CHAPTER IV

RESULTS AND DISCUSSION

This study was carried out in the batch experiment and was conducted in a laboratory. Feasibility of biohydrogen and biomethane production from food waste by a two-stage fermentation process and the environmental factors (pH, temperature and C/N ratio) affected on biohydrogen and biomethane production were investigated.

4.1 Characteristics of substrate and seed sludge

Food waste was collected from the central cafeteria at Mahidol University (Salaya campus), Nakhonpathom, Thailand. It was mixed with distilled water with a volume ratio of waste to distilled water of 1:2 to made the food waste slurry which was used as a substrate. Anaerobic sludge was used as seed sludge, which was taken from the anaerobic digestion excrement treatment plant of Bureau of Environment and Health, Nonthaburi, Thailand. The physical and chemical characteristics of substrate and seed sludge are shown in Table 4.1.

Table 4.1 Physical and chemical characteristics of substrate and seed sludge.

Parameter (Unit)	**Substrate**	**Seed sludge**
pH	4.73 ± 0.02	7.82 ± 0.02
Total solids (TS, g/L)	208.53 ± 9.44	74.81 ± 4.33
Volatile solids (VS, g/L)	193.72 ± 9.22	52.89 ± 2.86
Chemical oxygen demand (COD, g/L)	165.33 ± 9.24	37.33 ± 1.53
Total Kjeldahl nitrogen (TKN, g/L)	3.87 ± 0.35	1.54 ± 0.28
Carbohydrate (g/L)	142.87 ± 6.66	ND
Protein (g/L)	24.21 ± 2.20	ND
Fat oil and grease (FOG, g/L)	26.63 ± 1.10	ND

Note ND: Not determined.

Data indicated average value ± standard deviation (n = 3).

Regarding the Table 4.1, food waste contained the high concentrations of carbohydrate, protein, and FOG, since it mainly consisted of rice and noodle, and also included a small amount of vegetable and meat. This result demonstrated that food waste was a carbohydrate-based waste which is the main source for biohydrogen production. Carbohydrate-based waste can provide hydrogen production 20 times higher than protein-based waste and fat-based waste (Show et al., 2012). Moreover, it showed that substrate had high volatile solids of 193.72 ± 9.22 g/L (approximately 93%). Generally, food waste consists of 85 – 95% volatile solids thus it is simply hydrolysable (Kim et al, 2013). Therefore, it was possible to use food waste from cafeteria as a substrate in this study. As regards the anaerobic sludge, it was obtained from the anaerobic digestion excrement treatment plant. The excrement was stored in the anaerobic digester for 28 days before it was released to the sand trap which the liquid effluent and sludge were separated out (Bureau of Environment and Health Nonthaburi municipality, 2013). Excrement is the waste product from metabolism in human body. It consists of the mixed cultures of bacteria that have ability the degradation of organic matter to biogas in anaerobic process. Therefore, it was possible to use anaerobic sludge as seed sludge in fermentation process (Wang and Wan, 2009b).

4.2 Phase I: Feasibility of biohydrogen and biomethane production from food waste by a two-stage fermentation process

Cumulative hydrogen production and cumulative methane production from food waste were investigated from biohydrogen production (stage I) and biomethane production (stage II), respectively. The results were explained as follows:

(1) Biogas production, biogas component and COD removal

Biogas production and biogas component from food waste by a two-stage fermentation process were summarized in Table 4.2.

Table 4.2 Biogas production and biogas component from food waste by a two-stage fermentation process.

Treatment	Biogas (mL)	H_2 (%)	CO_2 (%)	N_2 (%)	CH_4 (%)
Control					
Stage I: Biohydrogen production	121.00 ± 15.56	8.85 ± 0.46	47.43 ± 0.66	43.72 ± 0.20	ND
Stage II: Biomethane production	ND	ND	ND	ND	ND
IA					
Stage I: Biohydrogen production	848.00 ± 60.81	33.61 ± 0.33	43.99 ± 0.74	22.40 ± 0.41	ND
Stage II: Biomethane production	ND	ND	ND	ND	ND
IB					
Stage I: Biohydrogen production	486.50 ± 40.31	27.35 ± 1.09	47.13 ± 0.82	25.52 ± 1.91	ND
Stage II: Biomethane production	ND	ND	ND	ND	ND
IC					
Stage I: Biohydrogen production	741.50 ± 16.26	32.83 ± 0.28	45.10 ± 0.38	22.07 ± 0.10	ND
Stage II: Biomethane production	2,420.00 ± 82.02	1.07 ± 0.09	44.75 ± 0.58	7.54 ± 0.26	46.64 ± 0.41

Note Control: Food waste.

IA: Food waste and pretreated seed sludge.

IB: Food waste and seed sludge without pretreatment method.

IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

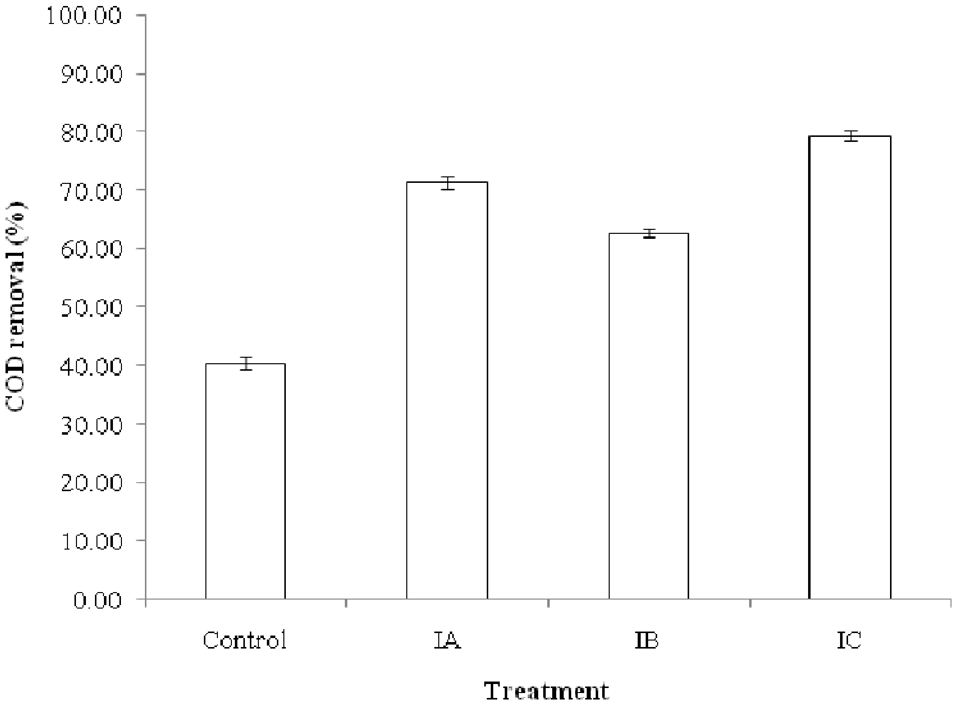
ND: Not detectable.

Data indicated average value ± standard deviation (n = 2).

The experiment of biohydrogen production (stage I) and biomethane production (stage II) were conducted for 7 and 21 days, respectively. The result of stage I showed that the maximum biogas production and percentage of hydrogen were obtained from the treatment IA (848.00 ± 60.81 mL, 33.61 ± 0.33 %H_2), IC (741.50 ± 16.26 mL, 32.83 ± 0.28 %H_2), IB (486.50 ± 40.31mL, 27.35 ± 1.09 %H_2) and control (121.00 ± 15.56 mL, 8.85 ± 0.46 %H_2), respectively. It was not found methane production in this stage. After biogas was not produced in stage I, biomethane production of byproducts was monitored in stage II. For stage II, only the treatment IC added the seed sludge without pretreatment method (10 mL) in byproducts from stage I and adjusted pH into 7.5 with $NaHCO_3$ (approximately 10 g). The result of stage II showed that biogas production was produced from only the treatment IC (2,420.00 ± 82.02 mL) and biogas component was 1.07 ± 0.09 %H_2, 44.75 ± 0.58 %CO_2, 7.54 ± 0.26 %N_2 and 46.64 ± 0.41 %CH_4. When biogas production from a two-stage fermentation process was summarized from biogas production of stage I and stage II. It found that the maximum total biogas production was obtained from the treatment IC (3,161.50 ± 65.76 mL), IA (848.00 ± 60.81 mL), IB (486.50 ± 40.31mL) and control (121.00 ± 15.56 mL), respectively.

In this study, the treatment IC was completely a two-stage fermentation process. It demonstrated that H_2 (32.83 ± 0.28 %) and CO_2 (45.10 ± 0.38 %) were the main biogas component in stage I, while CH_4 (46.64 ± 0.41 %) and CO_2 (44.75 ± 0.58 %) were the main biogas component in stage II. When it was compared with previous researches, the percentage of H_2, CH_4 and CO_2 might be differenced since the variation of substrate, seed sludge and environmental factors. However, the result of the main biogas component each stage was agree well with other researches (Liu et al., 2006; Wang and Zhao, 2009; Giordano et al., 2011; Chu et al., 2012). Wang and Zhao (2009) studied biohydrogen and biomethane production from food waste by a two-stage fermentation process, the research presented that the main biogas components from biohydrogen production process were 28 – 33 %H_2 and 47 – 51 %CO_2. For biomethane production process, the main biogas components were 58 – 71 %CH_4 and 26 - 41 %CO_2. Giordano et al. (2011) investigated the potential of biohydrogen and biomethane production from food industry waste by a two-stage fermentation process, the research presented that H_2 was produced in the range of 45 – 72% with CO_2 as the remaining portion from biohydrogen production process and CH_4 was produced in the range of 45 – 64% with CO_2 as the remaining portion from biomethane production process.

After the experiment finished, it found that COD of all the treatment decreased. COD removal efficiency each treatment is shown as Figure 4.1. The maximum COD removal efficiency was obtained from the treatment IC (79.41 ± 0.71 %), IA (71.32 ± 1.04 %), IB (62.78 ± 0.65 %) and control (40.45 ± 1.04 %), respectively.



Note Control: Food waste.

IA: Food waste and pretreated seed sludge.

IB: Food waste and seed sludge without pretreatment method.

IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

Bars indicated the standard deviation of data (n = 2).

Figure 4.1 COD removal efficiency from a two-stage fermentation process.

COD removal presented to degradation of organic matter. In dark fermentation process, the complex organic matter was degraded and converted to biogas by microorganisms. Thus, the process which presents the great biogas production will also provide the great COD removal. As a result of a two-stage fermentation process, the maximum total biogas production and COD removal were achieved from the treatment IC which occurred completely a two-stage fermentation process whereas the other treatments seem to be only a single stage of biohydrogen production. In previous researches from the literature, removal efficiency has been reported as the percentage of VS, carbohydrate and protein. Liu et al. (2006) studied biohydrogen and biomethane production from household solid waste by a two-stage fermentation process. The comparison of a single-stage and a two-stage process was studied. The result demonstrated that removal efficiency from a two-stage fermentation process (86% VS removal) was approximately 9% higher than its from a single-stage process (79% VS removal). Moreover, it found that removal efficiency from stage II (biomethane production, 83% VS removal) was higher than its from stage I (biohydrogen production, 19% VS removal). Chu et al. (2012) studied biohydrogen and biomethane production from food waste materials by a two-stage fermentation process. It found that a two-stage fermentation process presented low COD as the final product and removal efficiency of carbohydrate reached to 96%. Furthermore, removal efficiency of carbohydrate from stage II (biomethane production, above 96%) was higher than its from stage I (biohydrogen production, 50 – 69%).

(2) Cumulative hydrogen production and cumulative methane production

Cumulative hydrogen production and cumulative methane production were predicted by the modified Gompertz equation as shown in Table 4.3. The modified Gompertz equation has long been used as a kinetic model for study on hydrogen, methane and biogas production in a batch experiment (Nathao et al., 2013). This model presents some constants that have biological importance. Thus, it can be used for explanation and understanding of the fermentation process (Wang and Wan, 2009c). In previous study, it found that several researches used the modified Gompertz equation in order to study on biohydrogen production and biomethane production by a two-stage fermentation process (Yang et al., 2011; Liu et al., 2013; Nathao et al., 2013). In biohydrogen production process (stage I) as shown in Table 4.3, the lag phase time (λ) was 8 – 24 h. Biohydrogen production of food waste with the pretreated seed sludge presented the shortest lag phase time of 8 h, while that of food waste without seed sludge presented the longest lag phase time of 24 h. This duration was physiological adaptation of bacteria in environment conditions that depended on several factors such as pH, temperature, nutrient, germinant availability and others. The great conditions for bacterial growth provide the short lag phase time (Valdez-Vazquez and Poggi-Varaldo, 2009).

Table 4.3 Cumulative hydrogen production and cumulative methane production from a two-stage fermentation process by the modified Gompertz equation.

Treatment	**Stage I: Biohydrogen production**				**Stage II: Biomethane production**			
	H_{max} (mL)	**λ (h)**	**R_m (mL/h)**	**R^2**	**H_{max} (mL)**	**λ (h)**	**R_m (mL/h)**	**R^2**
Control	34.11	24	0.89	0.9687	ND	ND	ND	ND
IA	327.63	8	17.71	0.9865	ND	ND	ND	ND
IB	170.13	12	11.51	0.9811	ND	ND	ND	ND
IC	285.40	8	15.55	0.9838	1,570.58	24	22.99	0.9666

Note Control: Food waste.

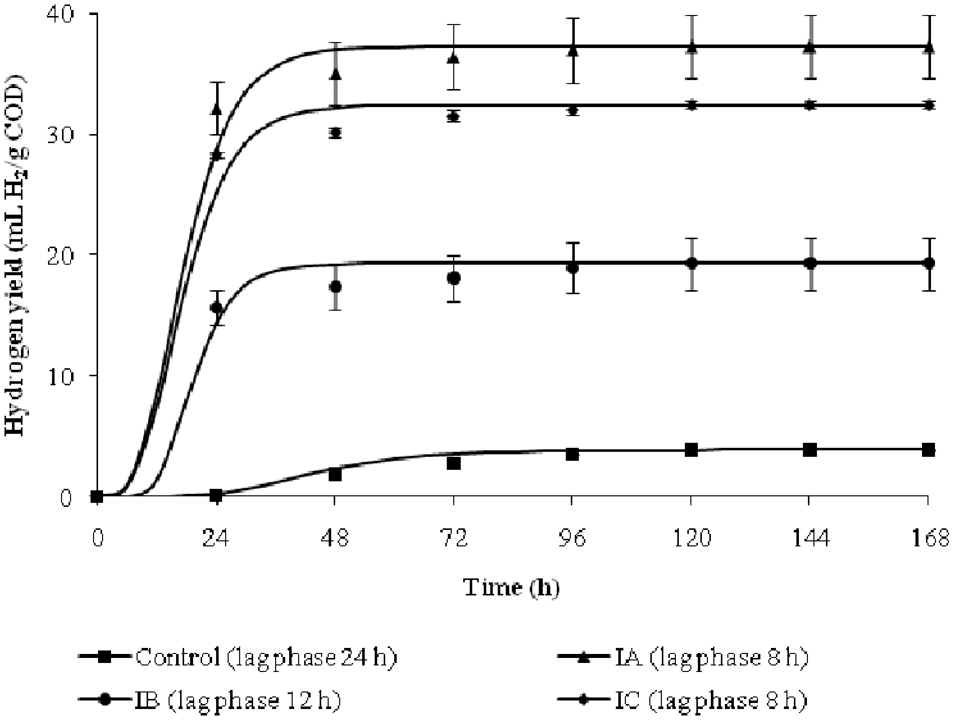
IA: Food waste and pretreated seed sludge.

IB: Food waste and seed sludge without pretreatment method.

IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

ND: Not detectable.

In this study, the maximum cumulative hydrogen production (H_{max}) and the maximum hydrogen production rate (R_m) were obtained from the treatment IA (327.63 mL, 17.71 mL/h), IC (285.40 mL, 15.55 mL/h), IB (170.13 mL, 11.51 mL/h) and control (34.11 mL, 0.89 mL/h), respectively. The correlation coefficient (R^2) was in the range of 0.96 – 0.98, which indicated that cumulative hydrogen production by the modified Gompertz equation was good consistent with the experimental results. Cumulative hydrogen production and COD of food waste were calculated for hydrogen yield (mL H_2/g COD). Hydrogen yield (stage I) is shown in Figure 4.2. The curves were plotted from the predicted values by the modified Gompertz equation and were fitted with the observed values from the experiment. The maximum hydrogen yields were obtained from the treatment IA (37.24 mL H_2/g COD), IC (32.43 mL H_2/g COD), IB (19.33 mL H_2/g COD) and control (3.88 mL H_2/g COD), respectively.



Note Control: Food waste.

IA: Food waste and pretreated seed sludge.

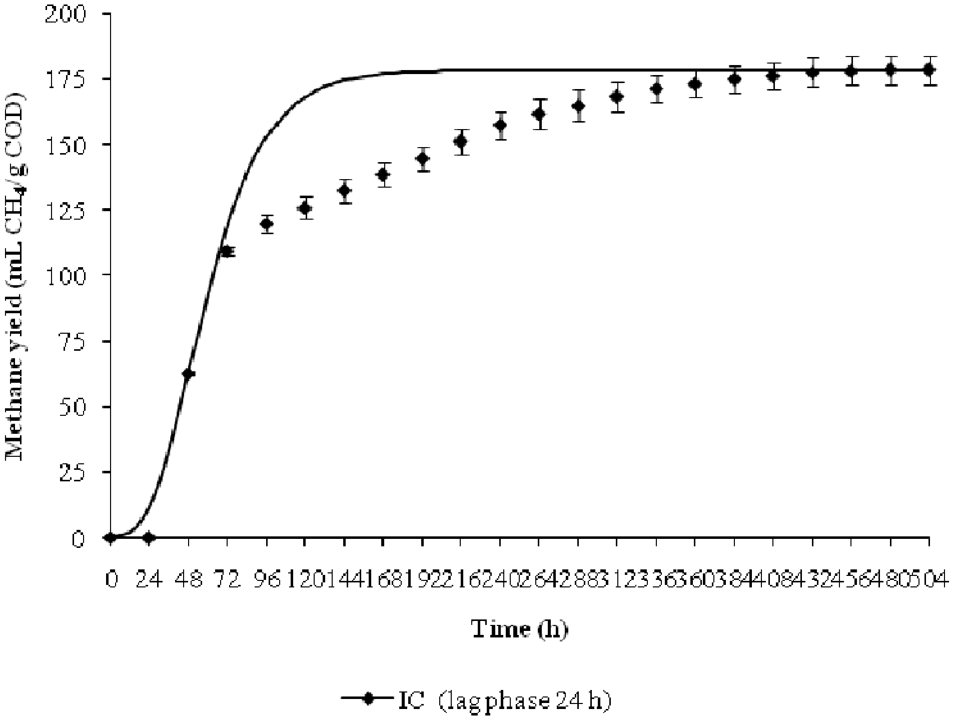
IB: Food waste and seed sludge without pretreatment method.

IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

Bars indicated the standard deviation of data (n = 2).

Figure 4.2 Hydrogen yield from biohydrogen production (stage I) by the modified Gompertz equation.

For biomethane production process (stage II), it had only the treatment IC which produced biogas. The lag phase time (λ) for methane production was 24 h. The maximum cumulative methane production (H_{max}) and the maximum methane production rate (R_m) were 1,570.58 mL and 22.99 mL/h, respectively. The correlation coefficient (R^2) was approximately 0.96 which indicated that cumulative methane production by the modified Gompertz equation was moderately consistent with the experimental results. Cumulative methane production and COD of food waste were calculated for methane yield (mL CH_4/g COD). Methane yield of the treatment IC is shown in Figure 4.3. The maximum methane yield was 178.49 mL CH_4/g COD.



Note IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

Bars indicated the standard deviation of data (n = 2).

Figure 4.3 Methane yield from biomethane production (stage II) of the treatment IC by the modified Gompertz equation.

As shown in Figure 4.2 and 4.3, the modified Gompertz equation provided S-shaped curve. Hydrogen (or methane) increased slowly with the increasing cultivation time from 0 to λ (lag phase), afterward it increased rapidly nearly at the rate of R_m (exponential growth phase), finally it reached to the value of H_{max} with an additional increasing the cultivation time (stationary phase). Moreover, this curve could indirectly explain the bacterial growth because hydrogen and methane were produced by hydrogen-producing bacteria and methanogenic bacteria, respectively. Thus, biogas was produced and increased rapidly when population of bacteria enlarged (Wang and Wan, 2009c).

As a result of stage I (Figure 4.2), it presented that addition of seed sludge in dark fermentation process was significantly ($p < 0.05$) positive effect on hydrogen yield (Appendix D, Table D1). Addition of seed sludge provided greater hydrogen yield than no addition of seed sludge. Moreover, addition of the pretreated seed sludge provided greater hydrogen yield than addition of the seed sludge without pretreatment. Seed sludge was added in dark fermentation process to provide the microorganisms which can convert organic matter to biogas. However, the control experiment which was not added any seed sludge but it found slight hydrogen yield. It indicated that food waste had some microorganisms, included hydrogen-producing bacteria. It was consistent with Wang and Zhao (2009), their study indicated that the original microbial cultures contained in food waste can used as the mixed cultures and biohydrogen production from food waste achieved in the first stage without addition of seed sludge. Generally, anaerobic sludge consists of the mixed cultures. During biohydrogen production process using the anaerobic sludge, hydrogen may be consumed by hydrogen-consuming bacteria. Thus, pretreatment of anaerobic sludge with some methods to suppress hydrogen-consuming bacterial activity whereas still preserving hydrogen-producing bacterial activity is necessary (Wang and Wan, 2009b).

In this study, the pretreated seed sludge was anaerobic sludge, which was pretreated at 100^{o}C for 15 min using the heat shock method. Heat shock method can inhibit hydrogen-consuming bacteria and select spores of acidogenic bacteria, which are a one of hydrogen-producing bacteria and to germinate and produce hydrogen when the environmental conditions are suitable for growth (Valdez-Vazquez and Poggi-Varaldo, 2009). Moreover, it has been reported that heat shock method could enrich hydrogen-producing bacteria from the mixed cultured (Sinha and Pandey, 2011). Therefore, hydrogen yield from food waste with the pretreated seed sludge of the treatment IA and IC were higher than that of the seed sludge without preheat method of the treatment IB.

A methanogenic bacterium is the microorganisms, which can produce methane and is a kind of hydrogen-consuming bacteria (Abbasi et al., 2012). Regarding all treatments of stage I, they were not found methane production due to the factors of initial pH (Table 4.5) and appropriate activity of methanogenic bacteria. For the treatment IA and IC, they were not found methane production because of using the pretreated seed sludge in experiment. For the result of stage II, biomethane production was not found from the treatment IA, IB and control because the initial pH (Table 4.5) was not suitable for methanogenic bacteria activity. The optimal pH for methanogenic bacteria should be in the range of 7.0 – 8.0 (Raposo et al., 2011). For the treatment IC, the seed sludge without pretreatment method was added to provide methanogenic bacteria and $NaHCO_3$ was added to adjust the initial pH into 7.5 which appropriate for the activity of methanogenic bacteria thus biomethane production achieved. This study was similar to other studies that addition of the seed sludge without pretreatment method and adjustment of pH in stage II was necessary (Giordano et al., 2011; Nathao et al., 2013).

Sodium bicarbonate ($NaHCO_3$) or baking soda is recognized as a bicarbonate buffer. Bicarbonate is often recommended using in biomethane production process to preserve a moderate pH because methanogenic bacteria prefer to active in the range of a neutral pH (Lin et al., 2013). It has been reported that addition of $NaHCO_3$ in thermophilic biomethane production from food waste and green waste (grass and wood chip) could enhance cumulative biogas production, biodegradation of organic matter and stability of pH (Abdulkarim and Abdullahi, 2010). However, it has been reported that the high concentration of $NaHCO_3$ (0.15 – 0.2 mol/L) could decrease methane production rate and acetate degradation rate from thermophilic biomethane production of glucose although it provided the great alkalinity and the short lag phase time (Lin et al., 2013).

A comparative study was carried out in other studies on biohydrogen and biomethane production from wastes by a two-stage fermentation process. The results were quite different as shown in Table 4.4. The possible reason for this variation was the difference in the characteristics of substrate and seed sludge, reactor type, temperature, pH and other environmental factors. In this study, hydrogen yield (32 mL H_2/g COD or 27 mL H_2/g VS) and methane yield (178 mL H_2/g COD or 150 mL H_2/g VS) were generally lower than the other reported values because the experiment was carried out under room temperature. However, methane yield in this study was higher than the maximum methane yield of 94 mL CH_4/g VS from mesophilic two-stage fermentation process as was reported by Nathao et al. (2013).

Another study, Chu et al. (2012) investigated a two-stage fermentation process from food waste materials under thermophilic temperature. There were three types of food wastes used as substrates: bean curd manufacturing waste (*okara*), kitchen garbage and potato. Hydrogen and methane yields were 17 - 59 mL H_2/g COD and 247 - 276 mL CH_4/g COD, respectively. It observed that hydrogen yield enhanced in the sequence of potato, kitchen garbage and *okara*, but inversely, the methane yield decreased. In stage I, potato provided the maximum hydrogen yield of 59 mL H_2/ g COD because it had the higher carbohydrate content than other substrates. However, it provided the minimum methane yield of 247 mL CH_4/g COD in stage II since most of substrate was degraded in the stage I.

Table 4.4 Biohydrogen and biomethane production from wastes by a two-stage fermentation process.

Substrate/ Inoculum	**Test type**	**Temperature**	**Stage I: Biohydrogen production**		**Stage II: Biomethane production**		**References**
			pH	**H_2 yield**	**pH**	**CH_4 yield**	
Food waste/ Anaerobic sludge	Batch	27^{o}C	5.87	32 mL H_2/g COD (or 27 mL H_2/g VS)	7.50	178 mL CH_4/g COD (or 150 mL CH_4/g VS)	This study*
Food waste/ Anaerobic activated sludge	Batch	37^{o}C	5.50	121 mL H_2/g VS	7.00	322 mL CH_4/g VS	Liu et al. (2013)
Food waste/ Anaerobic sludge	Batch	37^{o}C	6.00	55 mL H_2/g VS	7.00	94 mL CH_4/g VS	Nathao et al. (2013)
Wheat/ Anaerobic sludge	Batch	35^{o}C	7.00	47 mL H_2/g COD	7.00	202 mL CH_4/g COD	Giordano et al. (2011)
Household solid waste/ Anaerobic sludge	Continuous	37^{o}C	5.20	43 mL H_2/g VS	7.50	500 mL CH_4/g VS	Liu et al. (2006)
Potato/ Anaerobic digester sludge	Continuous	55^{o}C	5.50	59 mL H_2/g COD	7.10-7.60	247 mL CH_4/g COD	Chu et al. (2012)

Note * The results of treatment IC.

(3) pH and intermediate products

In this study, the initial pH and the final pH were measured using a pH meter. The pH values of a two-stage fermentation process are shown in Table 4.5. Moreover, concentration of intermediate products was measured during fermentation process as shown in Table 4.6. In stage I, the initial pH values of biohydrogen production process were in the range of 5.56 – 5.89. After biohydrogen production finished, the final pH decreased in the range of 3.86 – 4.10. When the maximum hydrogen production occurred, it found 340.47 – 1,909.28 mg/L of acetic acid and 3,065.28 – 3,230.73 mg/L of butyric acid, while it was not found propionic acid. The concentration of butyric acid and acetic acid were calculated for butyrate/acetate ratio (B/A ratio). B/A ratio was in the range of 1.62 – 1.80.

Table 4.5 pH values in each stage of a two-stage fermentation process.

Treatment	**Stage I: Biohydrogen production**		**Stage II: Biomethane production**	
	Initial pH	**Final pH**	**Initial pH**	**Final pH**
Control	5.56 ± 0.04	4.10 ± 0.01	4.10 ± 0.01	3.80 ± 0.01
IA	5.89 ± 0.01	3.90 ± 0.04	3.90 ± 0.04	3.63 ± 0.13
IB	5.82 ± 0.01	3.86 ± 0.06	3.86 ± 0.06	3.04 ± 0.02
IC	5.87 ± 0.01	4.05 ± 0.06	7.50 ± 0.00*	5.21 ± 0.03

Note * pH was adjusted with $NaHCO_3$.

Control: Food waste.

IA: Food waste and pretreated seed sludge.

IB: Food waste and seed sludge without pretreatment method.

IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

Data indicated average value ± standard deviation (n = 2).

Table 4.6 Concentration of intermediate products during a two-stage fermentation process.

Treatment	Acetic acid (mg/L)	Butyric acid (mg/L)	Propionic acid (mg/L)	B/A ratio	Ammonia (mg N/L)
Control					
D_{Hmax}	340.47 ± 15.23	ND	ND	ND	ND*
D_{Mmax}	ND*	ND*	ND*	ND*	328.61 ± 18.42
D_{L}	345.58 ± 23.68	ND	ND	ND	398.61 ± 3.87
IA					
D_{Hmax}	1,796.42 + 20.47	3,230.73 ± 95.45	ND	1.80 ± 0.07	ND*
D_{Mmax}	ND*	ND*	ND*	ND*	310.44 ± 10.42
D_{L}	1,864.14 ± 94.26	3,156.81 ± 195.99	ND	1.69 ± 0.02	367.92 ± 9.84
IB					
D_{Hmax}	1,867.75 + 29.60	3,065.28 ± 111.76	ND	1.64 ± 0.03	ND*
D_{Mmax}	ND*	ND*	ND*	ND*	218.72 ± 37.78
D_{L}	1,920.15 ± 9.65	3,225.43 ± 47.72	ND	1.68 ± 0.03	302.51 ± 16.10
IC					
D_{Hmax}	1,909.28 ± 60.35	3,089.74 ± 86.93	ND	1.62 ± 0.01	ND*
D_{Mmax}	ND*	ND*	ND*	ND*	146.49 ± 8.71
D_{L}	1,342.93 ± 31.93	4,549.32 ± 65.90	ND	3.39 ± 0.03	182.87 ± 23.48

Note Control: Food waste.

IA: Food waste and pretreated seed sludge.

IB: Food waste and seed sludge without pretreatment method.

IC: Food waste and pretreated seed sludge were operated in stage I. Then, seed sludge without pretreatment method was added in stage II.

D_{Hmax} and D_{Mmax}: The day that the maximum hydrogen and the maximum methane were produced, respectively.

D_{L}: The last day of the experiment.

ND: Not detectable.

ND*: Not determined.

Data indicated average value ± standard deviation (n = 2).

pH is a main factor that influences the activity of hydrogen-producing bacteria since pH affect to hydrogenase (Valdez-Vazquez and Poggi-Varaldo, 2009). In this study, the maximum hydrogen production was achieved from the treatment IA which the initial pH of 5.89 was in the range of biohydrogen production. The activity of hydrogen-producing bacteria can occur at pH range of 5.0 – 7.5. During biohydrogen production process, not only hydrogen and carbon dioxide are produced as the main products but organic acids and alcohols are also produced as the byproducts (Wei et al., 2010). It well known that volatile fatty acids (VFAs) are produced as the byproducts throughout biohydrogen production process. The kind of VFAs is strongly related to the quantity of hydrogen production (Wang and Zhao, 2009; Kargi et al., 2012). According to the stoichiometry, the maximum of 4 mole hydrogen is produced per mole glucose when the end product is acetic acid (Eq. 4-1), while only 2 mole hydrogen is produced per mole glucose when the end product is butyric acid (Eq. 4-2) (Liu et al., 2006; Ni et al., 2006; Holladay et al., 2009).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH \text{ (acetic acid)} + 2CO_2 \qquad \text{(Eq. 4-1)}$$
$$C_6H_{12}O_6 \rightarrow 2H_2 + CH_3CH_2CH_2COOH \text{ (butyric acid)} + 2CO_2 \qquad \text{(Eq. 4-2)}$$

In this study, the main VFAs were butyric acid and acetic acid, respectively. Butyric acid and acetic acid were calculated for B/A ratio. Butyric acid of 3,230.73 mg/L, acetic acid of 1,796.42 mg/L and B/A ratio of 1.80 were obtained from the treatment IA, which was accompanied with the maximum hydrogen yield (37.24 mL H_2/g COD). The B/A ratio has been used for evaluation of biohydrogen production. The effective biohydrogen production will provide a high B/A ratio. It has been reported that the variation of B/A ratio value depended on seed cultures and substrate, which effected on biohydrogen production via butyrate and acetate pathways (Han and Shin, 2004; Thanwised et al., 2012). This study was not found propionic acid because the metabolic pathway of glucose has no hydrogen production via propionate pathway (Chu et al., 2012). The accumulation of VFAs caused reduction of the final pH in the reactor. Wei et al. (2010) reported that the low pH and the large amount concentration of VFAs could inhibit the activity of hydrogen-producing bacteria. Hydrogen was not produced when the pH value was less than 4.0.

According to the previous result, biomethane production was achieved from only treatment IC in stage II. The possible reasons were the seed sludge without pretreatment method consisted of methanogenic bacteria and the initial pH of 7.50 was in the range of the optimal pH (7.0 – 8.0) for the activity of methanogenic bacteria (Raposo et al., 2011). It reported that the byproducts from biohydrogen production process can be used as a substrate in biomethane production process (Wang and Zhao, 2009; Luo et al., 2011; Chu et al., 2012; Guo et al., 2014). Methanogenic bacteria can produce methane from hydrogen with carbon dioxide (Eq. 4-3), acetic acid (Eq. 4-4) and methanol (Eq. 4-5) as follows (Chandra et al., 2012):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \text{(Eq. 4-3)}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \text{(Eq. 4-4)}$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad \text{(Eq. 4-5)}$$

After treatment IC finished, it found 1,342.93 mg/L of acetic acid and 4,549.32 mg/L of butyric acid. Propionic acid and methanol concentrations were not found in this treatment (Table 4.6). It noticed that acetic acid in biomethane production process decreased when was compared to its concentration in biohydrogen production process. This indicated that the byproduct of biohydrogen production process was converted to methane.

In biomethane production process, ammonia also was measured. When the maximum methane production was produced, it found 146.49 – 328.61 mg N/L of ammonia. After the experiment finished, it found 182.87 – 398.61 mg N/L of ammonia. Generally, ammonia is produced by biodegradation of protein and urea. It is considered as a main inhibitor of methanogenic bacteria growth. The activity of methanogenic bacteria was inhibited 56% by concentration of ammonia in the range of 4,051 – 5,743 mg/L. Moreover, it has been reported in literature that methane production was inhibited 50% by concentration of ammonia in wide range of 1,700 – 14,000 mg/L. However, concentration of ammonia lower 200 mg/L is useful to fermentation process because nitrogen is an important nutrient for microorganisms (Chen et al., 2008). Among all treatment in stage II, treatment IC which biomethane production achieved, it found the minimum concentration of ammonia (146.49

mg N/L) when the maximum methane production (178.49 mL CH_4/g COD) was produced. pH is an important factor, which involve toxicity of ammonia. Concentration of ammonia will enlarge with increasing of pH, but it can be reduced by accumulation of VFAs in system. However, the high concentration of VFAs leads to the low pH. If pH is lower than 6.0, methane production will be inhibited (Luo et al., 2011). Therefore, the maintenance of pH, VFAs and ammonia is important to avoid failure of biomethane production process (Chen et al., 2008).

Two-stage fermentation process is separation of acidogenesis/acetogenesis and methanogenesis for biohydrogen production in stage I and biomethane production in stage II, respectively. Acidogenesis/acetogenesis produces hydrogen and carbon dioxide as the main gas products and provides VFAs as the byproducts. Then, a part of VFAs and other biodegradable residues from stage I will be converted into methane and carbon dioxide by methanogenesis. There are several advantages from this process such as enhance efficient of substrate digestion and COD removal, high biogas yield, high energy recovery and reduce concentration of VFAs in the final product (Liu et al., 2006; Yang et al., 2011; Chu et al., 2012; Nathao et al., 2013; Gioannis et al., 2013). Moreover, carbon dioxide and carbon monoxide emission from a two-stage fermentation process are less than a single stage fermentation process (Luo et al., 2011). Thus, a two-stage fermentation process is an environmental friendly process. However, the previous research from the literature suggested that an adjustment of environmental factors for each stage is necessary because the activity of hydrogen-producing bacteria and methanogenic bacteria can work at the different optimum conditions (Liu et al., 2006; Giordano et al. 2011; Chu et al., 2012). As a result from this study, it was summarized that food waste could produced biohydrogen production and biomethane production by a two-stage fermentation process. The result from the treatment IC was applied in the next experiment. Food waste and the pretreated seed sludge were used for study the optimum of environmental conditions on biohydrogen production, while hydrogen fermentation effluent and the seed sludge without pretreatment method were used for study the optimum of environmental conditions on biomethane production.

4.3 Phase II: The optimum of environmental conditions on biohydrogen production

The optimum of initial pH, temperature and carbon to nitrogen ratio (C/N ratio) were investigated on biohydrogen production from food waste by dark fermentation. The pretreated seed sludge was used as inoculum (Phase I result). The results were explained as follows:

4.3.1 Phase IIA: Effect of initial pH and temperature on biohydrogen production

The batch reactor was set up with food waste solution and the pretreated seed sludge which its original pH value was 5.88 ± 0.01. Then, the batch reactor was adjusted with HCl or KOH to set the initial pH in the range from 5.0, 6.0, 7.0, 8.0 and 9.0. Each the initial pH test was controlled at mesophilic temperature (35 ± 2°C) and thermophilic temperature (55 ± 2°C). The results were explained as follows:

(1) Biogas production, biogas component and COD removal

Biogas production and biogas component at various initial pH values under the mesophilic and thermophilic temperatures were summarized in Table 4.7. All batch experiments were set up the working volume at 200 mL and were operated for 12 days. At mesophilic temperature (35 ± 2°C), the maximum biogas production and percentage of hydrogen were obtained at initial pH 7.0 (1,386.00 ± 31.75 mL, 54.66 ± 1.06 %H_2), initial pH 6.0 (1,243.00 ± 22.11 mL, 34.02 ± 0.61 %H_2), initial pH 8.0 (1,051.00 ± 33.41 mL, 31.05 ± 0.89 %H_2), initial pH 9.0 (8982.33 ± 2.52 mL, 30.40 ± 0.23 %H_2) and initial pH 5.0 (880.33 ± 12.22 mL, 24.87 ± 0.46 %H_2), respectively. For thermophilic temperature (55 ± 2°C), the trends of biogas production and percentage of hydrogen were similar to that's of mesophilic temperature. The maximum biogas production and percentage of hydrogen were obtained at initial pH 7.0 (2,406.00 ± 19.92 mL, 59.45 ± 0.48 %H_2), initial pH 6.0 (2,075.33 ± 13.05 mL, 46.88 ± 0.26 %H_2), initial pH 8.0 (1,803.33 ± 27.01 mL, 40.38 ± 0.52 %H_2), initial pH 9.0 (1,438.33 ± 29.70 mL, 40.89 ± 0.48 %H_2) and initial pH 5.0 (1,060.67 ± 19.50 mL, 26.10 ± 0.58 %H_2), respectively. All experiments were not found methane production in both of the mesophilic and thermophilic temperatures.

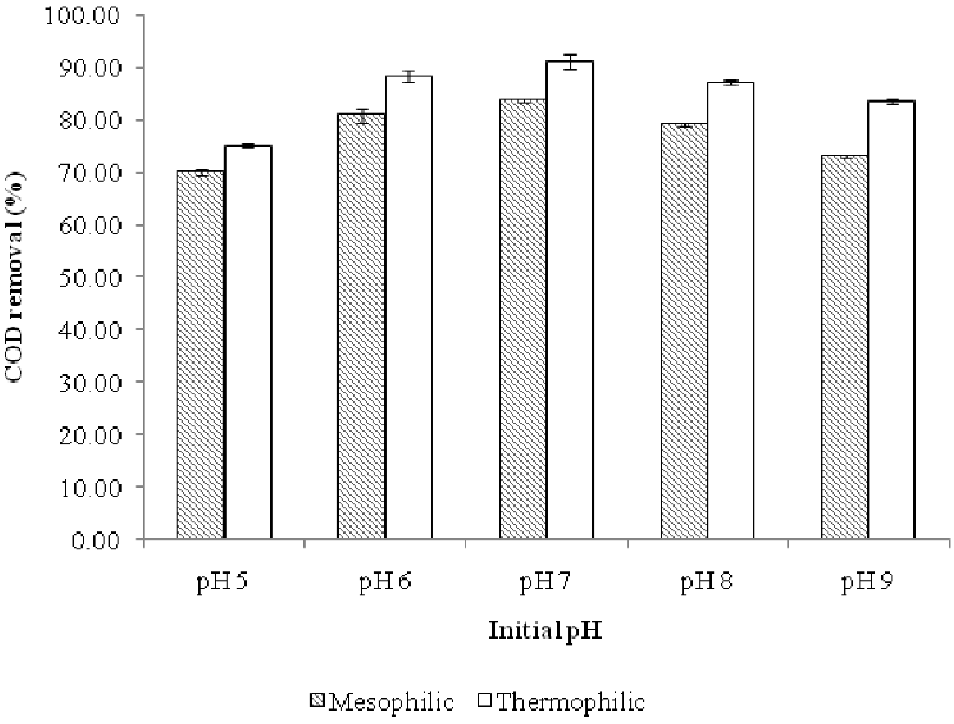
Table 4.7 Biogas production and biogas component at various initial pH values under the mesophilic and thermophilic temperatures.

Temperature	**Initial pH**	**Biogas (mL)**	**Biogas component**			
			H_2 (%)	**CO_2 (%)**	**N_2 (%)**	**CH_4 (%)**
Mesophilic (35 ± 2°C)	5.0	880.33 ± 12.22	24.87 ± 0.46	43.08 ± 0.82	32.05 ± 0.37	ND
	6.0	1,243.00 ± 22.11	34.02 ± 0.61	39.82 ± 0.78	26.16 ± 1.37	ND
	7.0	1,386.00 ± 31.75	54.66 ± 1.06	32.23 ± 0.16	13.11 ± 1.22	ND
	8.0	1,051.00 ± 33.41	31.05 ± 0.89	38.97 ± 0.75	29.98 ± 1.57	ND
	9.0	982.33 ± 2.52	30.40 ± 0.23	37.63 ± 0.36	31.97 ± 0.55	ND
Thermophilic (55 ± 2°C)	5.0	1,060.67 ± 19.50	26.10 ± 0.58	44.29 ± 1.88	29.61 ± 1.43	ND
	6.0	2,075.33 ± 13.05	46.88 ± 0.26	29.65 ± 0.59	23.47 ± 0.43	ND
	7.0	2,406.00 ± 19.92	59.45 ± 0.48	21.61 ± 0.04	18.94 ± 0.44	ND
	8.0	1,803.33 ± 27.01	40.38 ± 0.52	33.38 ± 0.43	26.24 ± 0.66	ND
	9.0	1,438.33 ± 29.70	40.89 ± 0.48	32.21 ± 0.56	26.90 ± 0.88	ND

Note ND: Not detectable.
Data indicated average value ± standard deviation (n = 3).

After the experiments finished, they found that COD of all batch reactors decreased. COD removal efficiency at various initial pH values under the mesophilic and thermophilic temperatures is shown in Figure 4.4. At mesophilic temperature (35 ± 2°C), the maximum COD removal efficiency was obtained at initial pH 7.0 (83.70 ± 0.32 %), initial pH 6.0 (80.93 ± 1.40 %), initial pH 8.0 (79.07 ± 0.32 %), initial pH 9.0 (73.15 ± 0.32 %) and initial pH 5.0 (70.00 ± 0.56 %), respectively. For thermophilic temperature (55 ± 2°C), the maximum COD removal efficiency was obtained at initial pH 7.0 (91.11 ± 1.47 %), initial pH 6.0 (88.33 ± 1.11 %), initial pH

8.0 (87.22 ± 0.56 %), initial pH 9.0 (83.66 ± 0.45 %) and initial pH 5.0 (75.19 ± 0.32 %), respectively.



Note Bars indicated the standard deviation of data (n = 3).

Figure 4.4 COD removal efficiency at various initial pH values under the mesophilic and thermophilic temperatures.

In dark fermentation, biogas was produced from degradation of organic matter by microorganisms. Therefore, biogas production and COD removal related each other. The batch reactor which presents the greatest biogas production will also provide the greatest COD removal. Among various initial pH in this study, the maximum biogas production and COD removal were achieved at initial pH 7.0. At this optimal initial pH, biogas production of 2,406.00 ± 19.92 mL and COD removal of 91.11 ± 1.47 % were obtained under thermophilic temperature (55 ± 2°C), while biogas production of 1,386.00 ± 31.75 mL and COD removal of 83.70 ± 0.32 % were obtained under mesophilic temperature (35 ± 2°C). This study indicated that initial pH and temperature influenced on biogas production and COD removal in biohydrogen production process. Wang and Wan (2011) reported that substrate degradation efficiency improved with increasing of initial pH and temperature to the optimal condition, and then it decreased with extra initial pH and temperature. In their study, biohydrogen production from glucose provided the maximum substrate degradation efficiency of 96.50% at initial pH 7.1 and temperature of 37.8°C, whereas the maximum substrate degradation efficiency of 98.10% at initial pH 7.0 and temperature of 40.0°C was reported from another study (Wang and Wan, 2008).

(2) Cumulative hydrogen production

Cumulative hydrogen production at various initial pH values under the mesophilic and thermophilic temperatures was predicted by the modified Gompertz equation as shown in Table 4.8. The correlation coefficient (R^2) was in the range of 0.98 – 0.99, which indicated that the modified Gompertz equation could successfully describe the progress of cumulative hydrogen production in this study.

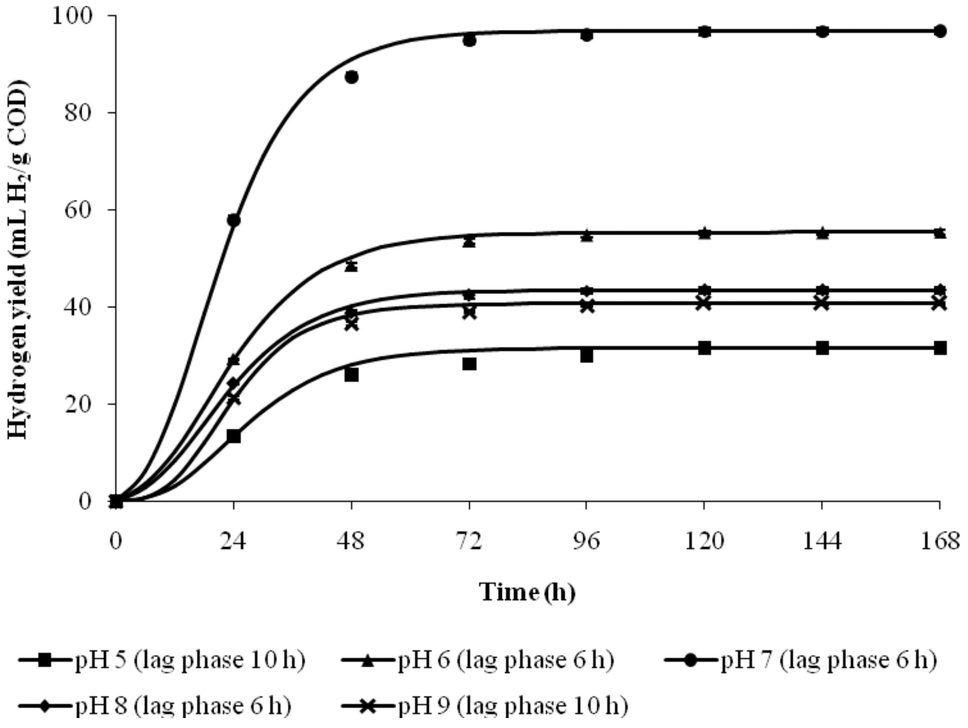
Table 4.8 Cumulative hydrogen production at various initial pH values under the mesophilic and thermophilic temperatures by the modified Gompertz equation.

Initial pH	**Mesophilic (35 ± 2 °C)**				**Thermophilic (55 ± 2 °C)**			
	H_{max} (mL)	**λ (h)**	**R_m (mL/h)**	**R^2**	**H_{max} (mL)**	**λ (h)**	**R_m (mL/h)**	**R^2**
5.0	278.34	10	8.43	0.9914	322.17	12	9.18	0.9870
6.0	487.80	6	14.34	0.9989	1,079.21	12	28.45	0.9852
7.0	853.65	6	28.35	0.9984	1,549.57	12	41.71	0.9808
8.0	383.17	6	11.85	0.9990	814.33	12	22.08	0.9826
9.0	358.74	10	13.40	0.9973	669.96	12	18.35	0.9837

The lag phase time (λ) was found in the range of 6 – 10 h under mesophilic temperature (35 ± 2°C), while it was found 12 h under thermophilic temperature (55 ± 2°C). Lag phase time is an adaptation period of microorganisms in the new environmental conditions. There are three main factors that effect on lag phase; environmental conditions, bacterial growth and seed sludge size. Among these factors, environmental conditions such as pH, temperature and nutrients are considered as the key factors (Valdez-Vazquez and Poggi-Varaldo, 2009; Kim et al., 2011). In this study, it found that the lag phase time under mesophilic temperature (35 ± 2°C) was shorter than that's of thermophilic temperature (55 ± 2°C). Moreover, the short lag phase of 6 h was found at initial pH in the range of 6.0 – 8.0, while the long lag phase of 10 h was found at initial pH 5.0 and 9.0 under mesophilic fermentation. These results resembled to other studies for example, Wang and Wan (2008) studied effect of temperature in the range of 20 – 55°C on biohydrogen production from glucose by mixed cultures. Their study presented that the lag phase time decreased when temperature increased from 20°C to 40°C, and then it increased when temperature increased from 40°C to 55°C. Kargi et al. (2012) studied biohydrogen production from chess whey powder solution under mesophilic temperature (35°C) and thermophilic temperature (55°C). The results presented that the lag phase time of thermophilic fermentation (136 h) was longer than that's of mesophilic fermentation (4 h) because the original seed sludge was taken from the anaerobic wastewater treatment plant, which was operated at 35°C. Thus, the mesophilic mixed cultures took long time for adaptation in thermophilic fermentation. Kim et al. (2011) investigated effect of initial pH ranging from 5.0 to 9.0 under mesophilic temperature (35°C). They reported that initial pH significantly affected on lag phase time. The short lag phase of 4 h was found at initial pH 7.0 and 8.0, while the long lag phase above 10 h was found at initial pH 5.0 and 9.0.

As a result in this study under mesophilic temperature (35 ± 2°C), the maximum cumulative hydrogen production (H_{max}) and the maximum hydrogen production rate (R_m) were in the range of 278.34 – 853.65 mL and 8.43 – 28.35 mL/h, respectively. For thermophilic temperature (55 ± 2°C), H_{max} and R_m were in the range of 322.17 – 1,549.57 mL and 9.18 – 41.71 mL/h, respectively. The results presented that H_{max} and R_m of thermophilic fermentation were higher than that's of mesophilic fermentation. Moreover, H_{max} and R_m increased with increasing initial pH from 5.0 to 7.0 after that they decreased with further initial pH from 7.0 to 9.0. As a result, the greatest H_{max} (1,549.57 mL) and R_m (41.71 mL/h) were obtained at initial pH 7.0 under thermophilic temperature (55 ± 2°C). Thus, this study demonstrated that initial pH and temperature influenced on lag phase time, cumulative hydrogen production and hydrogen production rate.

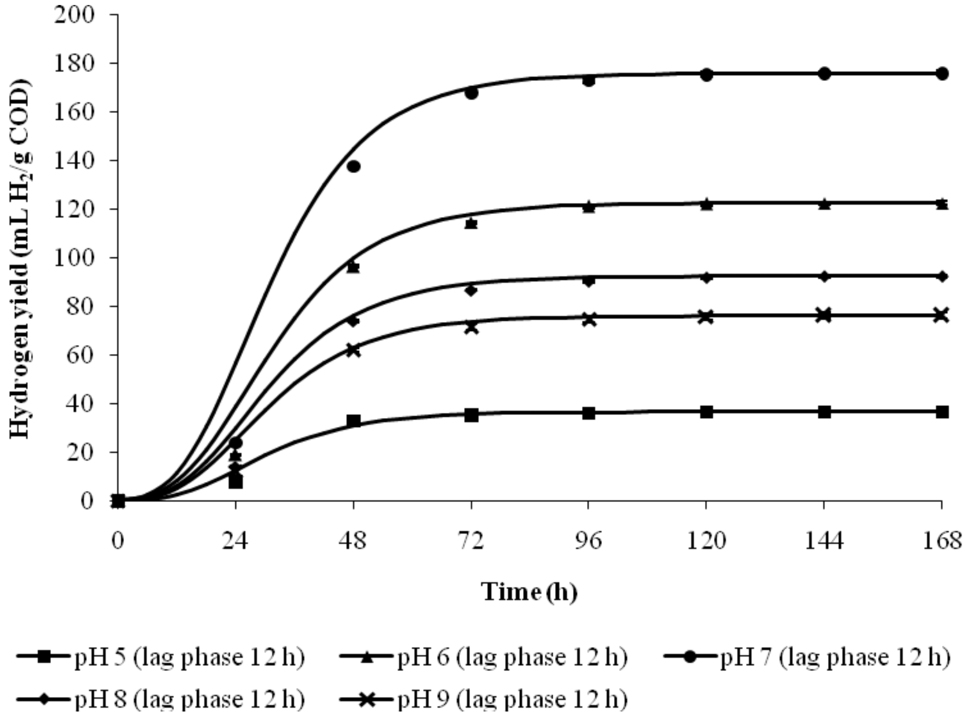
Cumulative hydrogen production and COD of food waste were calculated for hydrogen yield (mL H_2/g COD). Hydrogen yields at various initial pH values under mesophilic (35 $\pm$ 2°C) and thermophilic (55 $\pm$ 2°C) temperatures are shown in Figure 4.5 and 4.6, respectively. The curves were plotted from the predicted values by the modified Gompertz equation and were fitted with the observed values of the experiment.



Note Bars indicated the standard deviation of data (n = 3).

Figure 4.5 Hydrogen yield by the modified Gompertz equation at various initial pH values under mesophilic temperature (35 $\pm$ 2°C).

As shown in Figure 4.5, it found that initial pH was significantly ($p < 0.05$) effect on hydrogen yield under mesophilic temperature (35 $\pm$ 2°C) (Appendix D, Table D2). The maximum hydrogen yield was obtained at initial pH 7.0 (97.02 mL H_2/g COD), initial pH 6.0 (55.44 mL H_2/g COD), initial pH 8.0 (43.55 mL H_2/g COD), initial pH 9.0 (40.77 mL H_2/g COD) and initial pH 5.0 (31.63 mL H_2/g COD), respectively.



Note Bars indicated the standard deviation of data (n = 3).

Figure 4.6 Hydrogen yield by the modified Gompertz equation at various initial pH values under thermophilic temperature (55 ± 2°C).

As shown in Figure 4.6, initial pH was significantly ($p < 0.05$) effect on hydrogen yield under thermophilic temperature (55 ± 2°C) (Appendix D, Table D3). The maximum hydrogen yield was obtained at initial pH 7.0 (176.10 mL H_2/g COD), initial pH 6.0 (122.65 mL H_2/g COD), initial pH 8.0 (92.55 mL H_2/g COD), initial pH 9.0 (76.14 mL H_2/g COD) and initial pH 5.0 (36.61 mL H_2/g COD), respectively.

It noticed that the trends of hydrogen yields at various initial pH under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures were similarity. Hydrogen yield significantly increased with increasing initial pH from 5.0 to 7.0. Then, it significantly decreased with further increasing initial pH from 7.0 to 9.0. This result demonstrated that increasing initial pH could enhance the capability of hydrogen-producing bacteria to produce hydrogen. However, the higher or lower initial pH could decrease hydrogen production (Wang and Wan, 2009b). Initial pH is an important factor that can enhance biohydrogen production because it affects on lag phase time, spore germination, bacterial growth and synthesis of enzymes. Activity of hydrogen-producing bacteria and metabolic pathway are controlled by pH that influence on hydrogenase, which is an important enzymes for hydrogen production (Valdez-Vazquez and Poggi-Varaldo, 2009; Sinha and Pandey, 2011; Gioannis et al., 2013). In this study, the optimal initial pH of 7.0 was in the range of 5.0 – 7.5, which hydrogen-producing bacteria can active (Wei et al., 2010). As in literature, it has been reported that the maximum biohydrogen production from organic wastes achieved at the optimum pH in the range of 5.0 – 7.0, which the optimal pH of 5.5 was mostly presented (Chong et al., 2009). Moreover, this study presented that hydrogen yield from thermophilic fermentation was higher than that's of mesophilic fermentation. Temperature is a significant factor because it influences on the activity of hydrogen-producing bacteria and the fermentation process in hydrogen production. The optimum temperature can improve the shorten lag phase time, hydrogen production rate and hydrogen production potential (Wang and Wan, 2008; O-Thong et al., 2011). The capability of hydrogen-producing bacteria to generate hydrogen can enhance by increasing temperature. However, temperature at critically higher levels can reduce hydrogen production. It is well known that fermentation process under thermophilic temperature (around 55^{o}C) is more proficient than mesophilic temperature (around 37^{o}C) to produce biogas (Abbasi et al., 2012). Thermophilic temperature was appropriately considered for the activity of hydrogen-producing bacteria, while it could restrain the growth of lactate-forming bacteria and the activity of other hydrogen-consuming bacteria (Gioannis et al., 2013). Thermophilic temperature could decrease the solubility of hydrogen thus it relieved the effect of hydrogen partial pressure on biohydrogen production (Nazlina et al., 2009). The accumulation of hydrogen partial pressure in head space of the reactor caused the shifting of metabolic pathway from acidogenesis to solventogenesis. The solvent such as ethanol and buthanol was more produced instead hydrogen in solventogenesis (Valdez-Vazquez and Poggi-Varaldo, 2009). Moreover, it has been reported that the role of temperature was selection of microorganisms in the fermentation process (Gioannis et al., 2013). Thermophilic bacteria are microbial community that appears in biohydrogen production at high temperature. It was considered as the proficient hydrogen-producing bacteria because it could consume various organic wastes (Chong et al., 2009; O-Thong et al., 2011).

This study demonstrated that initial pH and temperature significantly influenced on hydrogen yield. The maximum hydrogen yield of 176.10 mL H_2/g COD was obtained at initial pH 7.0 under thermophilic temperature (55 $\pm$ 2°C). Moreover, it found that hydrogen yield was significantly decreased when initial pH extremely low or high at 5.0 or 9.0, respectively. It was compared with other researches on biohydrogen production from wastes at various initial pH values under the mesophilic (around 37°C) and thermophilic (around 55°C) temperatures as shown in Table 4.9. This study was similar to other studies, which demonstrated thermophilic fermentation provided higher hydrogen yield than mesophilic fermentation (Shin et al., 2004; Nazlina et al., 2009; Kargi et al., 2012). However, there are some studies reported that the maximum hydrogen yield was obtained under mesophilic fermentation (Kim et al., 2008; Lee et al., 2008a). Moreover, this study also resembled to several researches which presented the optimal initial pH of 7.0 provided the maximum hydrogen yield (Kim et al., 2008; Lee et al., 2008b; Nazlina et al., 2009; Kargi et al., 2012; Ramos et al., 2012). On the other hand, there were some studies reported that the maximum hydrogen yield was obtained at initial pH in the range of acidic or basal pH (Shin et al., 2004; Lee at el., 2008a; Kim et al., 2011). The optimal initial pH and temperature were still different. The possible reasons were the various characteristics of substrate and seed sludge, initial pH and temperature range studied, reactor type, nutrients, etc.

Nazlina et al. (2009) investigated the effect of initial pH (5.0 – 8.0) and temperature (35 – 60°C) on biohydrogen production from food waste. They reported that hydrogen yield was low obtained at mesophilic temperature in the range of 35 – 40°C, while it was higher obtained at thermophilic temperature in the range of 50 – 55°C. However, the extreme temperature of 60°C caused inhibition of hydrogen production. For the result of initial pH, it presented that the minimum hydrogen yield was obtained at initial pH 5.0 which was not appropriated for hydrogen-producing bacteria. The maximum hydrogen yield was obtained at initial pH 7.0, followed by initial pH 8.0 and 6.0, respectively. The results presented that the maximum hydrogen yield (593 mL/g carbohydrate) was obtained at initial pH 7.0 under thermophilic temperature of 55°C. Kim et al. (2011) investigated the effect of initial pH (5.0 – 9.0) under mesophilic temperature (35°C) on biohydrogen production from food waste. It was reported that initial pH of 5.0 was critical to the germination of hydrogen-producing bacteria and synthesis of hydrogenase which was the important enzymes for hydrogen production. Moreover, initial pH of 9.0 was also an inappropriate environment for the germination of hydrogen- producing bacteria but it was not affected on the synthesis and activity of hydrogenase after initial pH dropped from 9.0 to 5.0 during fermentation process. Their study demonstrated that initial pH in the range of 6.0 – 8.0 was a good environment for the germination of hydrogen- producing bacteria although it might be effect on the activity of hydrogenase in the different way as presented in the different results of hydrogen yield. The maximum hydrogen yield (1.92 mol H_2/g mol hexose) was obtained at initial pH 8.0. Shin et al. (2004) investigated the effect of pH (4.5 – 6.5) on biohydrogen production from food waste by the mesophilic and thermophilic acidogenic cultures. The maximum hydrogen yield (0.9 mol H_2/g mol hexose) was obtained at initial pH 4.5 under thermophilic fermentation. At this optimal condition, thermophilic saccharolytic microorganism of *Thermoanaerobacterium thermosaccharolyticum* was found. It is hydrogen-producing bacteria that can convert carbohydrate into the great quantity of hydrogen.

Table 4.9 Biohydrogen production from wastes at various initial pH values under the mesophilic and thermophilic temperatures.

Substrate/ Inoculum	**Test type**	**Experimental study**		**Optimal condition**		**H_2 yield**	**References**
		Initial pH	**Temperature (^oC)**	**Initial pH**	**Temperature (^oC)**		
Food waste/ Anaerobic sludge	Batch	5.0 - 9.0	35, 55	7.0	55	176 mL H_2/g COD or 148 mL H_2/g VS or 7.85 mmol H_2/g COD	This study
Food waste/ Anaerobic sludge	Batch	5.0 – 8.0	35 - 60	7.0	55	593 mL H_2/g carbohydrate	Nazlina et al. (2009)
Food waste/ Anaerobic granular sludge	Batch	5.5 - 7.0	36	7.0	36	183 mL H_2/g COD	Ramos et al. (2012)
Vegetable kitchen waste/ Compost	Batch	5.5 – 7.0	55	7.0	55	0.57 mmol H_2/g COD	Lee et al. (2008b)
Food waste/ Mesophilic and thermophilic acidogenic culture	Semi - Continuous	4.5 – 6.5	35, 55	4.5	55	0.90 mol H_2/mol hexose	Shin et al. (2004)

Table 4.9 Biohydrogen production from wastes at various initial pH values under the mesophilic and thermophilic temperatures (cont.).

Substrate/ Inoculum	**Test type**	**Experimental study**		**Optimal condition**		**H_2 yield**	**References**
		Initial pH	**Temperature (^oC)**	**Initial pH**	**Temperature (^oC)**		
Food waste/ No addition of inoculum	Batch	5.0 – 9.0	35	8.0	35	1.92 mol H_2/mol hexose	Kim et al. (2011)
Food waste/ *Clostridium beijerinckii* KCTC 1785	Batch	5.0 – 8.0	30 – 45	7.0	40	128 mL H_2/g COD	Kim et al. (2008)
Cassava starch/ Municipal sewage sludge	Batch	5.5 – 7.0	37, 55	6.0	37	9.47 mmol H_2/g starch	Lee et al. (2008a)
Chess whey powder/ Anaerobic sludge	Batch	7.0	35, 55	7.0	55	111 mL H_2/g sugar	Kargi et al. (2012)

(3) pH and intermediate products

After the experiment finished, the liquid in the batch reactor was examined the final pH value. Moreover, concentration of intermediate products was measured during fermentation process as shown in Table 4.10. It found that the final pH value under the mesophilic (35 ± 2^{o}C) and thermophilic (55 ± 2^{o}C) temperatures was around 4.0. After microorganisms active, initial pH would be decreased because accumulation of volatiles fatty acids was produced meanwhile with hydrogen (Kim et al., 2011). Then, the low pH can inhibit the activity of hydrogen-producing bacteria. It has been reported that cell growth was inhibited at pH less than 4.5 due to the high concentrations of acetic acid and butyric acid. Hence, hydrogen was not produced when the pH value was less than 4.0 (Chong et al., 2009; Wei et al., 2010). As shown in Table 4.10, the maximum acetic acid of 1,600.23 mg/L and the maximum butyric acid of 3,654.87 mg/L (B/A ratio of 2.31) were found when the maximum hydrogen production occurred at the optimal initial pH 7.0 under thermophilic temperature (55 ± 2^{o}C). After experiment finished, it found the increase of accumulation of acetic acid and butyric acid which were 2,972.94 mg/L and 8,743.48 mg/L, respectively. Propionic acid was not found in this study. It has been reported that the maximum acetic acid of 1,031 mg/L and butyric acid of 3,546 mg/L were observed at the optimal initial pH 7.0 and temperature of 40^{o}C on biohydrogen production from food waste (Kim et al., 2008). However, it found that biohydrogen production was inhibited at the high concentrations of acetic acid and butyric acid above 5,000 mg/L and 3,000 mg/L, respectively. Volatile fatty acids (VFAs) and alcohols are the metabolites which are produced during biohydrogen production. Acetic acid and butyric acid are produced at the same time with hydrogen by hydrogen-producing bacteria. Accumulation of acetic acid and butyric acid lead to the low pH. Then, activity of hydrogen-producing bacteria is inhibited. Generation of ethanol and buthanol is promoted since the metabolic pathway shift from acidogenesis to solventogenesis. Solventogenesis was considered as a detoxification route for avoid an adverse consequence from the high VFAs and the low pH (Kim et al., 2011)

Table 4.10 Final pH and concentration of intermediate products at various initial pH values under the mesophilic and thermophilic temperatures.

Temperature	Initial pH	Final pH	D_{Hmax}				D_L			
			Acetic acid (mg/L)	Butyric acid (mg/L)	Propionic acid (mg/L)	B/A ratio	Acetic acid (mg/L)	Butyric acid (mg/L)	Propionic acid (mg/L)	B/A ratio
Mesophilic (35 ± 2°C)	5.0	3.99 ± 0.08	1,334.96 ± 6.25	ND	ND	ND	1,387.80 ± 46.58	ND	ND	ND
	6.0	3.94 ± 0.13	1,387.79 ± 9.52	1,262.09 ± 37.43	ND	0.91 ± 0.02	1,356.80 ± 37.81	2,461.32 ± 36.34	ND	1.20 ± 1.04
	7.0	4.01 + 0.05	1,432.43 ± 11.20	1,573.14 ± 26.88	ND	1.10 + 0.02	1,422.10 ± 11.72	3,089.51 ± 8.03	ND	1.46 + 1.26
	8.0	4.03 ± 0.08	1,339.60 ± 10.38	1,292.19 ± 49.74	ND	0.97 ± 0.03	1,397.51 ± 55.52	2,391.33 ± 10.36	ND	1.15 ± 1.00
	9.0	4.10 ± 0.03	1,365.82 ± 14.68	ND	ND	ND	1,402.69 ± 89.64	ND	ND	ND
Thermophilic (55 ± 2°C)	5.0	4.02 ± 0.13	1,402.96 ± 82.26	2,609.11 ± 24.96	ND	1.86 ± 0.11	2,782.20 ± 85.54	7,013.50 ± 38.85	ND	2.52 ± 0.06
	6.0	4.10 ± 0.03	1,564.99 ± 87.72	3,399.24 ± 22.44	ND	2.17 ± 0.08	2,946.01 ± 89.43	8,424.33 ± 56.75	ND	2.86 ± 0.10
	7.0	4.19 ± 0.02	1,600.23 ± 81.39	3,654.87 ± 57.52	ND	2.31 + 0.18	2,972.94 ± 32.09	8,743.48 ± 61.28	ND	2.94 ± 0.05
	8.0	4.24 ± 0.03	1,551,92 ± 21.23	2,106.58 ± 75.49	ND	1.74 ± 0.08	2,654.96 ± 27.22	7,650.25 ± 48.62	ND	2.88 ± 0.01
	9.0	4.29 ± 0.01	1,591.17 ± 48.46	2,752.47 ± 14.49	ND	1.73 ± 0.07	2,960.85 ± 62.10	7,097.44 ± 85.17	ND	2.40 ± 0.04

Note D_{Hmax}: The day that the maximum hydrogen was produced. D_L: The last day of the experiment. ND: Not detectable.

Data indicated average value ± standard deviation (n = 3).

This study demonstrated that initial pH and temperature influenced on lag phase time, hydrogen production rate, hydrogen yield, COD removal and metabolic pathway. Biohydrogen production from food waste by mixed cultures enhanced with increasing initial pH from 5.0 to 7.0 but it reduced with the increasing initial pH from 7.0 to 9.0. Moreover, the lower or higher initial pH can significantly inhibited biohydrogen production. For the key factor of temperature, thermophilic fermentation provided higher hydrogen yield than mesophilic fermentation. Thus, the results of the optimal initial pH of 7.0 and thermophilic temperature ($55 \pm 2^{o}C$) from this study were installed in the next experiment.

4.3.2 Phase IIB: Effect of C/N ratio on biohydrogen production

The batch reactor was set up with food waste slurry and the pretreated seed sludge. Chemical oxygen demand (COD) of food waste slurry and ammonium hydrogen carbonate (NH_4HCO_3) were calculated to set C/N ratio ranging from 10, 20, 30, 40 and 50. The control group was not added NH_4HCO_3. Then, the optimal initial pH of 7.0 and thermophilic temperature ($55 \pm 2^{o}C$) (Phase IIA result) were installed in this experiment. The results were explained as follows:

(1) Biogas production, biogas component and COD removal

Biogas production and biogas component at various C/N ratios were summarized in Table 4.11. All batch experiments were set up the working volume at 200 mL and were operated for 14 days. Biogas production and percentage of hydrogen were obtained in the range of 5,066.67 – 1,055.67 mL and 50.50 – 28.13 $\%H_2$, respectively. All of the experiment was not found methane production. As a result, it observed that C/N ratio of 30 provided the maximum biogas production ($5,066.67 \pm 169.19$ mL), which consisted of 36.10 ± 0.85 $\%H_2$, 27.90 ± 1.08 $\%CO_2$ and 36.00 ± 1.83 $\%N_2$. The control group showed the maximum percentage of hydrogen (50.50 ± 0.59 $\%H_2$) but its volume of hydrogen ($1,202.21 \pm 4.91$ mL) was less than volume of hydrogen from the experiment at C/N ratio 30 ($1,828.04 \pm 29.14$ mL).

Table 4.11 Biogas production and biogas component at various C/N ratios.

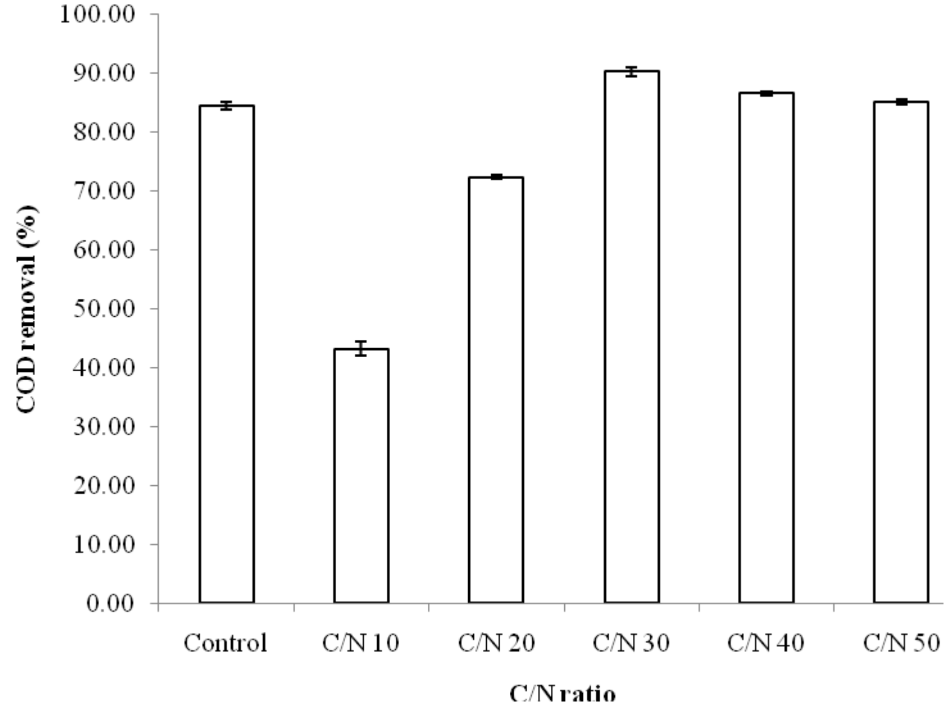
C/N ratio	Biogas (mL)	Biogas component			
		H_2 (%)	CO_2 (%)	N_2 (%)	CH_4 (%)
Control	2,380.67 ± 32.56	50.50 ± 0.59	23.74 ± 0.47	25.76 ± 0.41	ND
10	1,055.67 ± 19.76	28.13 ± 0.58	25.73 ± 0.24	46.14 ± 0.58	ND
20	4,265.33 ± 74.45	24.00 ± 0.07	30.14 ± 0.38	45.86 ± 0.39	ND
30	5,066.67 ± 169.19	36.10 ± 0.85	27.90 ± 1.08	36.00 ± 1.83	ND
40	3,111.33 ± 56.45	46.63 ± 0.78	24.42 ± 0.50	28.95 ± 0.82	ND
50	2,457.67 ± 6.66	49.69 ± 0.18	24.74 ± 0.27	25.57 ± 0.18	ND

Note Control: No addition of NH_4HCO_3.

ND: Not detectable.

Data indicated average value ± standard deviation (n = 3).

After the experiment finished, it found that COD of all the treatment decreased. COD removal efficiency at various C/N ratios is shown as Figure 4.7. The maximum COD removal efficiency was obtained at C/N ratio 30 (90.34 ± 0.70 %), C/N ratio 40 (86.61 ± 0.33 %), C/N ratio 50 (85.20 ± 0.45 %), C/N ratio 20 (72.41 ± 0.33 %) and C/N ratio 10 (43.23 ± 1.25 %) respectively. For the control group, COD removal efficiency was 84.55 ± 0.61 %. It well known that degradation of substrate correlated with biogas production. As a result, it found that C/N ratio of 30 provided the maximum COD removal that corresponded with the maximum biogas production. While the minimum biogas production and COD removal were obtained at C/N ratio 10. This study indicated that C/N ratio influenced on biogas production, volume of hydrogen and COD removal in biohydrogen production process.



Note Control: No addition of NH_4HCO_3.

Bars indicated the standard deviation of data (n = 3).

Figure 4.7 COD removal efficiency at various C/N ratios.

(2) Cumulative hydrogen production

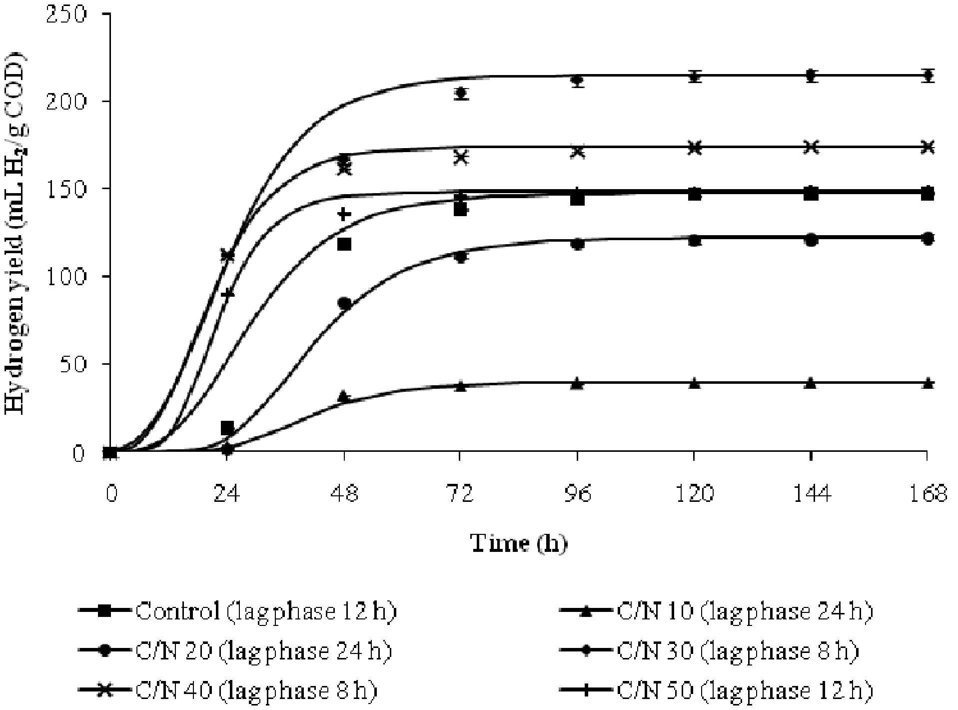
Cumulative hydrogen production at various C/N ratios was predicted by the modified Gompertz equation as shown in Table 4.12. In this study, C and N were calculated from COD of substrate and N of NH_4HCO_3, respectively. TKN of substrate was not considered in C/N ratio because the available nitrogen was not identified (Argun et al., 2008). The lag phase time (λ) was 8 – 24 h. As a result, the shortest lag phase time of 8 h was found at C/N ratio in the range of 30 - 40, while the longest lag phase time of 24 h was found at C/N ratio in the range of 10 - 20. This duration was physiological adaptation of bacteria in environment conditions. It demonstrated that addition of external nitrogen source might enhance bacterial growth and synthesis of hydrogenase (Argun et al., 2008).

Table 4.12 Cumulative hydrogen production at various C/N ratios by the modified Gompertz equation.

C/N ratio	**H_{max} (mL)**	**λ (h)**	**R_m (mL/h)**	**R^2**
Control	1,297.71	12	38.52	0.9624
10	351.00	24	10.69	0.9890
20	1,073.49	24	30.51	0.9964
30	1,890.81	8	60.32	0.9886
40	1,531.74	8	61.84	0.9973
50	1,303.50	12	65.45	0.9957

Note Control: No addition of NH_4HCO_3.

The maximum cumulative hydrogen production (H_{max}) and the maximum hydrogen production rate (R_m) were in the range of 351.00 – 1,890.81 mL and 10.69 – 65.45 mL/h, respectively. As a result, it found that the greatest H_{max} of 1,890.81 mL was obtained at C/N ratio 30, which its R_m was 60.32 mL/h. The minimum H_{max} of 351.00 mL was obtained at C/N ratio 10, which its R_m was 10.69 mL/h. It has been reported that C/N ratio significantly effect on hydrogen production and hydrogen production rate (Argun et al., 2008). In this study, the correlation coefficient (R^2) was mostly 0.99, which indicated that the modified Gompertz equation could describe the progress of cumulative hydrogen production in this study successfully. However, R^2 at C/N ratio 30 was 0.86 because the difference between the predicted values and the observed values, especially at 48 h as presented on curve in Figure 4.8. Cumulative hydrogen production and COD of food waste were calculated for hydrogen yield (mL H_2/g COD). Hydrogen yield at various C/N ratios is shown in Figure 4.8. The curves were plotted from the predicted values by the modified Gompertz equation and were fitted with the observed values from the experiment.



Note Control: No addition of NH_4HCO_3.

Bars indicated the standard deviation of data (n = 3).

Figure 4.8 Hydrogen yield at various C/N ratios by the modified Gompertz equation.

As shown in Figure 4.8, C/N ratio was significantly ($p < 0.05$) effect on hydrogen yield (Appendix D, Table D4). The maximum hydrogen yield was obtained at C/N ratio 30 (214.88 mL H_2/g COD), C/N ratio 40 (174.08 mL H_2/g COD), C/N ratio 50 (148.14 mL H_2/g COD), C/N ratio 20 (122.00 mL H_2/g COD), and C/N ratio 10 (39.89 mL H_2/g COD), respectively. For the control group, hydrogen yield was 147.48 mL H_2/g COD. The results showed that hydrogen yield significantly enhanced with increasing C/N ratio from 10 to 30. Then, it significantly decreased with further increasing C/N ratio from 30 to 50. It observed that hydrogen yield of 147.48 mL H_2/g COD from the control group (no addition of external nitrogen) was lower than the maximum hydrogen yield of 214.88 mL H_2/g COD from biohydrogen production at C/N ratio 30. However, the control group provided higher hydrogen yield than biohydrogen production at C/N ratio 10, which the minimum hydrogen yield of 39.89 mL H_2/g COD was obtained. It indicated that addition of the external nitrogen for considering of the optimal C/N ratio could improve hydrogen yield, but the excess nitrogen (low C/N ratio) could decrease hydrogen yield (Oztekin et al., 2008).

Food waste is a carbohydrate-based waste which the main composition is carbon (C), whereas the other nutrients such as nitrogen (N), phosphorus (P) are deficient. Nitrogen is an essential nutrient for microorganisms because it is a component in enzymes, nucleic acids and protein. Therefore, the appropriate amount of the external nitrogen was required for stimulation the activity of hydrogen-producing bacteria and enhancement of biohydrogen production (Argun et al., 2008; Wang and Wan, 2009b; Sreela-or et al., 2011b). The unappreciated concentration of nitrogen can reduce hydrogen yield and hydrogen production rate as a consequence of nutrient restriction, reduction of microorganism activity, variation of dominant microorganism, metabolic pathway shift and inhibition of some formation products (Oztekin et al., 2008). The shortage of nitrogen (high C/N ratio) could decrease biohydrogen production because nitrogen was rapidly consumed and then it will lack for microbial growth. On the other hand, the excess of nitrogen (low C/N ratio) also reduce biohydrogen production because nitrogen will be released and accumulated in form of ammonia that as a result to increasing of pH in system (Abbasi et al., 2012).

Generally, carbon utilization of microorganism is 25 – 30 times greater than nitrogen. Thus, C/N ratio of 20 – 30 is necessary for biological process and the optimal C/N ratio in the range of 10 – 45 is suggested for hydrolysis/acidogenesis (Chandra et al., 2012). In this study, the optimal C/N ratio of 30 was in the optimal range. When compared with the previous researches, it found that the variation of the optimal C/N ratio depended on substrate and seed sludge, nitrogen source, C/N range studied and other environmental factors as shown in Table 4.13. Inorganic nitrogen and organic nitrogen were used as an external nitrogen source to supplement N in C/N ratio. Inorganic nitrogen sources such as ammonium hydrogen carbonate (NH_4HCO_3), ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4)_2SO_4$), ammonium nitrate (NH_4NO_3) were used in several previous researches (Kalil et al., 2008). Moreover, organic nitrogen source such as sludge, manure also was used in co-digestion for biohydrogen production in recently study.

Table 4.13 Biohydrogen production from wastes at various C/N ratios.

Substrate/ Inoculum	Test type	Operational conditions		Nitrogen source	C/N ratio		H_2 yield	References
		Initial pH	Temperature (°C)		Range studied	Optimal value		
Food waste/ Anaerobic sludge	Batch	7.0	55	Ammonium bicarbonate; NH_4HCO_3	10 – 50	30	215 mL H_2/g COD or 181 mL H_2/g VS or 0.08 mol H_2/g COD	This study
Food waste/ Anaerobic sludge	Batch	5.5	37	Primary sludge	4 – 32	26	66 mL H_2/g COD	Zhou et al. (2013)
Food waste/ Anaerobic sludge	Batch	ND	30	Anaerobic sludge	10 – 50	33	103 mL H_2/g VS	Sreela-or et al. (2011b)
Cheese whey/ Anaerobic sludge	Batch	5.5	37	Vegetable fruit waste	7 – 46	21	450 mL H_2/g COD	Gomez-Romero et al. (2014)
Wheat starch/ Anaerobic sludge	Batch	7.0	37	Urea; $CO(NH_2)_2$	20 – 100	50	2.84 mol H_2/mol glucose	Oztekin et al. (2008)
Wheat powder solution/ Anaerobic sludge	Batch	7.0	37	Urea; $CO(NH_2)_2$	20 - 200	200	281 mL H_2/g starch	Argun et al. (2008)

Note ND: Not determined.

Oztekin et al. (2008) investigated the effect of C/N ratio on mesophilic biohydrogen production from hydrolyzed wheat starch. Nitrogen of the hydrolyzed wheat starch (3.4 mg/g total nitrogen) was not considered in C/N ratio because the available nitrogen was not known. Their study found that the maximum hydrogen yield (2.84 mol H_2/mol glucose) was obtained at C/N ratio 50. The hydrogen yield was significantly reduced at C/N ratio less than 50 because of the excess of nitrogen. As presented in their study, the batch reactor with the excess of nitrogen at C/N ratio 20 provided lower hydrogen yield (1.62 mol H_2/mol glucose) than the batch reactor without addition of the external nitrogen (1.92 mol H_2/mol glucose). It compatible with the results was reported by Argun et al. (2008) that biohydrogen production from wheat powder solution without addition of the external nitrogen presented the low hydrogen yield. The external nitrogen is required for improvement the activity of microorganisms and then it leads to effective hydrogen production. Their study presented that the maximum hydrogen yield (281 mL H_2/g starch) was obtained at C/N ratio 200. For another research, Sreela-or et al. (2011b) studied biohydrogen production from co-digestion of food waste and sludge. Food waste and sludge were mixed for considering the final C/N ratio in the batch reactor. The results indicated that C/N ratio was significantly impacted on hydrogen yield. Hydrogen yield increased with increasing C/N ratio from 20 to 33 and then it decreased when C/N ratio was greater than 33. The maximum hydrogen yield (102.63 mL H_2/g VS) was obtained at C/N ratio 33.

(3) pH and intermediate products

In this study, the experiment was installed at initial pH 7.0 under thermophilic temperature ($55 \pm 2^{o}C$). After the experiment finished, the liquid in the batch reactor was examined the final pH. Moreover, concentration of intermediate products was measured during fermentation process as shown in Table 4.14. It found that the initial pH of 7.0 decreased to the final pH in the range of 4.27 – 6.61. When the maximum hydrogen production occurred, it found 546.53 – 4,982.03 mg/L of acetic acid and 223.49 – 1,523.14 mg/L of butyric acid. B/A ratio was in the range of 0.09 – 0.95. All batch reactors in this period were not found propionic acid. After the experiment finished, it found the increase of accumulation of volatile fatty acids

(VFAs). The concentrations of acetic acid and butyric acid were found in the range of 1,500.35 – 19,107.32 mg/L and 355.79 – 8,967.58, respectively. B/A ratio was in the range of 0.21 – 2.11. Moreover, it found 213.13 - 633.47 mg/L of propionic acid from biohydrogen production at C/N ratio in the range of 20 – 40. VFAs are produced as the byproducts throughout hydrogen fermentation process. Theoretical of glucose fermentation, acetate pathway and butyrate pathway provide 4 mole and 2 mole of hydrogen, respectively. While propionate pathway is considered should be prevent because it is a hydrogen-consuming reaction (Argun et al., 2008). Acetic acid of 19,107.32 mg/L, butyric acid of 6,370.51 mg/L and propionic acid of 396.32 mg/L were accumulated from biohydrogen production at the optimal C/N ratio of 30. The high accumulation of VFAs leads to the reduction of the final pH and inhibition of the activity of hydrogen-producing bacteria. It has been reported that hydrogen production was not produced when the pH value was less than 4.0 (Wei et al., 2010). Moreover, it observed that C/N ratio influenced on the final pH. The maximum final pH of 6.61 presented at C/N ratio 10, while the minimum final pH of 4.27 presented at C/N ratio 50. At the low C/N ratio (excess of nitrogen), nitrogen was released in form of ammonia. The accumulation of the excess ammonia caused the increase of the pH in system (Wang and Wang, 2009b; Chandra et al., 2012). In this study, ammonium hydrogen carbonate (NH_4HCO_3) was used as the external nitrogen source. It has been reported that NH_4HCO_3 could avoid the oscillation of pH during biohydrogen production. However, if a large amount of NH_4HCO_3 was applied for considering in the low C/N ratio, the increase of carbon dioxide from the disintegration of bicarbonate might be result in reduction of the hydrogen content. Furthermore, the release of the high concentration of ammonium caused an adverse effect on the activity of microorganisms (Sreela-or et al., 2011b; Escamilla-Alvarado et al., 2013).

Table 4.14 Final pH and concentration of intermediate products at various C/N ratios under initial pH of 7.0 and thermophilic temperature (55 ± 2°C).

C/N ratio	Final pH	D_{Hmax}				D_L			
		Acetic acid (mg/L)	**Butyric acid (mg/L)**	**Propionic acid (mg/L)**	**B/A ratio**	**Acetic acid (mg/L)**	**Butyric acid (mg/L)**	**Propionic acid (mg/L)**	**B/A ratio**
Control	4.28 ± 0.14	1,242.54 ± 29.68	1,186.85 ± 82.92	ND	0.95 ± 0.04	1,500.35 ± 76.38	1,359.02 ± 40.05	ND	0.91 ± 0.07
10	6.61 ± 0.02	546.53 ± 19.58	280.48 ± 45.97	ND	0.51 ± 0.09	1,735.07 ± 15.82	355.79 ± 27.00	ND	0.21 ± 0.02
20	6.45 ± 0.19	2,096.23 ± 18.86	223.49 ± 78.91	ND	0.11 ± 0.01	12,486.58 ± 366.43	3,065.57 ± 25.08	633.47 ± 45.52	0.25 ± 0.01
30	5.64 ± 0.32	4,982.03 ± 68.73	465.05 ± 78.91	ND	0.09 ± 0.01	19,107.32 ± 215.73	6,370.51 ± 82.50	396.32 ± 66.06	0.33 ± 0.01
40	4.64 ± 0.01	4,653.75 ± 40.38	556.04 ± 20.15	ND	0.12 ± 0.01	7,093.39 ± 80.23	8,967.58 ± 29.62	213.13 ± 42.82	1.26 ± 0.02
50	4.27 ± 0.10	3,376.82 ± 98.96	1,523.14 ± 53.07	ND	0.45 ± 0.03	2,984.93 ± 39.84	6,290.65 ± 80.09	ND	2.11 ± 0.01

Note Control: Control: No addition of NH_4HCO_3. D_{Hmax}: The day that the maximum hydrogen was produced. D_L: The last day of the experiment.

ND: Not detectable. Data indicated average value ± standard deviation (n = 3).

This study demonstrated that C/N ratio influenced on hydrogen yield, COD removal, metabolic pathway and final pH. Addition of the external nitrogen for considering in C/N ratio could improve hydrogen yield. However, the excess of nitrogen (low C/N ratio) could decrease hydrogen yield. Therefore, the appropriate amount of the external nitrogen was required for stimulation the activity of hydrogen-producing bacteria and enhancement of biohydrogen production. In this study, the optimal C/N ratio of 30 was obtained. Thus, biohydrogen production from food waste by mixed cultures under the optimal environmental conditions from the results of Phase IIB; initial pH of 7.0, thermophilic temperature ($55 \pm 2^{o}C$) and C/N ratio of 30 were installed for collection of hydrogen fermentation effluent. The effluent was used as a substrate on stage II of biomethane production in the next experiment.

4.4 Phase II: The optimum of environmental conditions on biomethane production

The optimum of initial pH and temperature was investigated on biomethane production from hydrogen fermentation effluent by anaerobic digestion. The seed sludge without pretreatment method was used as inoculum. The results were explained as follows:

4.4.1 Characteristics of hydrogen fermentation effluent

In this study, the residue of biohydrogen production under the optimal environmental conditions (initial pH of 7.0, thermophilic temperature ($55 \pm 2^{o}C$) and C/N ratio of 30) was collected. Then, it was separated into the liquid phase and solid phase by filtration. The liquid phase was used as the hydrogen fermentation effluent. The physical and chemical characteristics of hydrogen fermentation effluent are shown in Table 4.15.

Table 4.15 Physical and chemical characteristics of hydrogen fermentation effluent.

Parameter (Unit)	**Hydrogen fermentation effluent**
pH	6.98 ± 0.12
Chemical oxygen demand (COD, mg/L)	7,786.67 ± 224.40
Total Kjeldahl nitrogen (TKN, mg/L)	25.20 ± 2.80
Ammonia-nitrogen (NH_3-N, mg/L)	1,985.86 ± 22.91
Acetic acid (mg/L)	14,378.53 ± 430.44
Butyric acid (mg/L)	4,978.02 ± 77.86
Propionic acid (mg/L)	530.11 ± 37.80
Methanol (mg/L)	ND

Note ND = Not detectable.

Data indicated average value ± standard deviation (n = 3).

Hydrogen fermentation effluent (COD 7,786.67 mg/L and TKN 25.20 mg/L) was calculated for C/N ratio, it found the high C/N ratio approximately 312. Generally, C/N ratio in the range of 20 – 30 is considered that appropriate for biomethane production (Chandra et al., 2012). Therefore, hydrogen fermentation effluent was adjusted C/N ratio around of 25 by addition of NH_4HCO_3. For concentration of ammonia, it found ammonia of 1,985.86 mg/L in hydrogen fermentation effluent. Ammonia is considered as a main inhibitor in biomethane production process. It has been reported that the activity of methanogenic bacteria lost 56.5% when concentration of ammonia ranging of 4,051 – 5,734 mg/L (Chen et al., 2008). Moreover, it found acetic acid of 14,378.53 mg/L, butyric acid of 4,978.02 mg/L and propionic acid of 530.11 mg/L which were produced as the byproducts from biohydrogen production process. Generally, methanogenic bacteria can consume hydrogen with carbon, formate, methanol and acetic acid as substrates and convert to methane (Chandra et al., 2012). However, methanol was not found in hydrogen fermentation effluent.

Solid phase was an organic matter from the residue of biohydrogen production under the optimal environmental conditions. It contained the biomass of microorganisms. It was not used as a substrate in biomethane production process. The purpose was avoidance of the nutrient competition for bacterial growth between hydrogen-producing bacteria and methanogenic bacteria. Moreover, the breakdown of microbial biomass might be caused the release of the light metal ions such as sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). The high concentration of light metal ions could inhibit or toxic to methanogenic bacteria (Chen et al., 2008).

4.4.2 Phase IIC: Effect of initial pH and temperature on biomethane production

The batch reactor was set up with hydrogen fermentation effluent and the seed sludge without pretreatment method which its original pH value was 7.43 ± 0.03. Then, the batch reactors were adjusted with HCl or KOH at initial pH values in the range of 6.0, 7.0, 8.0, 9.0 and 10.0 under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures. The results were explained as follows:

(1) Biogas production, biogas component and COD removal

Biogas production and biogas component at various initial pH values under the mesophilic and thermophilic temperatures were summarized in Table 4.16. All batch experiments were set up the working volume at 200 mL and were operated for 14 days. At mesophilic temperature (35 ± 2°C), biogas production and percentage of methane were obtained in the range of 33.67 – 256.33 mL and 0 – 37.81 %CH_4, respectively. The initial pH of 7.0 provided the maximum biogas production (256.33 ± 0.58 mL), which consisted of 1.76 ± 0.16 %H_2, 37.85 ± 0.59 %CO_2, 23.29 ± 0.56 %N_2 and 37.10 ± 0.15 %CH_4. Moreover, it noticed that biogas production (255.00 ± 3.61 mL) and percentage of methane (37.81 ± 0.52 %CH_4) of the control group (initial pH of 7.43) were similar to that's of the initial pH of 7.0. However, methane production was not produced at initial pH 6.0 and 10.0. For thermophilic temperature (55 ± 2°C), it provided higher biogas production and percentage of methane than mesophilic temperature (35 ± 2°C). Biogas production and percentage of methane were obtained in the range of 55.67 – 486.67 mL and 0 – 53.45 %CH_4, respectively. It observed that initial pH of 7.0 provided the maximum biogas production (486.67 ± 34.59 mL), which consisted of 1.62 ± 0.11 %H_2, 23.06 ± 1.10 %CO_2, 24.05 ± 1.42 %N_2 and 51.28 ± 1.39 %CH_4. Furthermore, the results of the initial pH of 7.0 were similar to that's of the control group (initial pH of 7.43), which provided biogas production

(474.00 ± 34.83 mL) and percentage of methane (53.45 ± 0.70 %CH_4). On the other hand, methane production was not produced at initial pH 6.0.

Table 4.16 Biogas production and biogas component at various initial pH values under the mesophilic and thermophilic temperatures from biomethane production process.

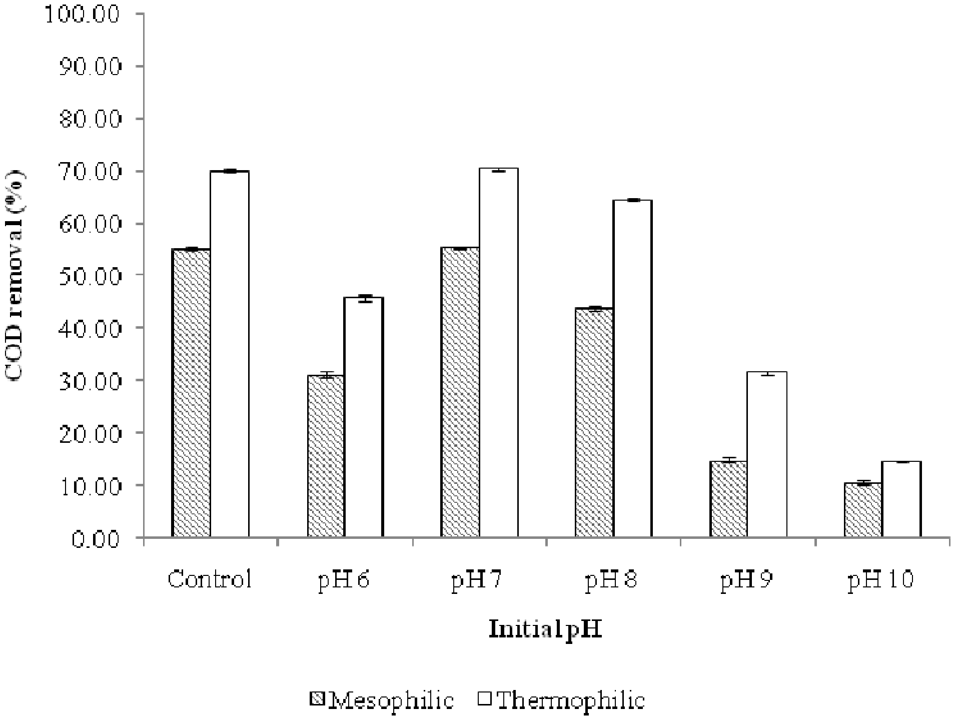
Temperature	Initial pH	Biogas (mL)	Biogas component			
			H_2 (%)	CO_2 (%)	N_2 (%)	CH_4 (%)
Mesophilic	Control	255.00 ± 3.61	1.43 ± 0.04	37.34 ± 0.19	23.42 ± 0.56	37.81 ± 0.52
(35 ± 2°C)	6.0	103.67 ± 15.18	4.28 ± 0.70	50.85 ± 1.00	44.88 ± 0.99	ND
	7.0	256.33 ± 0.58	1.76 ± 0.16	37.85 ± 0.59	23.29 ± 0.56	37.10 ± 0.15
	8.0	153.67 ± 5.51	1.08 ± 0.05	41.30 ± 0.84	28.82 ± 0.93	28.81 ± 0.46
	9.0	54.00 ± 4.58	1.06 ± 0.31	58.10 ± 3.62	28.76 ± 2.83	12.07 ± 1.06
	10.0	33.67 ± 3.51	1.09 ± 0.05	47.59 ± 2.28	51.33 ± 2.29	ND
Thermophilic	Control	474.00 ± 34.83	1.70 ± 0.10	19.60 ± 1.17	25.25 ± 0.68	53.45 ± 0.70
(55 ± 2°C)	6.0	154.67 ± 20.55	13.05 ± 1.01	41.79 ± 3.08	45.17 ± 2.08	ND
	7.0	486.67 ± 34.59	1.62 ± 0.11	23.06 ± 1.10	24.05 ± 1.42	51.28 ± 1.39
	8.0	362.33 ± 21.55	1.67 ± 0.11	30.28 ± 0.50	30.65 ± 1.08	37.40 ± 1.46
	9.0	102.33 ± 2.52	1.74 ± 0.24	47.78 ± 2.38	32.03 ± 2.58	18.46 ± 0.76
	10.0	55.67 ± 14.57	1.15 ± 0.58	41.21 ± 4.27	42.42 ± 0.62	15.23 ± 3.15

Note Control: Initial pH 7.43

ND = Not detectable.

Data indicated average value ± standard deviation (n = 3).

After the experiments finished, they found that COD of all batch reactors decreased. COD removal efficiency at various initial pH values under the mesophilic and thermophilic temperatures is shown in Figure 4.9. At mesophilic temperature (35 ± 2°C), the maximum COD removal efficiency was obtained at initial pH 7.0 (55.31 ± 0.21 %), initial pH 8.0 (43.85 ± 0.41 %), initial pH 6.0 (31.18 ± 0.62 %), initial pH 9.0 (14.81 ± 0.41 %) and initial pH 10.0 (10.63 ± 0.55 %), respectively. For the control group, COD removal was 55.16 ± 0.31 %. For thermophilic temperature (55 ± 2°C), the trend of COD removal was similar to that's of mesophilic temperature (35 ± 2°C) but the higher COD removal was obtained from this condition. The maximum COD removal efficiency was obtained at initial pH 7.0 (70.37 ± 0.21 %), initial pH 8.0 (64.52 ± 0.36 %), initial pH 6.0 (45.76 ± 0.55 %), initial pH 9.0 (31.54 ± 0.36 %) and initial pH 10.0 (14.58 ± 0.21 %), respectively. For the control group, COD removal was 70.01 ± 0.41 %. As a result, it noticed that COD removal of initial pH of 7.0 was close to COD removal of the control group in both of mesophilic and thermophilic temperatures because the pH value of the control group (7.43) closed to 7.0. In anaerobic digestion process for biogas production, organic matter is degraded by microorganisms. Therefore, it found the relation between biogas production and COD removal in term of quantity. Among various initial pH in this study, the maximum biogas production and COD removal were achieved at initial pH 7.0. At this optimal initial pH, the batch reactor provided biogas production of 486.67 ± 34.59 mL and COD removal of 70.37 ± 0.21 % under thermophilic temperature (55 ± 2°C), while biogas production of 256.33 ± 0.58 mL and COD removal of 55.31 ± 0.21 % were obtained under mesophilic temperature (35 ± 2°C). This study indicated that initial pH and temperature influenced on biogas production and COD removal in biomethane production process.



Note Control: Initial pH 7.43

Bars indicated the standard deviation of data (n = 3).

Figure 4.9 COD removal efficiency at various initial pH values under the mesophilic and thermophilic temperatures from biomethane production process.

(2) Cumulative methane production

Cumulative methane production from hydrogen fermentation effluent at various initial pH values under the mesophilic and thermophilic temperatures was predicted by the modified Gompertz equation as shown in Table 4.17. The correlation coefficient (R^2) was in the range of 0.95 - 0.99, which indicated that cumulative methane production by the modified Gompertz equation was good consistent with the experimental results. The lag phase time (λ) was 20 - 24 h. It was the duration for adaptation, growth and activity of methanogenic bacteria (Abbasi et al., 2012). The minimum lag phase time of 20 h was observed at initial pH in the range of 7.0 – 8.0, while the maximum lag phase time of 24 h was observed at the high initial pH in the range of 9.0 – 10.0. It was not found the activity of methanogenic bacteria at initial pH 6.0. In this study, cumulative methane production and methane production rate were in the range of 37.66 – 228.04 mL and 1.33 – 3.76 mL/h, respectively under mesophilic temperature (35 ± 2°C), whereas cumulative methane production and methane production rate were in the range 45.59 – 417.30 mL and 1.27 – 7.66 mL/h, respectively under thermophilic temperature (55 ± 2°C). It demonstrated that biomethane production was effective under thermophilic temperature (55 ± 2°C). Moreover, biomethane production at initial pH 7.0 and biomethane production from the control group (initial pH 7.43) provided the nearby results.

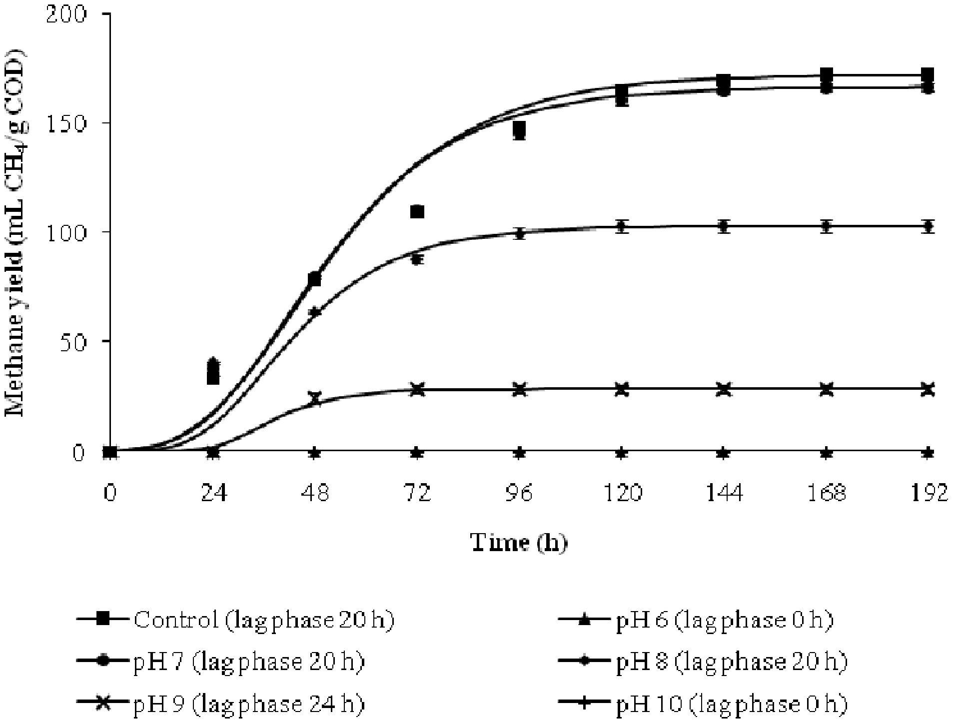
Table 4.17 Cumulative methane production at various initial pH values under the mesophilic and thermophilic temperatures by the modified Gompertz equation.

Initial pH	**Mesophilic (35 ± 2°C)**				**Thermophilic (55 ± 2°C)**			
	H_{max} (mL)	**λ (h)**	**R_m (mL/h)**	**R^2**	**H_{max} (mL)**	**λ (h)**	**R_m (mL/h)**	**R^2**
Control	228.04	20	3.70	0.9821	417.30	20	6.51	0.9825
6.0	ND	ND	ND	ND	ND	ND	ND	ND
7.0	220.60	20	3.76	0.9776	411.37	20	7.66	0.9831
8.0	136.17	20	3.02	0.9557	258.30	20	5.66	0.9729
9.0	37.66	24	1.33	0.9932	62.48	24	1.91	0.9966
10.0	ND	ND	ND	ND	45.59	24	1.27	0.9856

Note Control: Initial pH 7.43

ND: Not detectable.

Cumulative methane production and COD of hydrogen fermentation effluent were calculated for methane yield (mL CH_4/g COD). Methane yield at various initial pH under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures are shown in Figure 4.10 and 4.11, respectively. The curves were plotted from the predicted values by the modified Gompertz equation and were fitted with the observed values of the experiment.

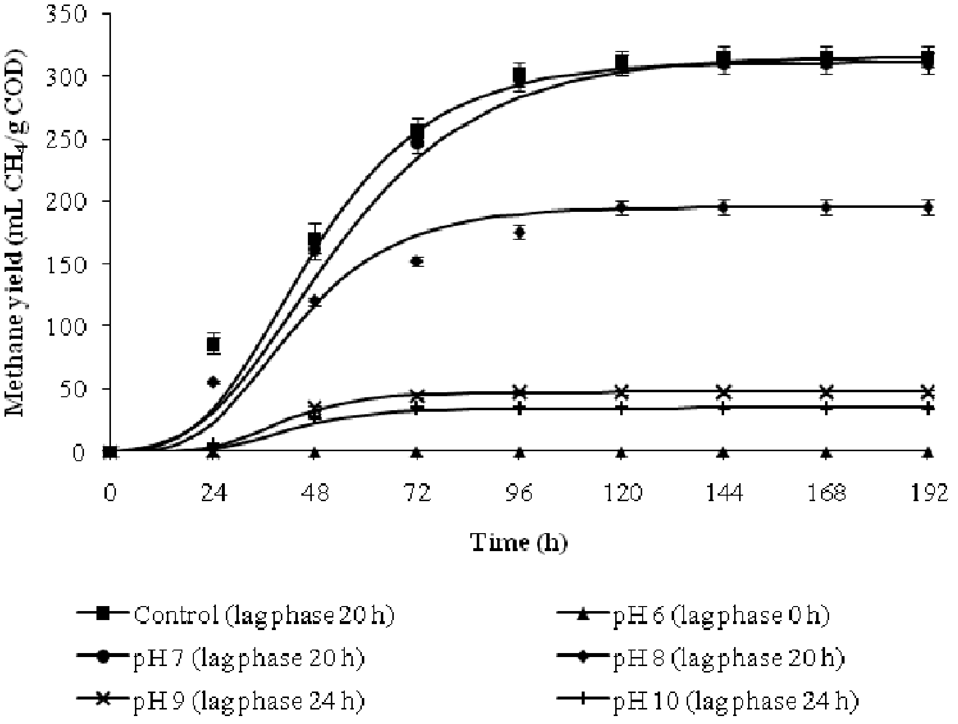


Note Control: Initial pH 7.43.

Bars indicated the standard deviation of data (n = 3).

Figure 4.10 Methane yield by the modified Gompertz equation at various initial pH values under mesophilic temperature (35 ± 2°C).

As shown in Figure 4.10, it found that initial pH was significantly ($p < 0.05$) effect on methane yield under mesophilic temperature (35 ± 2°C) (Appendix D, Table D5). The maximum methane yield was obtained at initial pH 7.0 (166.65 mL CH_4/g COD), initial pH 8.0 (102.87 mL CH_4/g COD) and initial pH 9.0 (28.45 mL CH_4/g COD), respectively. It was not found methane production at initial pH 6.0 and 10.0. The control group (initial pH of 7.43) provided methane yield of 172.27 mL CH_4/g COD. As shown in Figure 4.11, initial pH was significantly ($p < 0.05$) effect on methane yield under thermophilic temperature (55 ± 2°C) (Appendix D, Table D6). The maximum methane yield was obtained at initial pH 7.0 (310.77 mL CH_4/g COD), initial pH 8.0 (195.14 mL CH_4/g COD), initial pH 9.0 (47.20 mL CH_4/g COD) and initial pH 10.0 (34.44 mL CH_4/g COD), respectively. It was not found methane production at initial pH 6.0. The control group which had initial pH 7.43 provided methane yield of 315.25 mL CH_4/g COD.



Note Control: Initial pH 7.43.

Bars indicated the standard deviation of data (n = 3).

Figure 4.11 Methane yield by the modified Gompertz equation at various initial pH values under thermophilic temperature (55 ± 2°C).

It noticed that the trends of methane yields at various initial pH values under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures were similarity. Methane yield was greatly obtained from biomethane production at initial pH 7.0 (the control group with initial pH 7.43). Therefore, it presented that methane yield was effectively produced at initial pH around 7.0 - 7.5. Then, it significantly decreased when initial pH value was above 8.0. Moreover, the lower initial pH of 6.0 and the higher initial pH of 10.0 were toxicity to the activity of methanogenic bacteria. Initial pH is an important factor that can enhance biomethane production. The pH significantly influences on the growth of several microorganisms in the four steps of anaerobic digestion (hydrolysis, acidogenesis, acetogenesis and methanogenesis) (Abbasi et al., 2012; Chandra et al., 2012). The mostly methanogenic bacteria can active at the optimal pH in the range of 6.7 – 7.5, while hydrogen-producing bacteria can active at the lower optimal pH in the range of 5.2 – 6.3. However, it was reported that population of methanogenic bacteria was affected from toxicity of the pH value exterior the range of 6.0 – 8.5. The pH lower 6.6 could unfavorably influence on the activity of the methanogenic bacteria and the low pH of 6.2 was toxicity to methanogenic bacteria (Chandra et al., 2012). In this study, the optimal initial pH of 7.0 was in the range of the optimal pH for the activity of methanogenic bacteria.

Moreover, this study indicated that biomethane production under thermophilic temperature (55 $\pm$ 2°C) provided higher methane yield than its under mesophilic temperature (35 $\pm$ 2°C). Temperature influences on the activity of methanogenic bacteria and biomethane production process. Each species of methanogenic bacteria has the most suitable condition for their activity and growth in three different temperature ranges that are psychrophilic (< 20°C), mesophilic (20 - 40°C), and thermophilic (50 - 65°C). The temperature in the range of 40 - 50°C could inhibit the activity of methanogenic bacteria. The psychrophilic methanogens can produce a lowest of methane (Chandra et al., 2012). Biomethane production process under thermophilic temperature is more proficient than that's of mesophilic temperature to produce biogas (Abbasi et al., 2012). Thermophilic fermentation provides more rapid reaction rate, shorter duration of organic matter degradation, higher methane yield and higher destruction of pathogens than mesophilic fermentation. Nevertheless, thermophilic fermentation is more sensitive to environmental change and more difficult to control the process than mesophilic fermentation (Forster-Carneiro et al., 2008; Chandra et al., 2012).

Thus, this study demonstrated that initial pH and temperature significantly influenced on methane yield. The maximum methane yield of 310.77 mL CH_4/g COD was obtained at initial pH 7.0 under thermophilic temperature (55 $\pm$ 2°C). It was compared with other researches on biomethane production from wastes at various initial pH under the mesophilic (around 37°C) and thermophilic (around 55°C) temperatures as shown in Table 4.18.

Table 4.18 Biomethane production from wastes at various initial pH values under the mesophilic and thermophilic temperatures.

Substrate/ Inoculum	**Test type**	**Experimental study**		**Optimal condition**		**CH_4 yield**	**References**
		Initial pH	**Temperature (^oC)**	**Initial pH**	**Temperature (^oC)**		
Effluent from hydrogen fermentation of food waste/ Anaerobic sludge	Batch	6.0 - 10.0	35, 55	7.0	55	311 mL CH_4/g COD	This study
Effluent from hydrogen fermentation of sugarcane juice/ Anaerobic sludge	Batch	4.5 – 9.5	30	7.0	30	367 mL CH_4/g VS	Reungsang et al. (2012)
Food waste/ Anaerobic sludge	Batch	7.0	35, 50	7.0	50	380 mL CH_4/g VS	Chen et al. (2010)
Food waste/ Anaerobic sludge	Batch	7.0	35, 55	7.0	55	502 mL CH_4/g VS	Liu et al. (2009)

As shown in Table 4.18, the variation of methane yield under the optimal initial pH and temperature might be occurred from the different characteristics of substrate and seed sludge, substrate to inoculum ratio, nutrients, and other environmental factors (Abbasi et al., 2012). For example, Chen et al. (2010) investigated biomethane production from cafeteria food waste under mesophilic (35 ± 2°C) and thermophilic (50 ± 2°C) temperatures. C/N ratio of cafeteria food waste was 23. It found that methane yield from mesophilic fermentation was 320 mL CH_4/g VS, while the higher methane yield from thermophilic fermentation was 380 mL CH_4/g VS. It was agree with Liu et al. (2009) reported that biomethane production from food waste at the initial pH 7.0 produced the maximum methane yield of 502 mL CH_4/g VS under thermophilic temperature (50 ± 2°C), while it produced the lower methane yield of 245 mL CH_4/g VS under mesophilic temperature (35 ± 2°C). However, it has been observed that the initial pH of 8.1 presented the maximum methane yield of 180 mL CH_4/g VS from thermophilic biomethane production of food waste (Forster-Carneiro et al., 2008). As a literature review, it found that acidic effluent from hydrogen fermentation was mostly used as a substrate for studying on enhancement of hydrogen production by photo-fermentation, while biomethane production of acidic effluent from hydrogen fermentation was not widely investigated. Reungsang et al. (2012) investigated mesophilic biomethane production from acidic effluent. The effluent was collected from biohydrogen production of sugarcane juice. It consisted of the main VFAs of butyric acid (13,000 mg/L) and acetic acid (3,390 mg/L), including the high COD (18,500 mg/L). Their study reported that methane yield increased with increasing initial pH from 4.5 to 7.0, and then it decreased with further increasing initial pH from 7.0 to 9.5. The maximum methane yield of 367 mL CH4/g VS was obtained at the initial pH 7.0.

(3) pH and intermediate products

After the experiment finished, the liquid in the batch reactor was examined the final pH. Moreover, concentration of intermediate products was measured during biomethane production process as shown in Table 4.19.

Table 4.19 Final pH and concentration of intermediate products at various initial pH values under the mesophilic and thermophilic temperatures from biomethane production process.

Temperature	Initial pH	Final pH	D_{Mmax}	D_L				
			Ammonia (mg N/L)	**Ammonia (mg N/L)**	**Acetic acid (mg/L)**	**Butyric acid (mg/L)**	**Propionic acid (mg/L)**	**Methanol (mg/L)**
Mesophilic (35 ± 2°C)	Control	5.83 ± 0.03	1,333.36 ± 90.20	1,215.80 ± 118.91	157.27 ± 13.92	183.67 ± 25.85	ND	ND
	6.0	5.89 ± 0.04	1,387.58 ± 62.62	1,571.79 ± 6.18	150.73 ± 10.86	217.36 ± 36.40	ND	ND
	7.0	5.89 + 0.08	1,326.94 ± 41.06	1,090.46 ± 58.84	132.03 ± 8.67	138.20 ± 15.22	ND	ND
	8.0	6.92 ± 0.02	1,038.90 ± 39.33	1,045.28 ± 83.65	125.92 ± 5.82	120.51 ± 7.63	ND	ND
	9.0	8.25 ± 0.05	935.47 ± 81.57	1,126.75 ± 113.51	111.97 ± 3.63	99.89 ± 3.88	ND	ND
	10.0	9.53 ± 0.03	879.41 ± 95.48	1,158.31 ± 26.11	113.04 ± 7.86	ND	ND	ND

Note Control: Initial pH 7.43. D_{Mmax}: The day that the maximum methane was produced. D_L: The last day of the experiment.

ND: Not detectable. Data indicated average value ± standard deviation (n = 3).

Table 4.19 Final pH and concentration of intermediate products at various initial pH values under the mesophilic and thermophilic temperatures from biomethane production process (cont.).

Temperature	Initial pH	Final pH	D_{Mmax}	D_L				
			Ammonia (mg N/L)	Ammonia (mg N/L)	Acetic acid (mg/L)	Butyric acid (mg/L)	Propionic acid (mg/L)	Methanol (mg/L)
Thermophilic (55 ± 2°C)	Control	5.86 ± 0.02	2,000.09 ± 25.27	2,215.80 ± 118.91	3,389.59 ± 13.38	ND	ND	ND
	6.0	5.71 ± 0.02	2,096.18 ± 6.70	2,058.90 ± 31.48	1,064.22 ± 56.86	631.96 ± 22.18	ND	ND
	7.0	5.80 ± 0.01	1,955.43 ± 25.85	2,024.20 ± 77.13	3,245.19 ± 67.69	1,455.43 ± 84.21	320.88± 77.80	ND
	8.0	5.90 ± 0.02	2,170.68 ± 86.14	1,985.00 ± 45.98	5,346.50 ± 94.63	2,560.56 ± 99.30	465.37 ± 23.64	ND
	9.0	6.13 ± 0.03	2,346.30 ± 32.47	2,053.57 ± 76.31	6,795.44 ± 95.23	2,938.82 ± 53.38	532.39 ± 13.60	ND
	10.0	8.41 ± 0.03	2,004.73 ± 31.24	1,819.84 ± 27.98	1,830.66 ± 47.86	970.30 ± 36.97	ND	ND

Note Control: Initial pH 7.43. D_{Mmax}: The day that the maximum methane was produced. D_L: The last day of the experiment.

ND: Not detectable. Data indicated average value ± standard deviation (n = 3).

It found that the final pH was in the range of 5.83 – 9.53 and 5.71 – 8.41 from biomethane production under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures, respectively. When the maximum biomethane production was produced, it found concentration of ammonia in the range of 879.41 – 1,387.58 mg N/L and 1,955.43 – 2,346.30 mg N/L under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures, respectively. After experiment finished, it found the increase of ammonia in the range of 1,045.28 – 1,571.79 mg N/L and 1,819.84 – 2,215.80 mg N/L under mesophilic (35 ± 2°C) and thermophilic (55 ± 2°C) temperatures, respectively. Ammonia is produced from degradation of nitrogenous matter. It has been considered as a significant inhibitor in biomethane production because it is independently membrane-permeable. Diffusion of ammonia into microbial cell can cause the variation of intracellular pH, imbalance of proton and insufficiency of potassium. Concentration of ammonia in aqueous depend on several factors such as substrate, inoculum, acclimation period, pH and temperature. Ammonia will be more formed at the high pH value and high temperature. The high concentration of ammonia causes the increase of pH in system, which can toxic to methanogenic bacteria. Generally, concentration of ammonia lower 200 mg/L is considered useful for biomethane production because nitrogen is an essential nutrient for anaerobic microorganisms. However, it has been reported that the activity of methanogenic bacteria lost 56.5% when concentration of ammonia ranging of 4,051 – 5,734 mg/L (Chen et al., 2008).

Moreover, volatile fatty acids (VFAs) and methanol in the liquid phase were measured after the experiment finished. It found 111.97 – 157.27 mg/L of acetic acid and 99.89 – 217.36 mg/L of butyric acid, while it was not found propionic acid and methanol under mesophilic temperature (35 ± 2°C). For thermophilic temperature (55 ± 2°C), it found 1,064.22 – 6,795.44 mg/L of acetic acid, 631.96 – 2,938.82 mg/L of butyric acid and 320.88 – 532.39 mg/L of propionic acid but it was not found methanol. It has been reported that concentration of VFAs in the final effluent was high from thermophilic fermentation, especially propionic acid (Guo et al., 2014). It was observed that VFAs of hydrogen fermentation effluent (14,378.53 mg/L of acetic acid, 4,978.02 mg/L of butyric acid and 530.11 mg/L of propionic acid) decreased after through biomethane production process. Generally, methanogenic bacteria can produce methane from three types of substrate that are carbon dioxide type, acetate type and methyl type as follows (Deublein and Steinhauser, 2008; Chandra et al., 2012):

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (Eq.4- 6)

$CH_3COOH \rightarrow CH_4 + CO_2$ (Eq. 4-7)

$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$ (Eq. 4-8)

$2C_2H_5OH + CO_2 \rightarrow CH_4 + 2CH_3COOH$ (Eq. 4-9)

In this study, methane might be produced from the metabolic pathway of hydrogen with carbon dioxide (Eq. 4-6) and acetic acid (Eq. 4-7). For biomethane production from methyl type, methane could be produced from methanol (Eq. 4-8) and ethanol (Eq. 4-9). However, methanol was not found in hydrogen fermentation effluent and ethanol was not unfortunately measured in this study.

This study indicated that the variation of the final pH value in biomethane production process was a result from concentration of various products in aqueous solution, especially ammonia and VFAs. It has been reported that the failure of biomethane production process happened on ammonia, VFAs and pH value. Chen et al. (2010) suggested that alkalinity adjustment in system should be considered for the prevention of the failure of anaerobic digestion process. Alkalinity is also identified as buffering capacity, which is the ability to neutralize acids from fluctuation of the pH value in system. It was recommended that alkalinity of 2,500 mg/L as $CaCO_3$ should be adjusted in biomethane production process. There are various chemical compounds that can be used as a source of alkalinity, which consist of carbonate, bicarbonate, phosphate, biphosphate and hydroxides. However, using of chemical compounds may be increase investment cost and the management of the salt in effluent in case of commercial application. Otherwise, co-digestion biomethane production of organic waste and other nutrient rich resources such as animal manure, compost, meat-based waste can be considered instead (Chen et al., 2010; Raposo et al., 2011).

The optimal environmental conditions for the maximum biohydrogen and biomethane production from food waste by a two-stage fermentation process were obtained from the experiment of Phase II. Biohydrogen production (stage I) from food waste and the pretreated seed sludge provided the maximum hydrogen yield of 214.88 mL H_2/g COD and COD removal of 90.34% at initial pH 7.0, thermophilic temperature (55 ± 2°C) and C/N ratio 30. Biomethane production (stage II) from hydrogen fermentation effluent and the raw seed sludge provided the maximum methane yield of 310.77 mL CH_4/g COD and COD removal of 70.37% at initial pH 7.0 and thermophilic temperature (55 ± 2°C).