CHAPTER III
MATERIALS AND METHODS

This research was designed to investigate the optimum of environmental conditions on biohydrogen and biomethane production from food waste by a two-stage fermentation process. This study was carried out in the batch experiment and was operated in a laboratory at Faculty of Environment and Resource Studies, Mahidol University (Salaya campus), Nakhonpathom, Thailand. The diagrams of experimental setup are shown in Figure 3.1 and 3.2.

3.1 Experimental design

3.1.1 Chemical substances

1) Acetic acid standard solution
2) Ammonium hydrogen carbonate (NH_4HCO_3)
3) Ammonium chloride (NH_4Cl)
4) Butyric acid standard solution
5) Dipotassium hydrogen orthophosphate ($K_2HPO_4.3H_2O$)
6) Ferrous sulphate ($FeSO_4.7H_2O$)
7) Hydrochloric acid (HCl)
8) Hydrogen gas (60% H_2, 30% CO_2, 10% CH_4)
9) Magnesium chloride ($MgCl_2.6H_2O$)
10) Methane gas (70% CH_4, 30% CO_2)
11) Methanol standard solution
12) Nickel (II) chloride ($NiCl_2.6H_2O$)
13) Nitrogen gas (N_2)
14) Potassium hydroxide (KOH)
15) Propionic acid standard solution

16) Sodium bicarbonate ($NaHCO_3$)

17) Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)

3.1.2 Tools and materials

1) Air bag

2) Black plastic

3) Centrifuge

4) Duran bottle 250 mL

5) Gas chromatography - flame ionization detector (Agilent 7890A, China)

6) Gas chromatography - thermal conductivity detector (Varian STAR 3400, USA)

7) Gas tight syringe 1.0 mL

8) Glass syringe 50 and 100 mL

9) Laboratory glassware (cylinder, pipette, vial bottle, etc.)

10) pH meter

11) pH paper

12) Plastic syringe 5.0 mL

13) Rubber tube

14) Screw cap

15) Sieve screen 2 mm

16) Silicone rubber stopper

17) Stainless steel needle

18) Three-way valve

19) Water bath shaker

3.1.3 Preparation of seed sludge

Anaerobic sludge was taken from the anaerobic digestion excrement treatment plant of Bureau of Environment and Health, Nonthaburi, Thailand. The seed sludge was screened with a sieve (size 2 mm) to eliminate the large particles and was examined physical and chemical characteristics of pH, total solids (TS), volatile solids (VS), chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN) according

to standard methods in Table 3.1. The seed sludge was separated into two parts. A part of seed sludge was pretreated at 100°C for 15 min using the heat shock method to inactive hydrogen–consuming bacteria and select spores of acidogenic bacteria which a one of hydrogen-producing microorganisms (Valdez-Vazquez and Poggi-Varaldo, 2009). Another part is the seed sludge without pretreatment method.

3.1.4 Preparation of substrate

Food waste was collected from the central cafeteria at Mahidol University (Salaya campus), Nakhonpathom, Thailand. The hard materials such as animal bones and shells were removed from the food waste which was grinded into small particles by a blender and was screened with a sieve (size 2 mm). Then, food waste was mixed with distilled water with a volume ratio of food waste to distilled water of 1:2 (Tawfik et al., 2011). This food waste slurry was used as a substrate. The substrate was kept in refrigeration at 4°C for preservation of sample. Before the substrate was used in the experiment, it was thawed into slurry at ambient temperature. The substrate was determined physical and chemical characteristics of pH, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), fat oil and grease (FOG), carbohydrate and protein according to the methods in Table 3.1.

Table 3.1 Analytical methods of seed sludge and food waste characteristics.

Parameter	Seed sludge	Food waste*	Analytical method	References
pH	/	/	pH meter	APHA, 2005
TS	/	/	Dried at 103 – 105°C	APHA, 2005
VS	/	/	Ignited at 550°C	APHA, 2005
COD	/	/	Closed reflux titration method	APHA, 2005
TKN	/	/	Kjeldahl method	APHA, 2005
FOG	ND	/	Soxhelt extraction method	APHA, 2005
Carbohydrate	ND	/	Colorimetric method	Mecozzi, 2005
Protein	ND	/	Calculated from TKN	AOAC Int., 2005

Note * Food waste slurry.
ND: Not determined.

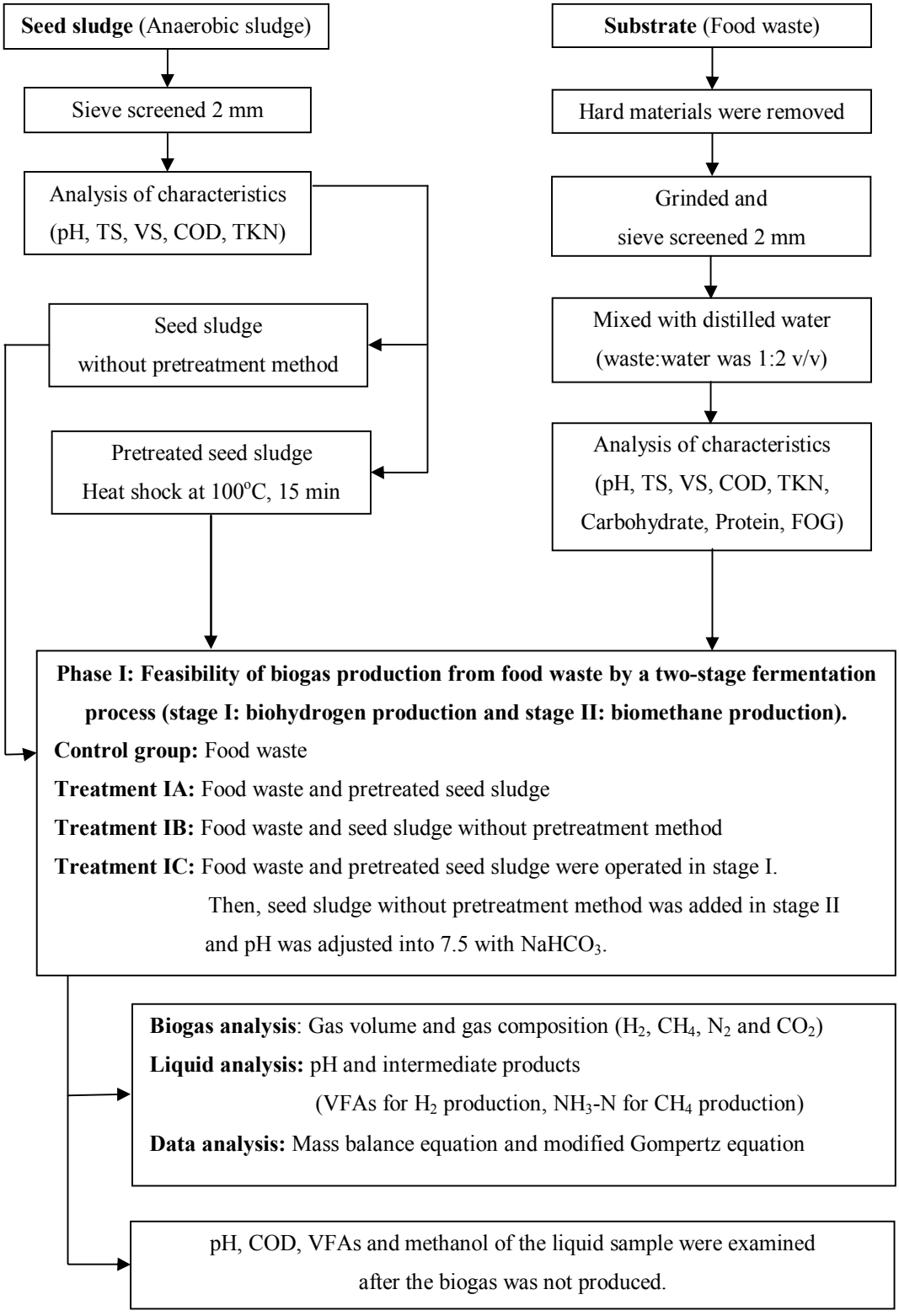


Figure 3.1 Diagram of experimental setup (Phase I).

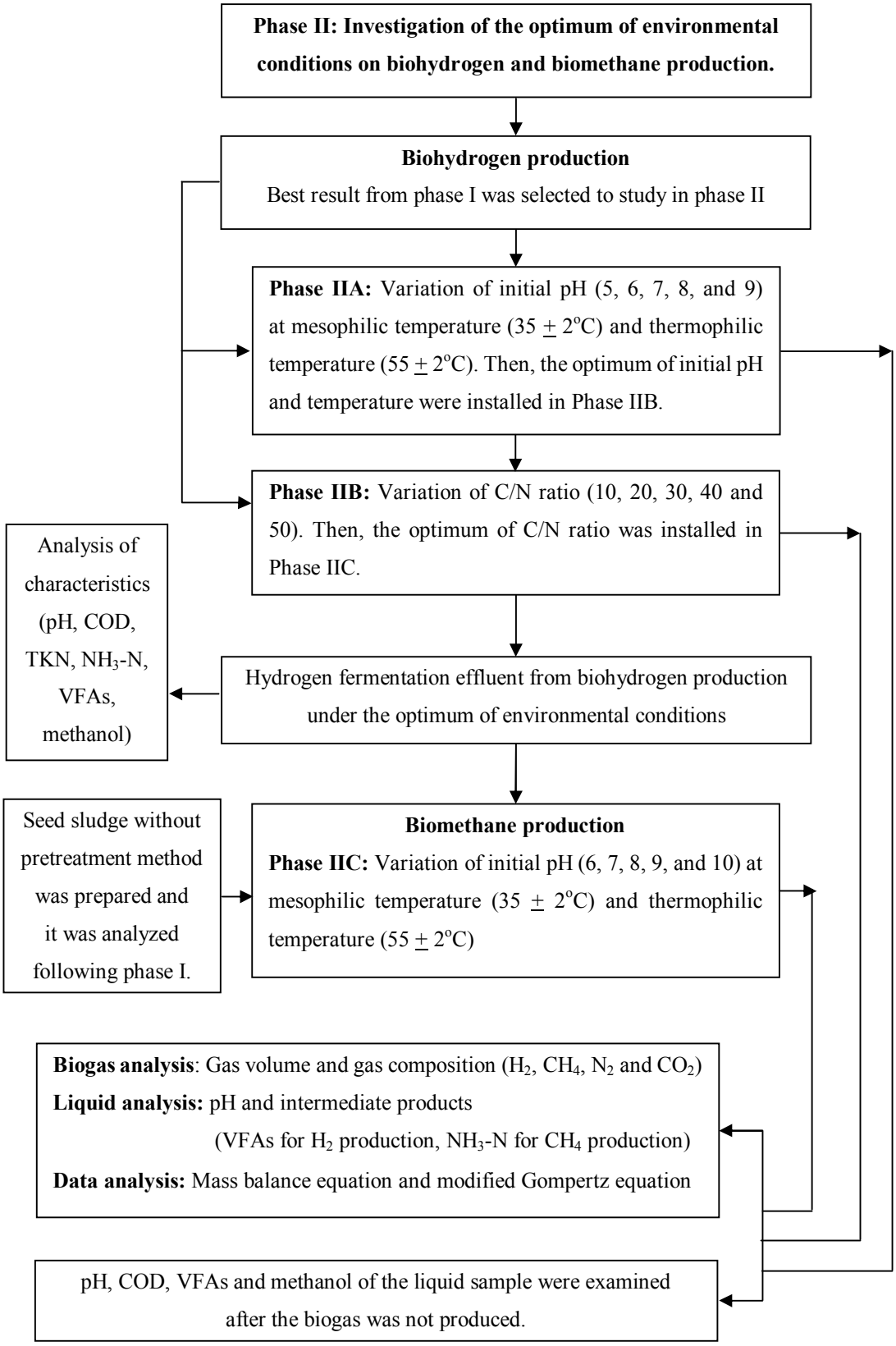


Figure 3.2 Diagram of experimental setup (Phase II).

3.1.5 Preparation of nutrient solution

In this study, the nutrient solution for bacteria growth contained 40 g $NaHCO_3$, 5 g NH_4Cl, 5 g $NaH_2PO_4.2H_2O$, 5 g $K_2HPO_4.3H_2O$, 15 g $FeSO_4.7H_2O$, 0.085 g $MgCl_2.6H_2O$ and 0.004 g $NiCl_2.6H_2O$ in 1,000 mL distilled water (Wang and Wan, 2008).

3.1.6 Experimental setup

The 250 mL glass bottle (Duran bottle) was used as a batch reactor in the experiment. The glass bottle was covered with a black plastic to make a dark condition. Each batch reactor has 200 mL working volume that consisted of 10 mL nutrient solution, 20 mL seed sludge (10% of working volume) and 170 mL food waste slurry. Next, the reactor was purged by nitrogen gas for 1 min to make an anaerobic condition. After that, a silicone rubber stopper and screw cap were used to avoid gas leakage from the bottle. Air bag was connected to the reactor for biogas collection that occurred during fermentation. Diagram of batch reactor is shown in Figure 3.3. Then, the reactor was placed in a water bath shaker with shaking speed at 100 rounds per minutes (rpm). The experiment was divided into two phases as follows:

3.1.6.1 Phase I: The experiment was conducted to study the feasibility of biohydrogen and biomethane production from food waste by a two-stage fermentation process under room temperature. The experiment was set up in duplication. This phase was divided into three treatments.

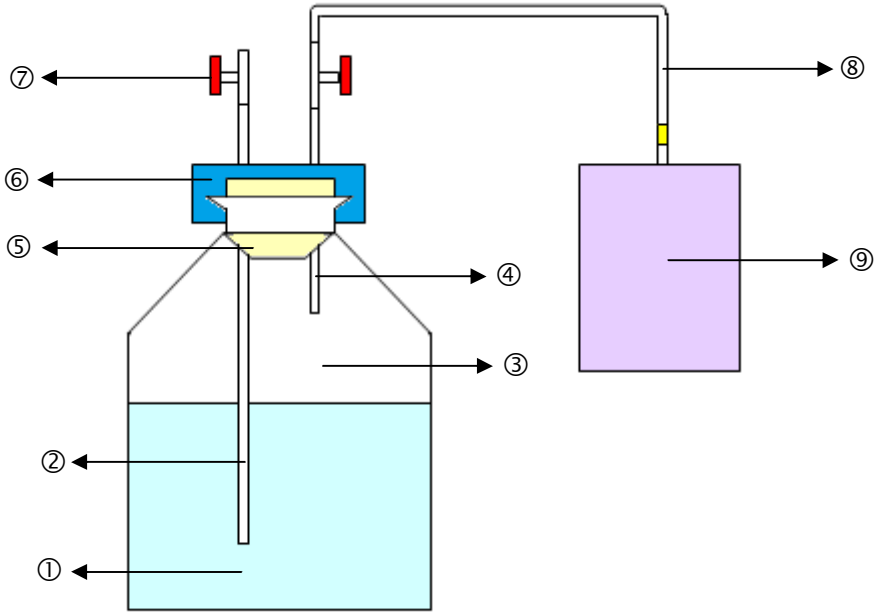
Treatment IA: The food waste and the pretreated seed sludge were used for biohydrogen production (stage I). After hydrogen gas was not produced, the experiment was carried out to monitor biomethane production (stage II).

Treatment IB: The experiment was operated in the same method of treatment IA, but the seed sludge without pretreatment method was added in stage I (biohydrogen production).

Treatment IC: The experiment was operated in the same method of treatment IA, but after hydrogen gas was not produced, the seed sludge without pretreatment method was added in byproducts from stage I (biohydrogen production) and pH was adjusted into 7.5 with $NaHCO_3$. Then, the reactor was purged with

nitrogen gas to provide an anaerobic condition before stage II (biomethane production) was carried out.

All treatment groups consisted of 10 mL of nutrient solution, 20 mL of seed sludge and 170 mL of food waste slurry. A control group consisted of 10 mL of nutrient solution, 170 mL of food waste slurry and 20 mL of distilled water. During the experiment, gas and liquid samples were sampled to analysis. After the biogas was not produced, the liquid sample in the batch reactor was examined the pH, COD, volatile fatty acids (VFAs) and methanol values.



Note: ① Mixed liquor of food waste, seed sludge and nutrient solution, ② Needle for sampling liquid, ③ Headspace, ④ Needle for sampling gas, ⑤ Silicone rubber stopper, ⑥ Screw cap, ⑦ Three-way valve, ⑧ Rubber tube, ⑨ Air bag

Figure 3.3 Diagram of batch reactor.

3.1.6.2 Phase II: The experiment was conducted to investigate the optimum of environmental conditions on biohydrogen and biomethane production. The important environmental conditions such as initial pH, temperature and carbon to nitrogen ratio (C/N ratio) were studied in biohydrogen production. For biomethane production, both of initial pH and temperature conditions were studied. The experiment was set up in triplication. This phase was divided into three phases. The experimental procedure was described as follows:

Phase IIA: The batch reactor was set up with 10 mL of nutrient solution, 20 mL of the pretreated seed sludge and 170 mL of food waste slurry. Then, it 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 ($35 \pm 2^{o}C$) and thermophilic temperature ($55 \pm 2^{o}C$).

Phase IIB: The experiment was operated in the same method of Phase IIA, but the optimal initial pH and temperature that provided the maximum biohydrogen production (Phase IIA result) was installed in this treatment. Moreover, NH_4HCO_3 was used as a nitrogen source to set the C/N ratio (Kalil et al., 2008), ranging from 10, 20, 30, 40 and 50 in the reactor. The control group was not added NH_4HCO_3.

Phase IIC: This treatment was to investigate the optimum of environmental conditions on biomethane production from hydrogen fermentation effluent by dark fermentation. The residue of biohydrogen production under the optimal environmental conditions from the experiment in Phase IIB was collected. Then, it was separated into liquid phase and solid phase by filtration. The liquid phase was used as the hydrogen fermentation effluent. The physical and chemical characteristics such as pH, COD, TKN, NH_3-N, VFAs and methanol of hydrogen fermentation effluent were examined. The 250 mL glass bottle was used as the batch reactor that consisted of 10 mL of nutrient solution, 20 mL of seed sludge without pretreatment method and 170 mL of hydrogen fermentation effluent. The batch reactor was adjusted with HCl or KOH to set the initial pH in the range from 6.0, 7.0, 8.0, 9.0 and 10.0. The batch reactor was purged with nitrogen gas for 1 min to provide an anaerobic condition. Each the initial pH test was controlled at mesophilic temperature ($35 \pm 2^{o}C$) and thermophilic temperature ($55 \pm 2^{o}C$).

During the experiment, gas and liquid samples were sampled to analysis. After the biogas was not produced, the liquid sample in batch reactor was examined the pH, COD, VFAs and methanol values.

3.2 Monitoring

3.2.1 Gas sampling

Biogas was produced from dark fermentation and collected in an air bag. Biogas volume was measured every 24 h using a glass syringe sized 50 - 100 mL. Biogas composition was measured after the experiment was started at 4, 6, 8, 10, 12 h and every 24 h until biogas was not produced in the batch reactor. Biogas was sampled using a gas tight syringe and biogas composition (hydrogen, methane, nitrogen and carbon dioxide) was analyzed using a gas chromatography (GC).

3.2.2 Liquid sampling

The liquid sample was monitored the pH value and the intermediate products during the experiment. The pH value was monitored every 48 h after the experiment was started until biogas was not produced in the batch reactor. It was measured using pH paper. Volatile fatty acids (VFAs; acetate, butyrate and propionate) were analyzed using a gas chromatography - flame ionization detector (GC-FID) when the maximum biohydrogen production was produced. Ammonia-nitrogen (NH_3-N) was analyzed using titrimetric method when the maximum biomethane production was produced. After the experiment finished, the liquid sample in batch reactor was examined the pH, COD, VFAs and methanol values.

3.3 Analytical method

3.3.1 Biogas analysis

Biogas volume was measured using a glass syringe sized 50 - 100 mL. Biogas was sampled using a gas tight syringe and biogas composition was measured using a gas chromatography (GC) was equipped with a thermal conductivity detector (TCD). The stainless steel packed column was Alltech, Molesieve 5A 80/100 10'x1/8''. Hydrogen, methane and nitrogen contents were analyzed using argon as a carrier gas and helium was applied as a carrier gas for carbon dioxide analysis (Selembo et al., 2009). The operating temperature of the injector, column and detector were 80ºC, 50ºC and 90^{o}C, respectively.

3.3.2 Liquid analysis

During the experiment, the liquid sample was measured pH value and analyzed the concentration of intermediate products. Volatile fatty acids (VFAs) was measured for biohydrogen production and ammonia-nitrogen (NH_3-N) was measured for biomethane production. After the experiment finished, the liquid sample in the batch reactor was examined the pH, COD, VFAs and methanol values. The method was described as follows:

3.3.2.1 The pH value: The liquid was sampled 0.2 mL using a plastic syringe and was dripped on pH paper to measure the pH value every 48 h. After the experiment finished, the liquid was examined the pH value using pH meter.

3.3.2.2 VFAs and methanol: The liquid was sampled 10 mL using a plastic syringe. The liquid samples were centrifuged at 10,000 rpm, 4ºC for 5 min. Then, they were filtrated through 0.45 µm membrane filter and acidified by 0.1 N HCl. The concentration of VFAs (acetate, butyrate and propionate) and methanol were analyzed using a gas chromatography - flame ionization detector (GC-FID). Helium was used as a carrier gas at flow rate of 3 mL/min. The temperature of oven was operated at 50ºC for 2 min and then it was increased at 50 ºC/min. After the temperature of the oven reached to 230ºC, it was controlled for steady stage at 230ºC for 3 min. The operating temperature of the injector and detector were 230ºC and 250ºC respectively.

3.3.2.3 NH_3-N: The liquid was sampled 10 mL using a plastic syringe. The concentration of ammonia was analyzed using titrimetric method (APHA, 2005).

3.3.2.3 COD: The liquid in the batch reactor was examined COD using closed reflux titration method (APHA, 2005).

3.3.3 Data analysis

Mass balance equation (Eq. 3-1) was calculated for hydrogen gas production and methane gas production (Sreela-or et al., 2011a).

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad \text{(Eq. 3-1)}$$

where $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volumes (or cumulative methane gas volumes) at the current (i), and the previous time interval (i-1), $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volume at the current and the previous time interval, $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen gas (or methane gas) in the headspace at the current and the previous time interval and V_H is the volume of the headspace in the batch reactor.

Modified Gompertz equation (Eq. 3-2) was calculated for cumulative hydrogen production and cumulative methane production (Wang and Wan, 2009c; Elbeshbishy and Nakhla, 2012).

$$H = H_{max}\exp\{-\exp[(R_m e/H_{max})(\lambda - t) + 1]\} \quad \text{(Eq. 3-2)}$$

where H (mL) is the cumulative hydrogen production (or cumulative methane production), H_{max} (mL) is the maximum hydrogen production (or the maximum methane production), R_m (mL/h) is the maximum hydrogen production rate (or the maximum methane production rate), λ (h) is the lag phase time, t (h) is the incubation time and e is 2.718281828.

3.4 Statistic analysis

The data of all experiments was calculated using mean ($\bar{x}$), standard deviation (SD) and percentage. The statistical significance ($\alpha = 0.05$) of the difference between hydrogen yield (mL H_2/g COD) of initial pH, temperature and C/N ratio and methane yield (mL CH_4/g COD) of initial pH and temperature were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test. Statistic analysis was carried out using a Microsoft Excel Software 2010.