CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Anaerobic Seed Sludge and Alcohol Wastewater

Seed sludge and alcohol wastewater are collected from the biogas plant of Sapthip Lopburi Co., Ltd., Thailand. The anaerobic seed sludge is black color, and has pH of 3.0–4.0, and total suspended solids (TSS) concentration of 10,000 mg/l. The characteristic of the alcohol distillery wastewater used in first part of the study are shown in Table 3.1. Table 3.2 shows characteristics of the alcohol wastewater used in a second part of the study. The slight differences in the characteristic of the alcohol wastewaters resulted from the change of the production process. Table 3.3 shows the characteristic of cassava wastewater used in a third part of the study. The anaerobic seed sludge, the alcohol wastewater, and the cassava wastewater were kept at 4 °C prior to use.

Table 3.1 Characteristics of the studied alcohol wastewater

Parameter	Unit	Value
pH	-	3.4
COD	mg/l	60,000
Total VFA	mg/l	5080
Ethanol concentration	mg/l	3120
Total solids (TS)	mg/l	10000
Total phosphorous	mg/l	800
Total nitrogen	mg/l	4400
Ammonia (NH_3)	mg/l	70
Nitrate (NO_3)	mg/l	400
Nitrite (NO_2)	mg/l	2
COD:N:P	-	100:7:1.3
Color	-	Blown

Table 3.2 Characteristics of the studied alcohol wastewater

Parameter	Unit	Value
pH	-	3.6
COD	mg/l	45,000
Total VFA	mg/l	4,000
Ethanol concentration	mg/l	534
Total solids (TS)	mg/l	12,000
Total phosphorous	mg/l	580
Total nitrogen	mg/l	600
Organic nitrogen (Org-N)	mg/l	208
Ammonia (NH_4^+-N)	mg/l	40
Nitrate (NO_3^--N)	mg/l	350
Nitrite (NO_2^--N)	mg/l	1.6
COD:N:P	-	100:1.33:1.23
Color	-	Blown

Table 3.3 Characteristics of the studied cassava wastewater

Parameter	Unit	Value
pH	-	4.1
COD	mg/l	14,500
Total VFA	mg/l	6,500.63
Total solids (TS)	mg/l	2,460
Total phosphorous	mg/l	295
Total nitrogen	mg/l	431
Organic nitrogen (Org-N)	mg/l	214.3
Ammonia (NH_4^+-N)	mg/l	75
Nitrate (NO_3^--N)	mg/l	140
Nitrite (NO_2^--N)	mg/l	1.7
COD:N:P	-	100:2.98:2.03
Color	-	Translucent yellow

3.1.2 Chemicals

All chemicals used in this study were in chemical grade and they were used without purification.

3.2 Equipment

3.2.1 Time-controlling System

Timers (OMRON model H5CX-A), as shown in Figure 3.1, were used to control the operation time of each steps: (1) feeding, (2) reacting, (3) settling, and (4) decanting for an anaerobic sequencing batch reactor (ASBR).

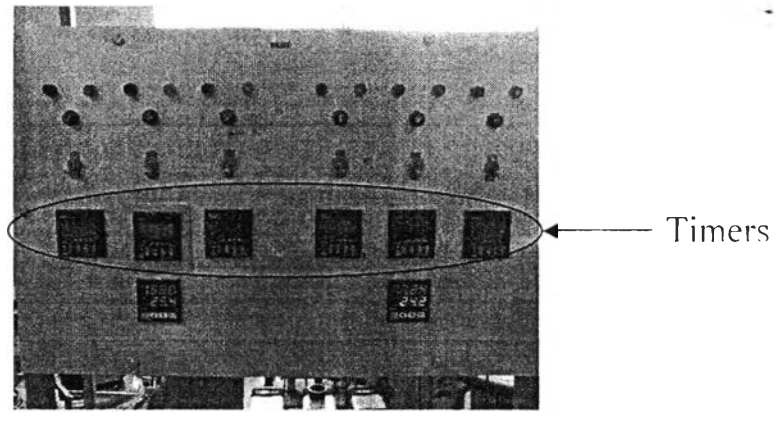


Figure 3.1 Time-controlling system.

3.2.2 Temperature-controlling System

This system comprising a heater rod, thermocouple, and control box (Figure 3.2) was used to control the temperature of each ASBR unit. The system temperature was adjusted to be around 55 °C.

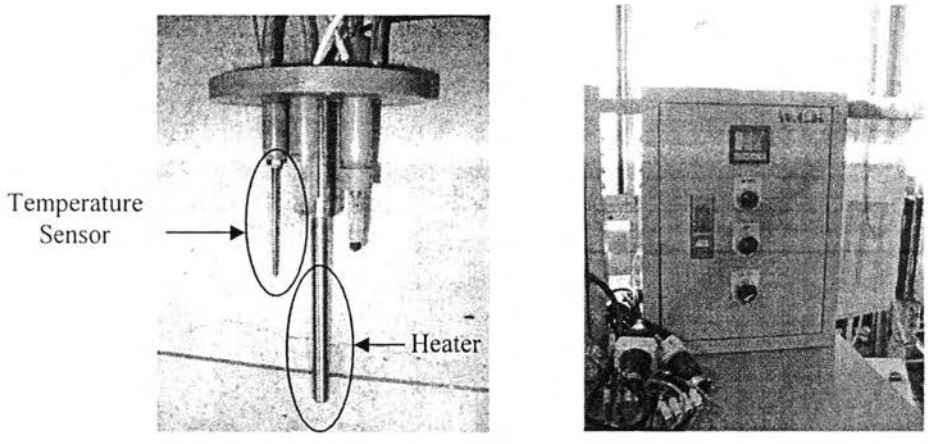


Figure 3.2 Temperature-controlling system installed at a cover of ASBR.

3.2.3 pH-controlling and Mixing Systems

This ASBR system consisted of a pH controller (Extech model 48PH2), a pH electrode (Cole-Parmer Double-Junction Electrode) (Figure 3.3), a diaphragm pump, a NaOH solution tank and a magnetic stirrer (40×20 mm, egg shape) for mixing. The pH of the mixed solution was controlled automatically by feeding a 1 M NaOH solution via the diaphragm pump regulated by the pH controller. The liquid in the bioreactor was homogeneously mixed using the magnetic stirrer at 400 rpm only during the react step.

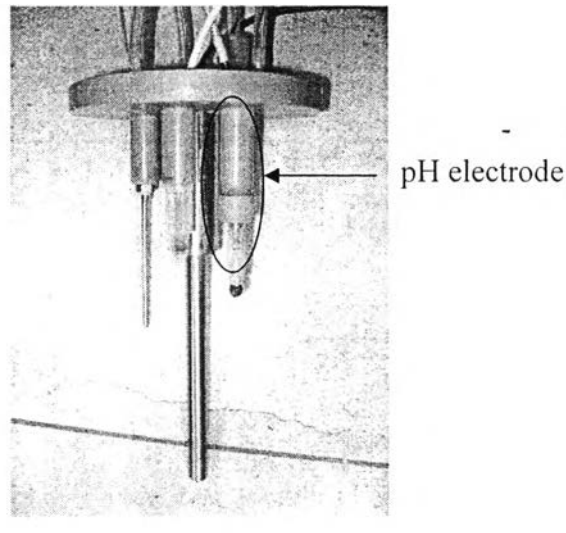


Figure 3.3 pH sensor installed at a cover of reactor.

3.2.4 Gas-measuring System

This system was composed of 2 flasks filled with a 1 M HCl solution, in order to prevent dissolution of the produced CO_2 gas (Ueno *et al.*, 1996), and a wet gas meter (Ritter, TGO5/5) (Figure 3.4) was used to measure the volume of produced gas at room temperature.

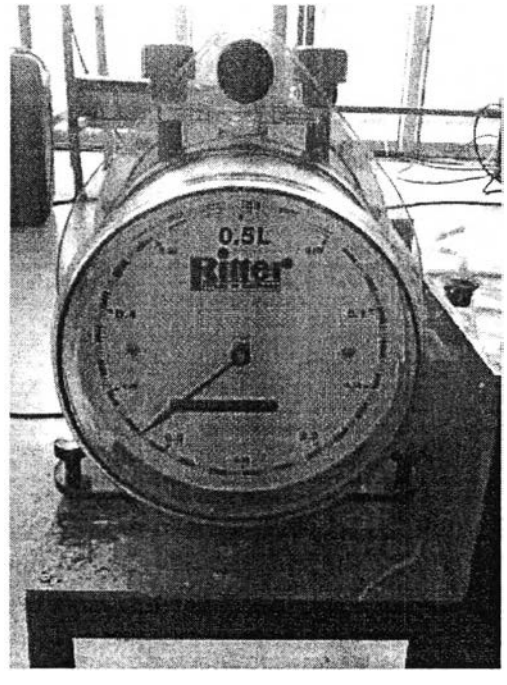


Figure 3.4 Wet gas meter.

3.3 Methodology

3.3.1 Seed Sludge Preparation

The seed sludge for hydrogen production experiments was first concentrated by sedimentation. Next, the concentrated sludge was ground and filtered through a sieve in the size of 1 mm in order to remove debris and large solid particles. Then, it was pretreated before being fed into the bioreactor by boiling at 95 °C for 15 min in order to enrich the hydrogen-producing acidogenic bacteria in spore forms and to eliminate hydrogen-consuming methanogens (Argun *et al.*, 2008).

3.3.2 Substrate Preparation

Alcohol distillery wastewater was filtered through a sieve size of 0.2 μm to remove debris and used to feed the bioreactor without dilution and addition of any nutrient further first two parts of the study.

3.3.3 Bioreactor Design and Operation

Two identical ASBR reactors are used in order to perform the biohydrogen production experiments at different COD loading rate. To inhibit the activity of photosynthetic bacteria, the system was operated without light exposure in 5 L opaque PVC reactors. Each of them had an inner diameter of 13 cm and a height of 30 cm. The reactors were operated with a working volume of 4 L under a thermophilic temperature of 55 °C. The schematic of the ASBR process is shown in Figure 3.5.

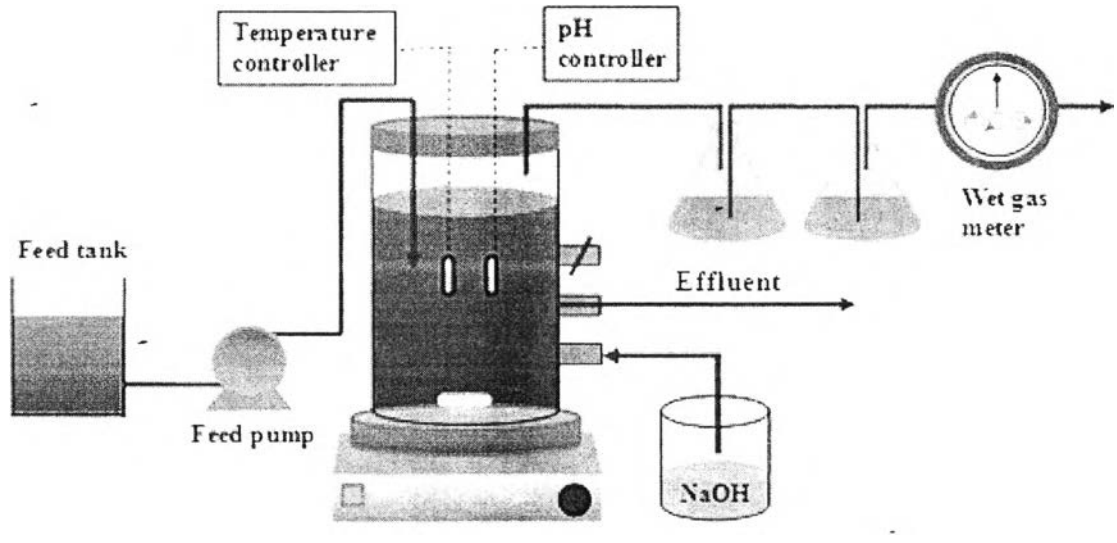


Figure 3.5 Schematic of the studied ASBR process.

The ASBR operation consisted of four sequencing steps: feed, react, settle, and decant. During the operation, the time for each step was controlled by a set of timers to allow the feed pump to pump wastewater during the feeding period. Mixing was achieved by using a magnetic stirrer at 400 rpm during the reacting

phase (Chen and Chen 2009). The pH-controller and heater were used to maintain a constant pH and temperature of the system, respectively.

During the start-up, 1000 ml of the pretreated seed sludge was completely mixed with wastewater at an initial feed COD of 60,000 mg/l and a COD loading rate of 45 kg/m^{3}d, which corresponded to a hydraulic retention time (HRT) of 32 h. The COD loading rate was then increased stepwise by reducing HRT. Under any studied conditions, the reactor was operated until the system reached steady state in which took around two weeks. Steady state conditions were justified when the variation in the production of produced gas was nearly constant (standard deviation less than 5%). Then, the samples of effluent and the produced gas are collected for analyses and measurement. Chatsiriwatana (2009) reported that the system operated at 6 cycles per day showed higher process performance in terms of hydrogen production rate and yield than that operated at 4 cycles per day. Hence, 6 cycles per day was selected to operate the ASBR in this study. The operation times of four steps, i.e. feed, react, settle, and decant, in the ASBR operation are shown in Table 3.4. Table 3.5 shows the operational condition used for the firdt part of this study

Table 3.4 Operation conditions for the ASBR system at 6 cycles per day

Operating step	Cyclic time (min)
Feed	15
React	90
Settle	120
Decant	15
Total	**240**

The feed and decant flow rates were varied at the constant feed COD values of 60,000 mg/l to achieve any COD loading rate, as expressed in Equation (3.1):

$$\text{COD loading rate (kg/m}^3\text{d)} = \frac{\text{(Feed COD)} \times \text{(Feed Flow Rate)}}{\text{(Working Volume)}} \qquad (3.1)$$

Table 3.5 Operation conditions for the ASBR system at 6 cycles per day

Feed and Decant (l/d)	HRT (h)	Feed or Decant (l/cycle)	COD loading rate (kg/m^3d)
3	32	0.5	45
3.75	25.6	0.625	56
4.5	21.3	0.75	68
5.25	18.3	0.875	79

The upflow anaerobic sludge blanket (UASB) reactors used in the third part of this study were constructed from borosilicate glass with a 4 and 24 L working volume for hydrogen and methane UASB bioreactors, respectively. The temperatures inside both bioreactors were controlled constant at 55 °C by circulating water through a water jacket of each bioreactor by a circulating/heating bath. The cassava wastewater was fed continuously to the bottom of the hydrogen UASB bioreactor (in upward direction) at any desired flow rate by using a peristaltic pump in order to obtain different COD loading rates (30, 60, 90, 120, and 150 kg/m^3d based on the hydrogen UASB bioreactor or 5, 10, 15, 20 and 25 kg/m^3d based on the methane UASB bioreactor). The pH of hydrogen UASB unit was maintained at 5.5 by using a pH controller. The effluent from the hydrogen UASB unit was directly pumped into the methane UASB bioreactor by a peristaltic pump with a level control probe. The effluent pH of the methane UASB unit was not controlled. In order to minimize the consumption of NaOH for the pH control of the hydrogen UASB unit, a recycle ratio of the methane UASB effluent flowrate-to-feed flowrate of 1:1 was used in this study. The schematic of the two-stage UASB unit is shown in Figure 3.6.

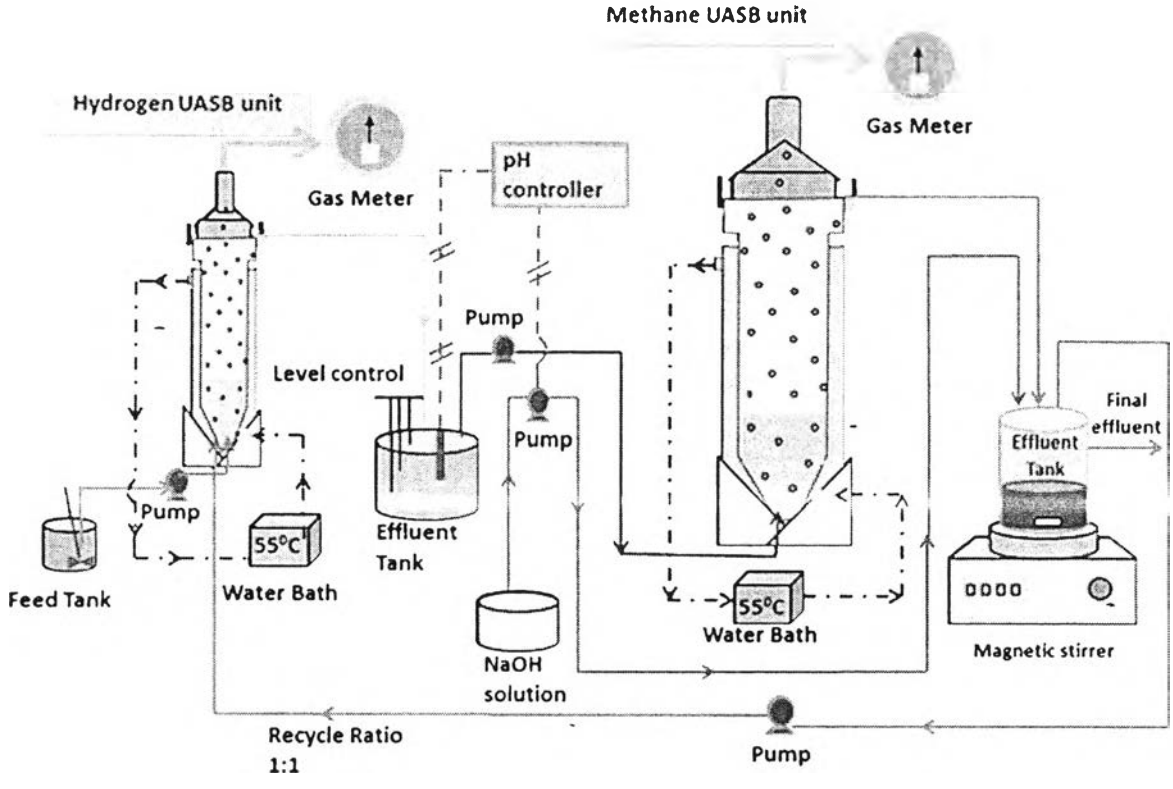


Figure 3.6 Schematic of the studied two-stage UASB process.

3.4 Analytical Methods

3.4.1 Total Suspended Solids (TSS) Analysis

A glass-fiber filter disk (Pall-61631 A/E, 47 mm, 1 μm) was used for analysis of TSS according to the standard methods. Figure 3.7 shows the filtation unit used for analysis

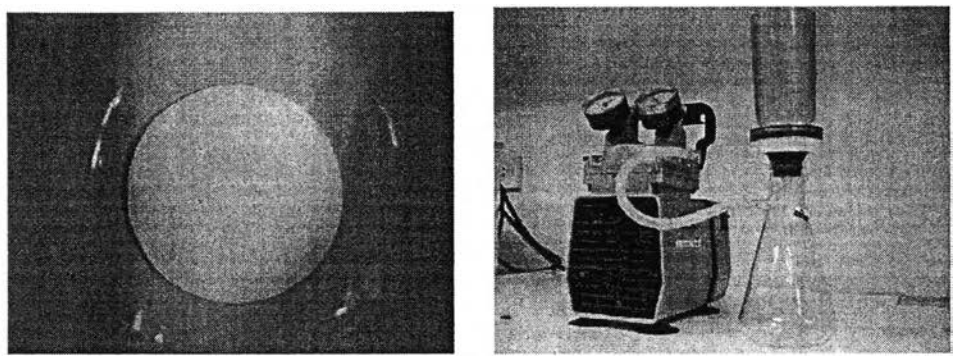


Figure 3.7 (a) glass-fiber filter disk and (b) filtration apparatus.

The calculation of SS is shown in the following equation:

$$\frac{\text{mg total suspend solids (TSS)}}{\text{1 l of sample volume}} = \frac{(A - B) \times 10^6}{\text{Sample volume, (mL)}} \qquad (3.2)$$

A = Weight of filter + dried residue [g]

B = Weight of filter [g]

3.4.2 Volatile Suspended Solids (VSS) Analysis

The residue produced by TSS method is ignited in a furnace at a temperature of 500 ± 50 °C for 1 hour. A furnace was heated up to 500 °C for 1 h after inserting sample. The filter disk was then left to partially cool in air until most of the heat was dissipated. The disk was finally transferred to a desiccator, and weighed as soon as it is cooled to balance temperature.

The Calculation of VSS is shown in the following equation:

$$\frac{\text{mg suspened solids (VSS)}}{\text{1 l of sample volume}} = \frac{(A - B) \times 10^6}{\text{Sample volume, (mL)}} \qquad (3.3)$$

A = Weight of residue + disk before ignition [g]

B = Weight of residue + disk after ignition [g]

3.4.3 COD Analysis (Closed Reflux, and Colorimetric Method)

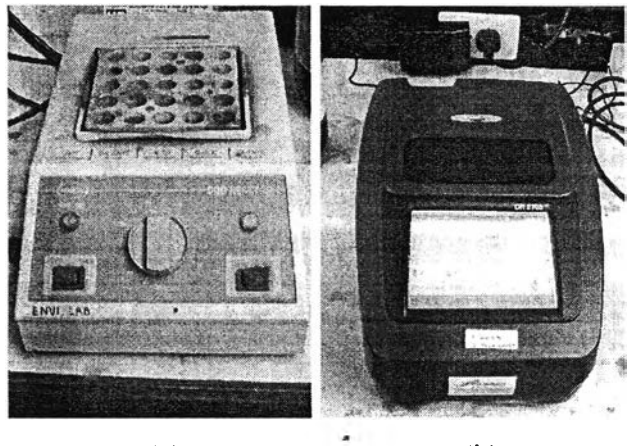
3.4.3.1 Reagents

- *Digestion solution.* The following reagents added into 500 ml distilled water were: 10.216 g of $K_2Cr_2O_7$ (primary standard grade) previously dried at 103 °C for 2 h, 167 ml of 98% H_2SO_4, and 33.3 g of $HgSO_4$. The mixture was left for complete dissolution, cooled to room temperature, and finally diluted to 1 L.

- *Sulfuric acid reagent.* Ag_2SO_4 (reagent grade, crystals or powder) was added to 98% H_2SO_4 at ratio of 5.5 g Ag_2SO_4/kg H_2SO_4. The mixture was left to stand for 1 to 2 d to completely dissolve the Ag_2SO_4.

3.4.3.2 Procedure

A Sample (dilute 100 times) of 2.5 ml was added to a digestion vial (HACH, 16×100 mm). The Digestion reagent of 1.5 ml was added to the vial. Afterwards, the sulfuric acid reagent was slowly dropped for 3.5 ml into the vial. The vial with a screw cap is inverted several times to homogeneously mix the contents, and the vial is then placed in a preheated COD reactor (HACH) (Figure 3.8(a)). The vial was heated for 2 h, and then left for about 20 min to be cooled. The vial is finally placed into a spectrophotometer (HACH DR 2700) for reading for absorbance, as shown in Figure 3.7(b).The COD value of the sample was obtained from the calibration curve plotting COD and absorbance



(a) (b)

Figure 3.8 (a) COD reactor and (b) spectrophotometer.

3.4.4 Total VFA Analysis

The amount of VFA was determined by the distillation-titration method. This technique covers all organic acids containing up to six carbon atoms and reports the results in terms of equivalent acetic acid (Greenberge *et al.*, 1992).

3.4.5 VFA Composition Analysis

The organic acid composition of the distilled organic acid sample obtained from the total VFA analysis was further determined by a gas chromatograph (PR2100, Perichrom) equipped with a flame ionization detector and a 50 m x 0.32 ID, 0.25 μm film thickness DB-WAXetr (J & W Scientific) capillary column in the split mode (10 mL/min) with helium at a pressure of 82 kPa as a carrier gas, H_2 at 50 kPa as a combustion gas, and air zero at 50 kPa as a combustion-supporting gas. The column temperature program is started at 60 °C, heated to 125 °C at a ramping rate of 10 °C min^{-1}, held for 2 min, then heated to 180 °C at a ramping rate of 15 °C min^{-1}, and held for 15 min. The temperatures of injector and detector were 250 and 270 °C, respectively.

3.4.6 Phosphorous Analysis

The total phosphorous in feed and effluent samples was determined by the molybdovanadate method with acid persulfate digestion (Hach Company). The sample cell was placed into the spectrophotometer (HACH DR 2700) for determining phosphorous content.

3.4.7 Nitrogen Analysis

The nitrogen concentrations (in terms of organic-nitrogen by the diazotization, and cadmium reduction method and inorganic nitrogen by the salicylate method) in feed and effluent samples were determined via the TNT persulfate digestion. The sample cell was placed into the spectrophotometer (HACH DR 2700) for determining nitrogen content.

3.4.8 Gas Composition Analysis

The gas compositions of the produced gas samples under different COD loading rate were determined by a gas chromatograph (AutoSystem GC, Perkin-Elmer) equipped with a thermal conductivity detector (TCD) and a stainless-steel 10′ x 1/8″ x .085″ HayeSep D 100/120 mesh (Alltech) packed column. Injector,

column, and detector temperatures were kept at 60, 35, and 150 °C, respectively. Argon was used as the carrier gas at pressure of 345 kPa.

3.4.9 Preparation of Residue and Composition Analysis

The fermentation residue sample provided by Sapthip Lopburi Co., Ltd., Thailand. was dried at 105 °C and stored in sealed plastic bags. The dried sample was milled to reduce the particle sizes and sieved to sizes between 40 and 60 mesh. To identify the physical properties, chemical composition and the particle size distributions of the dried sample were analyzed, as following methods.

An elemental analyzer (TruSpec-CHN) was used to determine C, H, O, N and S contents in the sample. Combustion and burner temperatures were kept at 950 °C and 850 °C, respectively, with oxygen, helium, and air used as carrier gases. The concentrations of glucose in the dried sample of fermentation residue and the effluent were determined by the enzymatic method with a glucose (HK) assay kit (Sigma-Aldrich, Inc).

To determine the amount of extractives in the residue sample, solvent extraction (60 ml acetone for 1 g of dried residue sample) was used, and the extraction step was performed at 90 °C for 2 h. After that, the sample was dried at 105 °C until a constant weight was obtained. The weight difference before and after the acetone extraction was defined as the amount of extractives.

To determine the amount of hemicellulose, 10 ml of a 0.5 M sodium hydroxide solution was added to 1 g of the extractive-free dried residue, and the mixture was held at 80 °C for 3.5 h. After that, the sample was washed using distilled water until a neutral pH value of 7 was reached. Next, it was dried to obtain a constant weight. The weight difference before and after this alkaline dissolution step was defined as the hemicellulose content. The starch fraction in the NaOH dissolution solution was then determined by the amylase/amyloglocosidase method using a starch assay kit (Sigma-Aldrich, Inc).

To determine the amount of lignin, 30 ml of a 72 wt% sulfuric acid was added to the dried residue after the NaOH dissolution step. The mixture was kept at 8–15 °C for 24 h. Then, it was transferred into a flask and diluted with 300 ml of distilled water. After that, the sample was boiled at 100 °C for 1 h. The mixture was filtered, and then the residue was washed until the sulfate ion in the filtrate is not

detected (via titration with a 10% barium chloride solution). The remaining solid was finally dried to obtain a constant weight. The weight of the remaining residue was defined as the lignin content.

Next, the dried residue after the H2SO4 dissolution step was placed in a furnace at 550 °C. The weight loss was defined to represent the cellulose fraction. The weight of ash was used to represent the sand fraction in the fermentation residue sample (Lin *et al.*, 2008).

3.4.10 Microbial Concentration (MLVSS)

The microbial concentration in the system can be simply measured in terms of microbial concentration or MLVSS. At steady state, the whole liquid was drained out from the reactor and then stirred homogeneously. A volume of 5 ml of welled-mixed sample was filtrated through a glass fiber filter, washed with distilled water, and dried in an oven at 105 °C for 1 h. The dried filtered sample was used to represent for MLSS. The MLVSS was the weight difference between the dried sample at 105 °C and the burnt sample at 550 °C (for 1 h.).

3.4.11 Microbial Washout (Effluent VSS)

The microbial washout from the system can be measured in terms of Effluent VSS. After steady state for each studied COD loading rate, effluent samples were through a glass fiber filters, the filtrated solid were washed with distilled water, and dried in an oven at 105 °C for 1 h. The dried sample weight was used to represent as TSS. The effluent VSS was the weight difference between the dried filtered solids at 105°C and the burnt sample at 550 °C (for 1 h.).

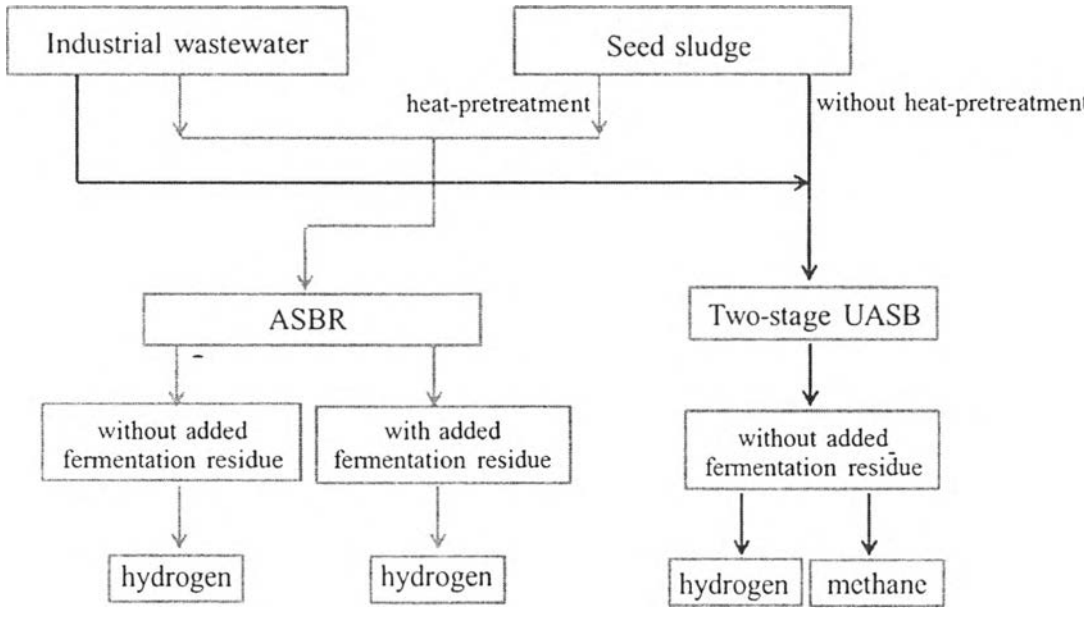


Figure 3.9 Hydrogen and methane production in various processes used in this research.