic mixed cultures were studies. The optimum mixing ratio of SLS to POME was 55:45 (%v/v) with the hydrogen content, cumulative hydrogen, and hydrogen production yield was 27.2±1.5%, 25.1±0.6 mL H_2, and 71.8±1.7 mL H_2/g-VS_{added}, respectively. Then, it was optimized through employing response surface methodology (RSM) with a central composite design (CCD). Although, the hydrogen production yield achieved from optimization is higher than that obtained from non-optimization, approx. 22%. However, the increased yield is not uneconomical for the industrial scale when considering cost of the external buffer and nutrients supplemented. Acetic and propionic acids were the major soluble end-products with concentrations of 26.83±0.40 mM and 7.59±1.14 mM, respectively under the optimum mixing ratio of SLS to POME condition. Afterwards, the effluents achieved from H_2 production phase which consisting of SLS and POME at the mixing ratio of 55:45 (%v/v) was further used as substrate for methane production in the second stage. The result shows that the methane production yield obtained from co-digestion of SLS and POME was 2 times greater than that achieved from sole fermentation of SLS.

3.2 Introduction

Nowadays, higher energy demands used for transportation, industries, power plant and as well as household which was achieved mostly from fossil fuels are comprehensive coal, oil and natural gas. Nevertheless, global fossil fuels storage was gradually decrease which a contrary in prices. Moreover, the extensive use of fossil fuel which is caused of global climate change due to rapidly increasing concentrations of greenhouse gas especially carbon dioxide during the combustion of fossil fuels. Due to the depletion of limited fossil fuels is inevitable, there is an urgency to search for replacement source of energy. Among several options, biohydrogen and biomethane generated from organic wastes mainly achieved from various industries by applying a two-stage anaerobic digestion process is one of the promising routes that can contribute to sustainable biofuel in a form of biohythane. Biohydrogen is clean energy; high energy content, rapid burning speed, high-octane number and it is considered to be promising fuel since it can be produced using renewable sources. Additionally, gas mixture blending of hydrogen at 10 – 60% by volume with methane could be considered as an efficient fuel for the vehicles using an internal combustion engine (Alavandi and Agrawal, 2008).

Thermophilic mixed cultures has been examined for their potential as biohydrogen producers and they are able to utilize a wide range of organic wastes. In our previously research using two-stage anaerobic digestion process of skim latex serum in batch experiments and it was operated under thermophilic condition. Sole fermentation of skim latex serum in batch experiments, satisfactory results in term of biohydrogen and biomethane yield of 1.57±0.06 L H_2/L-SLS and 12.20±0.31 L CH_4/L-SLS were achieved, respectively with initial organic concentration of 22.8 g-VS/L. However, less hydrogen and methane production yield achieved from sole fermentation of SLS compared with sole fermentation of POME was 4.2 L H_2/L-POME and 15.2 L CH_4/L-POME, respectively was obtained (Mamimin *et al*., 2012). The possible reasons for less both hydrogen and methane production yield are: (i) relatively high concentration of ammonia (rubber preservation) of 1213±81 mg/L which was significant factor affecting on both hydrogen producing bacteria and methanogen archaea; (ii) relatively high sulfate content (rubber coagulation) of 258±1 mg/L which has inhibitory effect on both hydrogen and methane production.

Thus, to enhance in both hydrogen and methane production yield, co-digestion of SLS which is nitrogen rich substrate with other carbon rich substrate such as palm oil mill effluent (POME) and waste glycerol is simplest method and suitable approach. Beside rubber, palm oil is one of the most agricultural crops cultivated in Southern Thailand. Moreover, there are many researches were successfully achieved in both hydrogen and methane generations using POME as substrate. To overcome overload the process and inhibitory effect on both hydrogen producing bacteria and methanogenic archaea and as well as improved biogas yield, various mixing ratio of SLS and POME has been investigated.

The sufficient amounts of macro-nutrients and buffer are playing a vital role on microbial growth and to resist the pH in the anaerobic digester change. There are several components of macro-nutrients such as sulphur, phosphorus, potassium, calcium, magnesium, and iron are required for specific proteins and cofactor for enzyme activity (Batstone *et al*., 2002). However, among them in this research was focused on phosphorus from $Na_2HPO_4.12H_2O$ and potassium obtained from the empty fruit brunch (EFB) ash as phosphorus and potassium sources. A study from O-Thong *et al*. (2008) found that the optimal C/P ratio for hydrogen production by using single substrate fermentation of POME was 559 with high hydrogen production yield of 6.33±0.14 L H_2/L-POME. Meanwhile, the reason for using of EFB ash as potassium source due to it relatively high potassium content was 139.35 mg/kg (Udoetok, 2012) which could utilize for potassium source and as well as to utilize of solid organic wastes generated in palm oil mill. Bicarbonate (HCO_3^-) is often the main buffer in anaerobic digesters to resist the pH in the anaerobic digester change. However, digesters present at high concentration of bicarbonate supplemented in substrate, resulting low in hydrogen and methane with both quantity and quality in biogas generated as CO_2 was released from buffer supplemented.

This study was conducted with the aims to investigate the optimum mixing ratio of SLS to POME, to investigate the optimum concentration of buffer and nutrients concentrations including $NaHCO_3$, $Na_2HPO_4.12H_2O$ and empty fruit brunch (EFB) ash concentrations, respectively for biohydrogen production in the first phase, and to investigate the potential of the sequential methane production in the second phase under thermophilic temperature (55°C).

3.3 Materials and methods

3.3.1 Anaerobic seed sludge

The anaerobic seed sludge originating employed in this research was collected from mesophilic wastewater treatment pond of Palm Pattana Southern Border Co, Ltd., Pattani Province; Southern Thailand was used as inoculum for hydrogen and methane production. Hydrogen producing seed used in this work was obtained from a lab-scale continuously stirred tank reactor (CSTR) that generated biohydrogen from co-digestion of SLS and POME. The H_2-CSTR reactor was operated by using the mixture consisting of SLS and POME at the volumetric mixing ratio of 1:1 under thermophilic temperature. At the same time, the sludge used for produced methane was enriched for methanogens by using basic anaerobic (BA) medium supplemented with sucrose 3 g/L for a week in batch reactor. Afterwards, the enriched sludge was used as inoculum in batch tested to determining its ability to generate hydrogen and methane from co-digestion of SLS and POME.

3.3.2 Skim latex serum

The SLS was collected from Chana Latex Co, Ltd., Songkhla Province; Southern Thailand. **Table 3.1** shows that there are several limitations of using SLS as substrate to generate biogas such as it still having low C/N ratio about 3 along with it also still having relatively high sulfate contaminated was 258±0 mg/L which could potentially toxic to methanogens. Nevertheless, SLS is one of the interest substrate used to generate biogas, thanks to its sufficient in the macronutrients in nitrogen and phosphorus which is crucial for microbial growing. The physical and chemical characteristics of SLS are summarized in **Table 3.1**. The SLS was kept at 2±1°C and was used within a month in order to minimize self-biodegradation and acidification.

3.3.3 Palm oil mill effluent

The POME was collected from the receiving tank of Palm Pattana Southern Border Co, Ltd., Pattani Province; Southern Thailand. The POME has brown color, pH 4.68±0.00, a temperature of 70-80°C which could be applied to operate under thermophilic temperature. POME is deficient in the macronutrients in nitrogen as well as it contains phenolic compounds which is antibacterial and phytotoxic properties. The physical and chemical characteristics of POME are summarized in **Table 3.1**. The POME was kept at 2±1°C and was used within a month

3.3.4 Empty fruit bunch (EFB) ash

The bottom EFB ash used in this research was collected from a boiler of Palm Pattana Southern Border Co, Ltd., Pattani Province; Southern Thailand. The EFB ash was then ground in a blender and passed through a 1.75 mm sieve. In the present study, the EFB as was used as potassium source since its high potassium content of 175±8 mg/kg which coincided with a study from Udoetok (2012) found that high potassium content in the EFB ash was 139.35 mg/kg. The mixtures consisting of SLS and POME at the mixing ratio of 55:45 (%v/v) with initial organic concentration of 7 g-VS_{added}/L were supplemented with the EFB ash and were then shaken at 130 rpm for 60 min to ensure the potassium contained in the EFB ash was maximal dissolved into the mixtures. Subsequently, the supernatants were then used as substrate for nutrients optimization to generate biohydrogen.

3.3.5 Optimization of SLS and POME mixing ratio in biohydrogen production

The enriched sludge was used as inoculum in batch tested to determine its ability to generate hydrogen from co-digestion of various mixing ratios of SLS and POME including 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45 and 50:50 (%v/v) with initial organic concentration of 7 g-VS_{added}/L and 21 g-VS_{added}/L, respectively. The assay was conducted as batch cultivations in 120 mL serum bottles with 50 mL of working volume. In each bottle, 15 mL of inoculum was added; corresponding to 30% of working volume and the rest of the working volume active was filled up with 35 mL of substrate/water mixture. All experiment without supplementation of additional nutrients and no initial pH adjustment. The mixtures were then purged with N_2 (100%) to ensure anaerobic conditions. Afterwards the bottles were closed with butyl rubber stoppers and aluminum seal and then placed in a 55°C incubator for 5 days. Hydrogen production in the headspace of the vials was monitored. The headspace gas was collected for hydrogen determination daily. All experiment was done in triplicate for each replication at each time point. At each time interval for each biogas volumes and biogas composition were determined. Then, the optimum mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L was further investigated by using RSM with CCD, for buffer and nutrients optimization. Subsequently, the effluent from H_2 potential phase with optimal conditions was further used as substrate for the sequential methane production in the second stage.

3.3.6 Effect of $NaHCO_3$, $Na_2HPO_4.12H_2O$ and EFB ash concentrations on biohydrogen production

A factorial central composite experimental design was used to investigate the effect of $NaHCO_3$, $Na_2HPO_4.12H_2O$, and empty fruit brunch's (EFB) ash concentrations on hydrogen production. In this experiment was conducted in 16 runs and each run was performed in triplicates. The concentration levels of the variables and the experimental design are shown in **Table 3.2** and **Table 3.3**. The concentrations of $NaHCO_3$ ranged from 3 to 9 g/L, $Na_2HPO_4.12H_2O$ ranged from 0 to 20 mg/L, and EFB ash ranged from 0 to 21 g/L, respectively. A quadratic model (Sreela-or et al., 2011; Sittijunda and Reungsang, 2012) was used to evaluate the optimization of key factors.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{22} X_2^2 + \beta_{23} X_2 X_3 + \beta_{33} X_3^2 \quad (3.1)$$

Where Y is the predicted response; X_1, X_2 and X_3 are the parameters; β_0 is the offset term; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the squared coefficients; and β_{12}, β_{13} and β_{23} are the interaction coefficients. The response variable was fitted using a predictive polynomial quadratic equation (**Eq. (3.1)**) in order to correlate the response variable to the independent variables (Lay, 2000). **Table 3.2** and **Table 3.3** illustrate the code values of the variables, the conditions of each run and the corresponding results.

Table 3.2 Experimental variables and concentration levels investigated by using central composite design.

Variable	**Parameter value**		
	-1	**0**	**1**
X_1: $NaHCO_3$ concentrations (g/L)	3	6	9
X_2: $Na_2HPO_4.12H_2O$ concentrations (mg/L)	0	10	20
X_3: EFB ash concentration (g/L)	0	15	30

Table 3.3 Central composite experimental design matrix defining $NaHCO_3$, $Na_2HPO_4.12H_2O$, and EFB ash concentrations on hydrogen production yield.

Run	Parameter		
	X_1: $NaHCO_3$ (g/L)	**X_2: $Na_2HPO_4.12H_2O$ (mg/L)**	**X_3: EFB ash (g/L)**
1	6	10	0
2	3	0	0
3	9	10	15
4	9	20	30
5	3	0	0
6	6	20	15
7	9	0	0
8	9	20	0
9	6	0	15
10	3	20	0
11	6	10	30
12	9	0	30
13	6	10	15
14	3	10	15
15	3	20	30
16	6	10	15

3.3.7 Methane potential from co-digestion of SLS with POME

The enriched methanogenic inoculum was used to determine methane potential from co-digestion of SLS and POME. The batch assay was carried out in 500 mL serum bottles with 300 mL working volume and was conducted in triplicates. Each bottle contained 250 mL enriched methanogenic inoculum and 50 mL substrate/water mixture achieved from H_2 batch production stage of our previous studied with the optimal mixing ratio of SLS to POME of 55:45 (%v/v) and without supplementation of additional nutrients and no initial pH adjustment. Each bottle was then flushed with nitrogen gas to ensure anaerobic conditions and was then closed with butyl stoppers, sealed with aluminum cap and subsequently was placed in a 55°C incubator for 27 days. The headspace gases were collected at time intervals for biogas determination until biogas production ceased. The biohydrogen and biomethane produced was followed the method described by Zheng and Yu (2005).

3.3.8 Analytical methods

The volume of biogas produced was measured using water displacement method. The hydrogen content was measured by gas chromatography (Shimadzu GC 14A equipped with thermal conductivity detector, TCD) fitted with a 1.5 m stainless steel column paced with molecular sieve 58 (80/100 mesh). Argon was used as a carrier gas at a flow rate of 15 mL/min. The temperature of the injection port, oven and detector were 100, 50, and 100°C, respectively. 0.5 mL of sampling gas was injected in triplicate (Akutsu *et al*., 2009; O-Thong *et al*., 2008).

Volatile fatty acids (VFAs) including acetic acid, propionic acid, butyric acid, and alcohols such as ethanol (EtOH) were analyzed by a gas chromatography (Shimadzu, GC 8A) equipped with a flame ionization detector (FID). A column capillary packed with nitroterephthalic acid-modified polyethleneglycol (DB-FFAP) and with a length of 30 m was used. The chromatography was performed using the following program: 100°C for 5 min, 100-250°C with a ramping of 10°C/min, 250°C for 12 min. The detector temperature was set at 300°C (O-Thong *et al*., 2008; Prasertsan *et al*., 2009). Lactic acid (HLa) was analyzed by a high performance liquid chromatography (HP1100, Hewlett-Packard GMGH) with the following operating conditions; Pinnacle® II C18 Columns, ultraviolet (UV) detector at 210 nm, 2.5 mM of H_2SO_4 was used as a mobile phase with a flow rate of 0.8 mL/min, and an oven temperature of 45°C (Castelló *et al*., 2009). The liquor samples were first centrifuged at 10,000 rpm for 10 min, and were then filtered through 0.45 µm nylon membrane. Calculation of chemical oxygen demand (COD) balance was followed the method described by Sittijunda and Reungsang (2012).

Chemical oxygen demand (COD), pH, total solid content (TSC), Volatile solid content (VSC), alkalinity, total Kjeldahl nitrogen (TKN), protein content, total organic nitrogen (TON), carbohydrate content, sulfate content, oil and grease and soluble phosphorus were determined in accordance with the procedures described in the standard methods (APHA, 1999).

3.4 Results and discussion

3.4.1 Characteristics of substrates used

Physical and chemical characteristics of raw SLS and raw POME were summarized in **Table 3.1**. The result shows that SLS was a concentrated substrate with high concentration of total Kjeldahl nitrogen (TKN) and low concentration of chemical oxygen demand (COD),

resulting in low C/N ratio about 3. O-Thong *et al*. (2008) investigated the effect of C/N ratio, C/P ratio and iron concentration in POME on fermentative biohydrogen production. The results found that the optimum C/N ratio of 74 with the hydrogen production yield of 6.33±0.14 L H_2/L POME. On the other hand, POME contains C/N ratio about 28, which is much higher than that of SLS. However, it was a concentrated substrate with high concentration of oil and grease of 13.70±0.12 g/L, which could potentially inhibit the process (O-Thong *et al*., 2012). Therefore, adding POME into SLS could definitely have more suitable C/N ratio for hydrogen production by using dark fermentation. Furthermore, they also high concentration of carbohydrate with concentrations were 9.00±0.00 g/L for POME, and 0.60±0.00 g/L for SLS, respectively, which is the real substrate for hydrogen production by dark fermentation (Abraham *et al*., 2009; Prasertsan *et al*., 2009; Ismail *et al*., 2010; Mamimin *et al*., 2012).

Table 3.1 Physical and chemical characteristics of skim latex serum and palm oil mill effluent

Parameters	Unit	SLS	POME
pH		4.83±0.01	4.68±0.00
TSC	g/L	49.65±0.01	42.07±0.16
VSC	g/L	43.46±0.39	32.24±0.89
Alkalinity	mg/L $CaCO_3$	56±1	28±0
COD	g/L	29.22±5.67	42.55±6.14
TKN	g/L	5.18±0.00	1.25±0.01
Protein	g/L	7.58±0.51	7.78±0.07
C/N ratio		3.27*	27.59*
Carbohydrate	g/L	0.60±0.00	9.00±0.00
Oil and grease	g/L	0.15±0.01	13.70±0.12
Sulfate	mg/L	258±1	-
Soluble phosphorus	mg/L	44±0	96±0

*C/N ratio was determined by CHN analysis using CHNS/O analyzer (Thermo Quest Flash EA 1112)

3.4.2 Optimization of SLS and POME mixing ratio in biohydrogen production

Cumulative hydrogen production under thermophilic condition obtained from individual fermentations of SLS and POME with initial organic concentration of 7 g-VS_{added}/L were 7.1±1.7

mL H_2 and 38.8±0.8 mL H_2, respectively as showed in **Fig. 3.1**, at the same time, cumulative hydrogen production obtained from individual fermentations of SLS and POME with initial organic concentration of 21 g-VS_{added}/L were 21.5±0.9 mL H_2 and 1.3±0.2 mL H_2, respectively (**Fig. 3.2**). Nevertheless, less than one day lag phase and the stationary phase had been reached at the 4^{th} day's fermentation of all mixing ratio of both initial organic loads. Low hydrogen production yield achieved from individual fermentations of SLS and POME with initial organic concentration of 21 g-VS_{added}/L (13.9±0.4 mL H_2/g-VS_{added} and 0.6±0.1 mL H_2/g-VS_{added}, respectively) when compared to individual fermentations of SLS and POME with initial organic concentration of 7 g-VS_{added}/L were 28.8±7.0 mL H_2/g-VS_{added} and 158.4±3.3 mL H_2/g-VS_{added}, respectively. The hydrogen potential of individual fermentations of SLS and POME with initial organic concentration of 21 g-VS_{added}/L decreased due to SLS was a concentrate substrate with high concentrations of ammonia, sulfate and also low pH, similarly high concentration of oil and grease and also low pH in POME, which could potentially inhibit or overload the process and lead to decrease in biodegradability (O-Thong *et al*., 2012).

In case of co-digestion, when the POME composition in the fermentation broth was increased, the hydrogen concentration, cumulative hydrogen and hydrogen production yield increased. The best results of hydrogen production with initial organic concentration of 7 g-VS_{added}/L achieved at the mixing ratio of SLS to POME at 50:50 %v/v with the highest hydrogen concentration, cumulative hydrogen and hydrogen production yield were 29.4±0.1%, 31.0±0.5 mL H_2 and 85.7±4.9 mL H_2/g-VS_{added} (**Fig. 3.3**), respectively possibly correlates to existing appropriate C/N ratio around 15. On the other hand, the optimal mixing ratio of SLS to POME under the initial organic concentration of 21 g-VS_{added}/L was 65:35 %v/v with the hydrogen production yield was 36.8±0.8 mL H_2/g-VS_{added}. The possible reason for high hydrogen production yield was achieved due to the inhibitants content in the SLS was diluted and lipid content in the POME fraction was still low as well as increasing in C/N ratio in the mixture around 12. It should be noted that the hydrogen production yield obtained from sucrose control with initial concentration of 7 g-VS_{added}/L was 321.1±10.5 mL H_2/g-COD_{added} which is 64% of the theoretical biohydrogen production yield (498 mL H_2/g-COD_{added}) (Kongjan *et al*., 2011). Although, the hydrogen production yield achieved from SLS: POME mixing ratio of 50:50 %v/v was different significant with the hydrogen production yield achieved from SLS: POME mixing ratio of 55:45 %v/v with $P \leq 0.05$ which was analyzed by using t-Test: Two-Sample Assuming Unequal Variances. In the present study, however, SLS was chosen as the main substrate and POME was chosen as a co-substrate. Thus, we wish to utilize a large proportion of SLS in the mixture along with high in hydrogen production yield. The resulted shows that a relatively high hydrogen production yield was achieved from SLS: POME mixing ratio of 55:45 %v/v of 71.8±1.7 mL H_2/g-VS_{added} was obtained. Therefore, in this work should be chosen the optimal mixing ratio of SLS to POME to generate both biohydrogen and biomethane was 55:45 %v/v.

Co-digestion of SLS with POME resulted in better than individual fermentation of SLS because inhibitants in SLS include, NH_3, SO_4^{2-}, and ZnO/TMTD were diluted and C/N ratio in

the mixtures was increased. The biohydrogen production yield obtained from the optimum mixing ratio of SLS and POME was 4 times greater than that of individual fermentation of SLS. Subsequently, it was further transferred into the BMP test in the second stage.

In the **Fig. 3.1** shows that cumulative hydrogen production achieved from sole fermentation of POME is dramatically increase from the 1st to the 2nd days of fermentation. The possible reasons is that the microorganisms can be easily broken down the complex organic contained in the POME such as carbohydrates to generate biohydrogen. Subsequently, cumulative hydrogen production is gradually increased during the 2nd to the 4th days of fermentation. The possible cause for gradually increased of biohydrogen was generated due to the microorganisms are broken down lipid contained in POME was slow, this is due to lipid is a large molecules which makes it difficult to degrade. At the end of fermentation, cumulative hydrogen production is dramatically increased possibly correlates to reduced products which expected mostly achieved from lipid hydrolysis was utilized to generate biohydrogen. Similarly to co-digestion of SLS and POME with a relatively high proportion of POME in the mixture such as SLS: POME mixing ratio of 50:50 %v/v as shown in **Fig. 3.1**.

On the other hand, the lowest cumulative hydrogen production achieved from individual fermentation of POME with initial organic concentration of 21 g-VS_{added}/L due to it is a concentrated substrate with high lipid content which could potentially inhibit or overload the process and lead to decrease in biodegradability. At the same time, cumulative hydrogen production achieved from sole fermentation of SLS around 17 times greater than that achieved from sole fermentation of POME. The possible reason is that the complex organics contained in SLS would be easily broken down as compared with POME. In the **Fig. 3.2** shows that when the proportion of POME was increased in the mixture, resulting in increase in cumulative hydrogen production was obtained. Nevertheless, when the proportion of POME in the mixture was higher than 40%, cumulative hydrogen production is gradually decreased as previously mentioned.

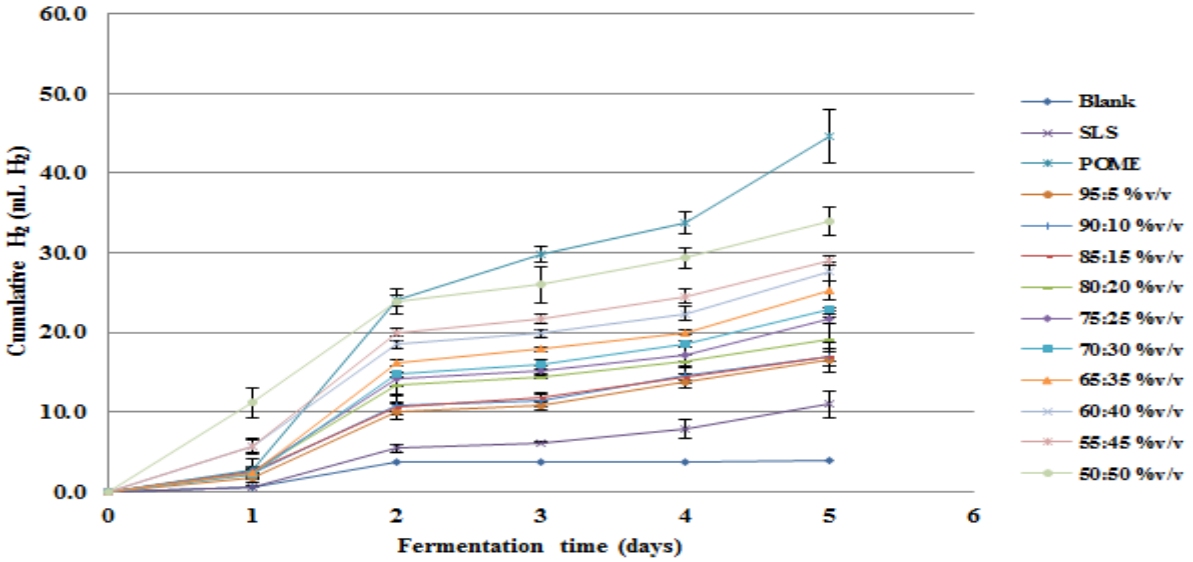

Fig. 3.1 Cumulative hydrogen achieved from different mixing ratio of SLS and POME with initial organic concentration of 7 g-VS_{added}/L.

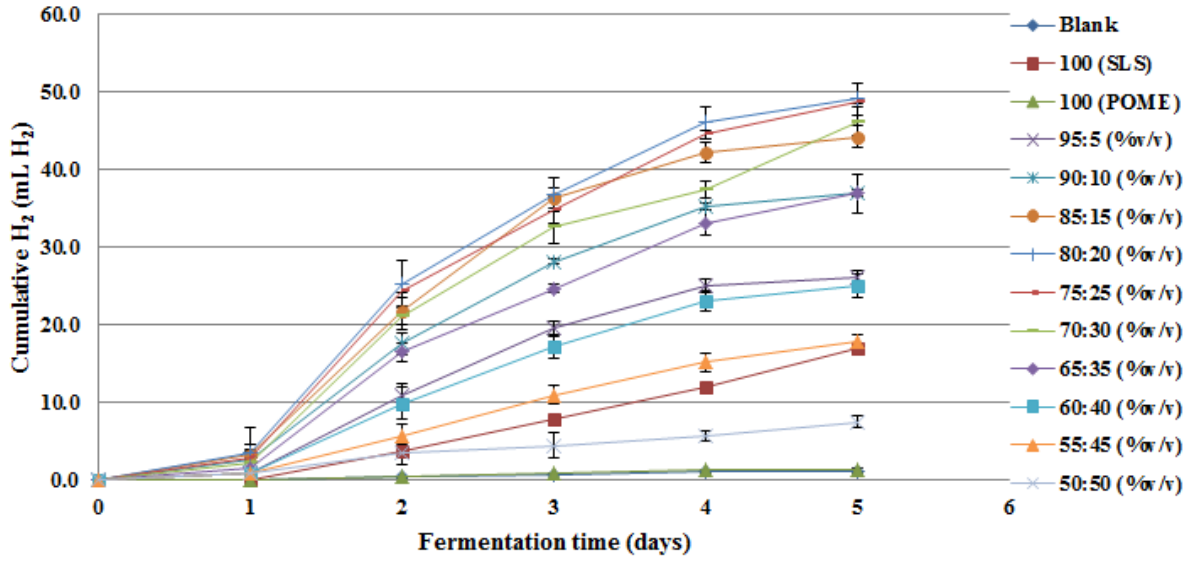

Fig. 3.2 Cumulative hydrogen achieved from different mixing ratio of SLS and POME with initial organic concentration of 21 g-VS_{added}/L.

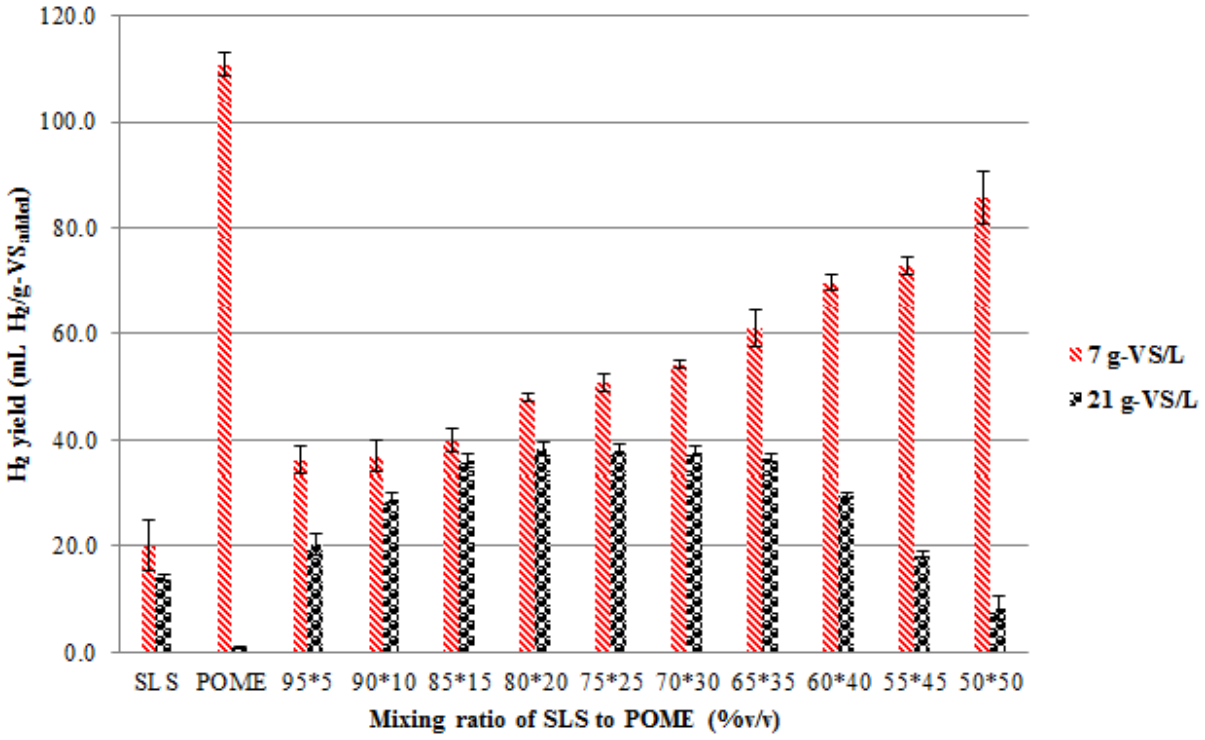

Fig. 3.3 Hydrogen production yield achieved from different mixing ratio of SLS and POME with initial organic concentration of 7 g-VS_{added}/L and 21 g-VS_{added}/L, respectively.

3.4.3 Effect of $NaHCO_3$, $Na_2HPO_4.12H_2O$ and EFB ash concentrations on biohydrogen production

Batch experiments was conducted to investigate the effects of key factors including $NaHCO_3$, $Na_2HPO_4.12H_2O$, and empty fruit brunch (EFB) ash concentrations on hydrogen production yield using response surface methodology (RSM). Regression analysis of the data from **Table 3.5** resulted in the quadratic equation (**Eq. (3.2)**) as follows:

$$Y = 246.32 - 67.86X_1 + 1.446X_2 - 0.158X_3 + 4.504X_1^2 - 0.04795X_1X_2 + 0.03099X_1X_3 - 0.04966X_2^2 + 0.00096X_2X_3 - 0.00363X_3^2 \quad (3.2)$$

The model presented a high determination coefficient ($R^2 = 0.994$) explaining 99% of the variability in the response and a high value of the adjusted determination coefficient (adjusted $R^2 = 0.986$) suggested a high significance of the model as shown in **Table 3.4**. In a good model, R^2, adjusted R^2 and R^2 for prediction should not be too different from each other. These results showed that only $NaHCO_3$ concentration had significant individual effect on hydrogen production yield ($P \leq 0.05$). The quadratic model term of X_1^2 variable was highly significant ($P < 0.0001$) as shown in **Table 3.5**. The statistical analysis was carried out based on the experimental data using a full quadratic model which was fitted to the data to obtained the regression equation using the multiple regression tool in Essential Regression software version 2.210 (Saelee, 2010).

High hydrogen production yield achieved from weak and moderate conditions (runs 2, 5, 10, 14 and 15) with the hydrogen production yield ranged from 79.0±3.8 to 91.7±3.9 mL H_2/g-VS_{added}. The highest hydrogen production yield was 91.7±3.9 mL H_2/g-VS_{added} (run 14) achieved from moderate additions of $NaHCO_3$, $Na_2HPO_4.12H_2O$ and EFB ash as shown in **Table 3.6**. At the same time, the hydrogen production yield achieved from substrate control of 75.0±4.6 mL H_2/g-VS_{added} was obtained. Although, the hydrogen production yield achieved from the optimal conditions (run 14) and achieved from substrate control was different significant with $P \leq 0.05$. Nevertheless, it is increased just only 22% which is still not uneconomic for applying in the industrial scale when considered the economic cost of the external buffer and nutrients supplemented. Thus, in this work was chosen co-fermentation of SLS: POME mixing ratio of 55:45 %v/v which without supplementation of additional nutrients as the optimal conditions for both biohydrogen and biomethane production. The possible reasons for still having low hydrogen production yield achieved from the optimal conditions is that the mixture would rather contain sufficient nutrients are comprehensive phosphorus and potassium. Corresponding to the results achieved from regression model which model coefficients related to X_1 and X_2 had not significant individual effect on hydrogen production yield which was estimated by multiples linear regression as shown in **Table 3.5**. Moreover, the other reason is that not initial pH suitable for biohydrogen production as summarized in **Table 3.6**. The optimum pH for hydrogen production was 5.4-5.7 (O-Thong *et al*., 2008; Mamimin *et al*., 2012), It can be concluded that initial pH adjustment plays an important role in improving co-digestion of SLS to POME by using dark fermentation for hydrogen production.

Table 3.4 Analysis of variance (ANOVA) for the regression model

Source of variation	Sum of squares	% Sum of squares	Mean squares	F-value	F Significant	Degree of freedom
Regression	23272.0	99	2585.8	116.85	4.82308E-06	9
Residual	132.78	1	22.13			6
LOF Error	54.45	0 (41)	13.61	0.3476	0.832	4
Pure Error	78.33	0 (59)	39.16			2
Total	23404.7	100				15

R = 0.997, R^2 = 0.994, R^2 adjusted = 0.986, R^2 for prediction = 0.881, Standard Error = 4.704, Coefficient of variation = 15.836, Precision Index = 18.049

Table 3.5 Model coefficients estimated by multiples linear regression (significance of regression coefficients), where X_1 =$NaHCO_3$ concentration (g/L), X_2 =$Na_2HPO_4.12H_2O$ concentration (mg/L) and X_3 = EFB ash concentration (g/L)

Factor	Coefficient	P value	Std Error	-95%	95%	t Stat	VIF
Intercept	246.32	3.99235E-07	10.53	220.57	272.08	23.40	
X_1	-67.86	2.81281E-06*	4.032	-77.73	-57.99	-16.83	66.13
X_2	1.446	0.09882	0.741	-0.367	3.258	1.952	24.79
X_3	-0.158	0.775	0.527	-1.449	1.133	-0.299	27.58
X_1^2	4.504	8.96892E-06*	0.326	3.706	5.302	13.81	63.74
X_1X_2	-0.04795	0.502	0.06711	-0.212	0.116	-0.715	10.62
X_1X_3	0.03099	0.531	0.04664	-0.08315	0.145	0.664	12.29
X_2^2	-0.04966	0.142	0.02935	-0.121	0.02216	-1.692	17.03
X_2X_3	0.000962	0.947	0.01399	-0.03328	0.03520	0.06878	5.574
X_3^2	-0.00363	0.790	0.01304	-0.03555	0.02829	-0.278	15.47

*Significant level at 95%

Table 3.6 pH and results on hydrogen production yield achieved from buffer and macro-nutrients optimization stage.

Run	pH		H_2 yield (mL H_2/g-VS_{added})	
	Initial	Final	Observed	Predicted
1	6.31±0.00	6.96±0.03	8.9±2.6	7.9
2	5.79±0.00	5.96±0.01	79.0±3.8	83.3
3	6.65±0.00	7.48±0.07	7.9±0.1	6.7
4	6.65±0.00	7.52±0.02	0.7±0.0	1.7
5	5.81±0.00	6.01±0.02	87.8±1.1	83.3
6	6.43±0.00	7.25±0.05	8.6±0.2	4.5
7	6.60±0.00	7.45±0.09	0.9±0.0	0.4
8	6.63±0.00	7.47±0.04	0.0±0.0	0.8
9	6.44±0.00	7.30±0.06	0.0±0.0	0.9
10	5.81±0.00	5.96±0.01	88.5±0.0	89.5
11	6.51±0.00	6.94±0.04	8.0±0.4	5.8
12	6.69±0.00	7.56±0.07	0.9±0.0	0.8
13	6.44±0.00	7.18±0.13	0.0±0.0	7.7
14	5.98±0.00	6.28±0.03	91.7±3.9	89.7
15	6.09±0.00	6.37±0.02	83.5±0.1	84.8
16	6.50±0.00	7.31±0.05	8.9±1.6	7.7

3.4.4 Soluble metabolite products and COD balance

Fig. 3.4 and Table 3.7 shows soluble metabolites obtained from different mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L. Most of treatments, acetic and lactic acids were the major submerged fermentation products. Under the optimum mixing ratio of SLS to POME at 50:50 (%v/v), acetic, propionic, and butyric acids concentrations were 26.83±0.40 mM, 7.59±1.14 mM, and 4.79±0.08 mM, respectively. In addition, small amount of

ethanol and lactic acid (1.58±0.25 mM and 3.12±0.56 mM, respectively) was detected. Moreover, when the proportion of POME in the mixture was increased were 40, 45 and 50% as well as sole fermentation of POME, less lactic acid was generated. The possible reason might be due to existing appropriate C/N ratio in the mixture for biohydrogen fermentation as previously mentioned. Moreover, a study from Kalil *et al*. (2008) found that proper C/N ratio enhance the bacteria for more growth as well as substrate utilization. On the contrary the mixture consisting of high proportion of SLS, lactic acid was the major soluble metabolite products. The possible cause might be due to relatively high inorganic nitrogen contained in the SLS such as ammonium nitrate and ammonium sulfate are affected on microbial growth (Kalil *et al*., 2008). These inorganic salt will usually produce acid condition due to the ammonium ion is utilized and the free acid will be liberated. Moreover, they are reported that inorganic nitrogen source contained in fermentation broth will did not change. This could be affected on microbial growth due to improper C/N ratio along with fermentation time. Similarly lactic and acetic acids were the major soluble end-products for initial organic concentration of 21 g-VS_{added}/L are summarized in **Table 3.8 and Fig. 3.5**. Moreover, other reason is that relatively high initial ammonia content in SLS of 1213±81 mg/L which was significant factor affecting on microbial growth, resulting shift in metabolic pathway to lactic acid formation pathway.

O-Thong *et al*. (2008) used *Thermoanaerobacterium*-rich sludge as inoculum for producing biohydrogen from POME; however, it was collected from a palm oil mill wastewater treatment plant that the same source of seed sludge used in this experiment. They are reported lactic acid was produced through glucose fermentation. Furthermore, hydrogen was generated from acetic acid, butyric acid and ethanol fermentations, not from propionic acid and lactic acid fermentations (Chan and Holtzapple, 2003; Angenent *et al*., 2004). This is a drawback for using anaerobic mixed cultures from palm oil mill wastewater treatment plant as inoculum to produce biohydrogen. However, these VFA can be further converted to acetic and following methane in the second methane stage.

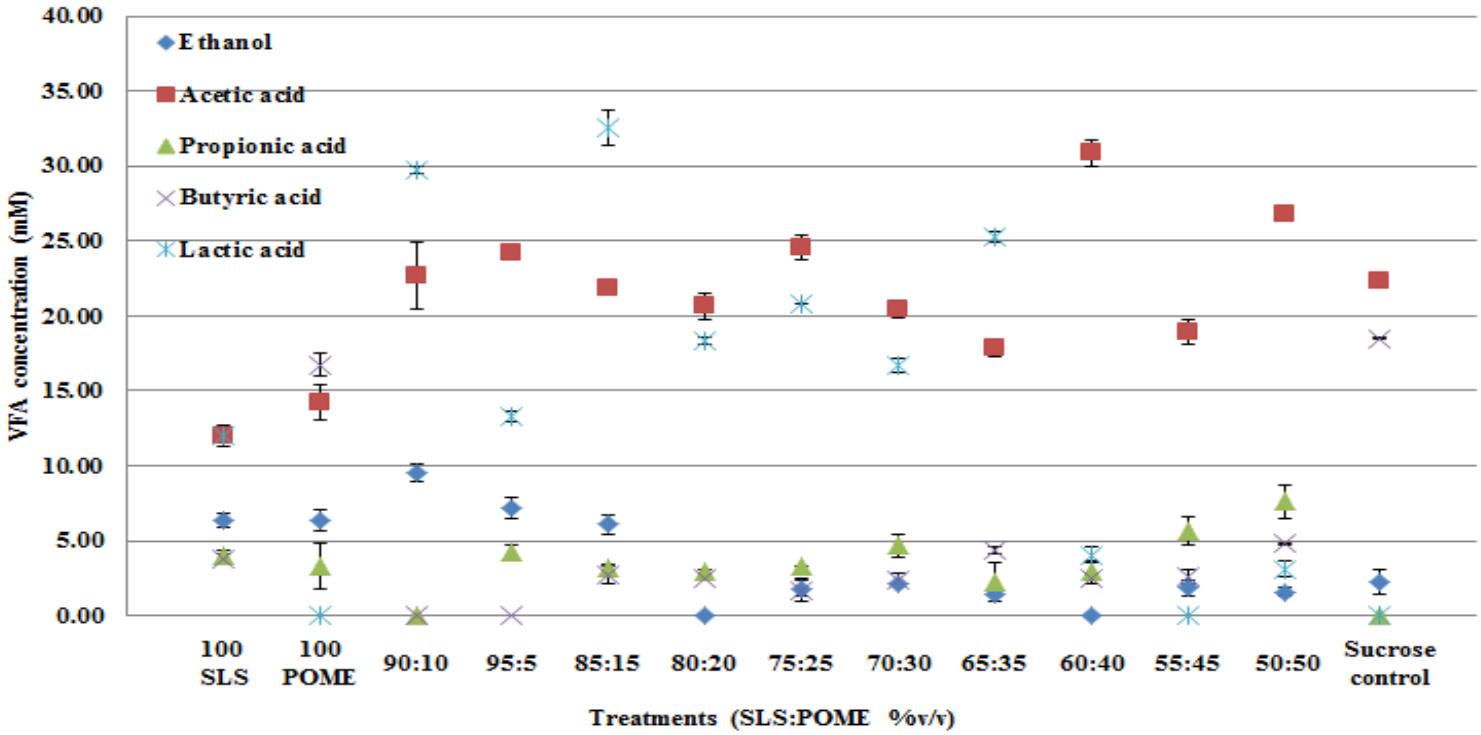

Fig. 3.4 Soluble metabolites obtained from different mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L.

Table 3.7 Soluble metabolites obtained from different mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L.

Treatments (SLS:POME %v/v)	Ethanol (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)	Lactic acid (mM)	C/N ratio	H_2 yield (mL H_2/g-VS_{added})
100 (SLS)	6.37±0.46	11.97±0.70	3.95±0.45	3.76±0.31	12.04±0.28	3.3	20.1±4.9
100 (POME)	6.39±0.71	14.22±1.20	3.34±1.52	16.72±0.79	0.00±0.00	27.6	110.9±2.3
95:5	9.56±0.58	22.71±2.24	0.00±0.00	0.00±0.00	29.72±0.28	4.5	36.3±2.5
90:10	7.18±0.71	24.20±0.28	4.28±0.47	0.00±0.00	13.28±0.37	5.8	36.9±3.0
85:15	6.08±0.62	21.84±0.24	3.16±0.25	2.72±0.55	32.50±1.17	6.2	39.9±2.1
80:20	0.00±0.00	20.65±0.85	2.93±0.12	2.51±0.07	18.32±0.28	8.2	48.1±0.8
75:25	1.80±0.51	24.55±0.78	3.31±0.03	1.71±0.82	20.77±0.02	9.4	50.8±1.8
70:30	2.16±0.01	20.42±0.57	4.65±0.79	2.39±0.42	16.65±0.47	10.6	54.2±0.7
65:35	1.37±0.02	17.82±0.53	2.24±1.37	4.32±0.24	25.26±0.37	11.8	61.1±3.4
60:40	0.00±0.00	30.85±0.84	2.94±0.76	2.50±0.33	4.05±0.51	13.0	69.7±1.5
55:45	1.87±0.52	18.92±0.78	5.69±0.95	2.56±0.49	0.00±0.00	14.2	71.8±1.7
50:50	1.58±0.25	26.83±0.40	7.59±1.14	4.79±0.08	3.12±0.56	15.5	85.7±4.9

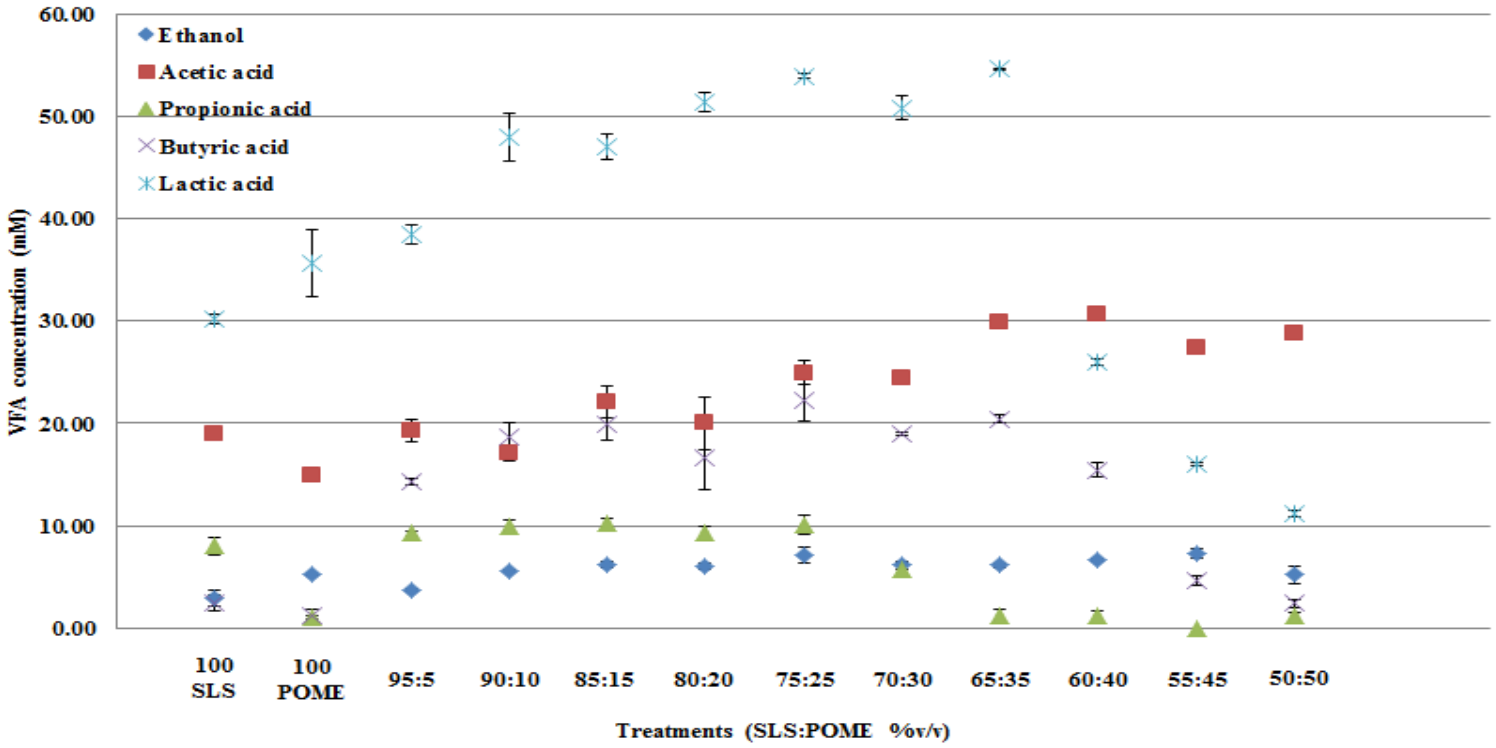

Fig. 3.5 Soluble metabolites obtained from different mixing ratio of SLS to POME with initial organic concentration of 21 g-VS_{added}/L.

Table 3.8 Soluble metabolites obtained from different mixing ratio of SLS to POME with initial organic concentration of 21 g-VS_{added}/L.

Treatments (SLS:POME %v/v)	Ethanol (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)	Lactic acid (mM)	C/N ratio	H_2 yield (mL H_2/g-VS_{added})
100 (SLS)	2.90±0.74	19.00±0.46	8.06±0.86	2.44±0.79	30.16±0.51	3.3	14.1±0.6
100 (POME)	5.22±0.12	14.91±0.33	1.08±0.20	1.22±0.57	35.65±3.28	27.6	0.9±0.1
95:5	3.70±0.10	19.28±1.08	9.25±0.29	14.36±0.33	38.45±0.96	4.5	20.4±2.0
90:10	5.62±0.06	17.06±0.81	10.00±0.58	18.69±1.41	47.91±2.37	5.8	29.0±0.9
85:15	6.14±0.31	22.11±1.54	10.32±0.40	19.92±1.56	47.03±1.22	6.2	36.1±1.2
80:20	6.05±0.29	19.99±2.59	9.34±0.66	16.65±3.13	51.34±0.91	8.2	38.4±1.3
75:25	7.09±0.76	24.95±1.13	10.09±0.90	22.20±2.00	53.93±0.21	9.4	38.2±0.9
70:30	6.15±0.35	24.39±0.35	5.80±0.63	18.99±0.19	50.77±1.17	10.6	37.8±0.9
65:35	6.13±0.18	29.88±0.36	1.25±0.52	20.42±0.39	54.58±0.05	11.8	36.8±0.8
60:40	6.68±0.21	30.70±0.60	1.14±0.47	15.45±0.74	25.99±0.28	13.0	29.6±0.5
55:45	7.29±0.50	27.37±0.65	0.00±0.00	4.65±0.47	16.00±0.19	14.2	18.3±0.6
50:50	5.20±0.89	28.75±0.65	1.17±0.39	2.39±0.44	11.17±0.33	15.5	8.5±2.1

Table 3.9 shows soluble metabolite products and COD balance obtained from different mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L. Under this condition the major submerged fermentation products were acetic acid (22.38±0.49 mM) butyric acid (18.48±0.04 mM) and ethanol (2.21±0.80 mM). Results indicated that hydrogen was generated pass through acetic acid, butyric acid and ethanol fermentations with the present of these organic acids in fermentation broth. The biomass concentration (assumed formula $C_5H_7O_2N$) used in COD balance was assumed to be 15% if the sugars degraded (Kotsopoulos *et al*., 2006; Kongjan, 2010). The COD balance of individual fermentations of SLS and POME, sucrose control and the mixture consisting of SLS and POME at the mixing ratio of 55:45 (%v/v) were 30.33, 31.85, 16.07 and 50.33% error, respectively. The possible reason for high measurement of degraded metabolic products error achieved from all treatment is other metabolite products such as 1,3-propanediol (Sittijunda and Reungsang, 2012), formic acid (Prasertsan *et al*., 2009) and butanol (Khamtib *et al*., 2011) was produced, which is not determined.

Table 3.9 Soluble metabolite products and COD balance obtained from different mixing ratio of SLS to POME with initial organic concentration of 7 g-VS_{added}/L at the end of fermentation.

Products	Concentration (g-COD/L) Treatments (SLS:POME (%v/v))				COD distribution (%) Treatments (SLS:POME (%v/v))			
	SLS	**POME**	**55:45**	**Sucrose control**	**SLS**	**POME**	**55:45**	**Sucrose control**
Initial COD added	***6.72***	***10.11***	***8.25***	***10.00***	***6.72***	***10.11***	***8.25***	***10.00***
Substrate consumption					-100	-100	-100	-100
Hydrogen	0.10	0.80	0.42	2.29	1.49	7.91	5.09	22.9
Ethanol	0.61	0.61	0.18	0.21	9.08	6.03	2.18	2.10
Acetic acid	0.77	0.91	1.21	1.43	11.46	9.00	14.67	14.30
Lactic acid	1.16	0.00	0.00	0.00	17.26	0.00	0.00	0.00
Propionic	0.44	0.37	0.63	0.00	6.55	3.66	7.64	0.00
Butyric acid	0.60	2.68	0.41	2.96	8.93	26.51	4.97	29.60
Biomass	1.01	1.52	1.24	1.50	15.03	15.03	15.03	15.00
Sum	4.68	6.89	4.10	8.39				
Soluble COD - A					***-30.33***	***-31.85***	***-50.33***	***-16.07***

*A = soluble metabolite products (including hydrogen, acetic, propionic and butyric acids) and biomass

3.4.5 Methane potential of co-fermentation of SLS and POME

The CH_4 potential in the second phase used effluent achieved from the BHP test under the optimal conditions as substrate, the highest cumulative methane and methane production yield achieved from sole fermentation of POME which was used as substrate control was 433±6 mL CH_4 and 618±8 mL CH_4/g-VS_{added}, respectively at the end of 27 days of fermentation because its high lipid content when compared with other treatments (O-Thong *et al*., 2012; Luo *et al*., 2011). Meanwhile, cumulative methane and methane production yield achieved from co-digestion of SLS and POME was 293±7 mL CH_4 and 418±10 mL CH_4/g-VS_{added}, respectively which is 41% of methane theoretical yield (1014 mL CH_4/g-VS) (Batstone *et al*., 2002). Low methane production yield achieved from co-digestion of SLS to POME as compared to sole fermentation of POME due to SLS was a concentrated substrate with high concentrations of nitrogen compounds and sulfate as shown in **Table 3.1**, corresponding to sole fermentation of SLS (substrate control) with the lowest methane production yield was 230±10 mL CH_4/g-VS_{added} as shown in **Fig. 3.6** and **Fig. 3.7**. The initial pH of all treatment ranged 7.06-7.55 and at the end of fermentation the final pH increased to 7.47-7.79. The results indicate that VFA produced in H_2 production stage was converted to methane, corresponding to soluble metabolite products were small amount in all treatment as shown in **Table 3.10**.

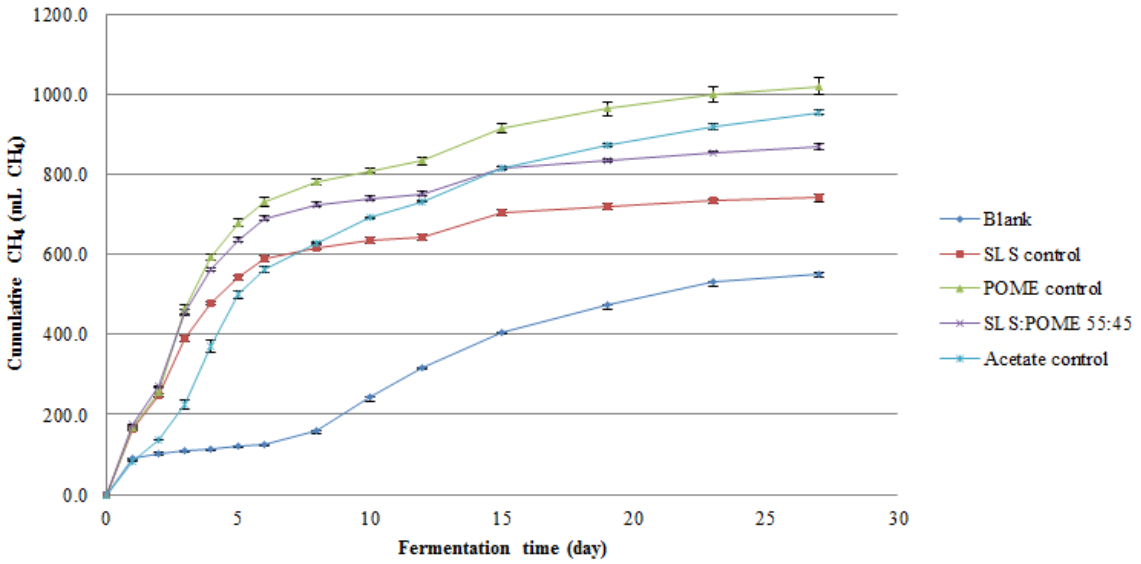

Fig. 3.6 Cumulative methane production achieved from the sequential methane production in batch experiment at the end of the 27 days of fermentation.

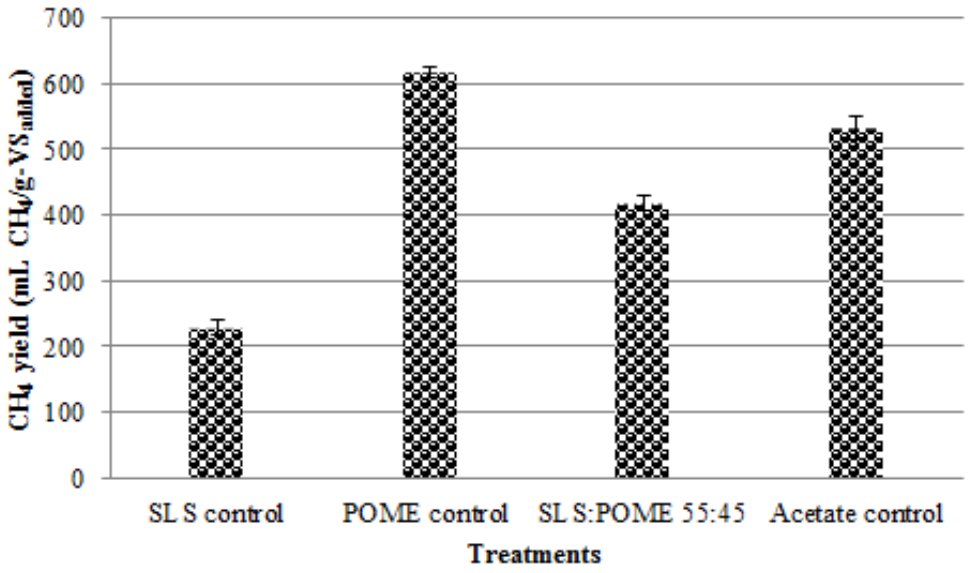

Fig. 3.7 Methane production yield achieved from the sequential methane production in batch experiment at the end of the 27 days of fermentation.

Table 3.10 Soluble metabolite products achieved from methane production in batch experiment at the end of the 27 days of fermentation.

Treatments	Initial pH	Final pH	Acetate (mM)	Propionate (mM)	Butyrate (mM)
Blank solution*	7.34±0.01	7.47±0.03	ND	1.48±0.05	ND
SLS*	7.24±0.02	7.49±0.01	ND	7.78±0.17	2.34±0.43
POME*	7.06±0.01	7.52±0.01	6.63±0.58	1.27±0.03	ND
SLS:POME 55:45 (%v/v)*	7.15±0.02	7.48±0.01	11.26±0.32	1.86±0.01	1.66±0.38
Acetate control	7.55±0.02	7.76±0.01	0.65±0.06	ND	ND

*Used effluent withdrawn from related BHP stage as substrate

ND = Non-detectable

3.5 Conclusions

The generating of biohydrogen and biomethane from two-stage dark co-digestion of SLS and POME was successfully achieved. The highest hydrogen production yield achieved from SLS: POME mixing ratio of 50:50 (%v/v) with the hydrogen production yield was 85.7±4.9 mL H_2/g-VS_{added}. The response surface methodology (RSM) results indicated that only $NaHCO_3$ concentration had significant individual effect on hydrogen production yield. The maximal hydrogen production yield achieved from moderate condition (run 14) with the hydrogen production yield was 91.7±3.9 mL H_2/g-VS_{added}. Nevertheless, it is increased just only 22% which is still not uneconomic for applying in the industrial scale when considered the economic cost of the external buffer and nutrients supplemented. This experiment is still having low of hydrogen production yield was obtained, the possible cause is that due to they are not initial pH suitable for biohydrogen production. Moreover, the other reasons is that using of anaerobic mixed cultures, its able to produce lactate resulted lead to not hydrogen production from lactate formation pathway. Meanwhile, higher of methane production yield achieved from co-digestion of SLS and POME of 418±10 mL CH_4/g-VS_{added} was obtained. Which are correlates to the lower of VFA concentration at the end of the 27 days of methane fermentation, indicated that VFA produced in the H_2 potential stage was converted to CH_4 in the second stage of the sequential processes.