CHAPTER III
MATERIALS AND METHODS

This research was an experimental research using a batch reactor to examine the optimal condition which is the maximum biohydrogen yield of bio-hydrogen production from dark fermentation of agricultural food wastewater by anaerobic microbes. The study was operated in laboratory of Faculty of Environment & Resource Studies at Mahidol University (Salaya campus), Nakhonpathom, Thailand. The diagram of experimental procedures is shown in Figure 3.1.

3.1 Equipment and chemicals

3.1.1 Equipment

1) Air bag

2) Black bag

3) Cylinder

4) Dropper

5) Flame atomic adsorption spectrophotometry (FAAS) (AA240FS, Varian, USA)

6) Gas chromatography (GC-TCD) (Varian STAR 3400, USA)

7) Gas chromatography/flame ionization detector (GC-FID) (Agilent 7890A, USA)

8) Glass syringe

9) Laboratory bottle

10) Needle

11) Pipette

12) pH meter

13) Rubber tube

14) Scanning electron microscope (SEM) (JEOL JSM-5600LV, USA)

15) Sieve screener

16) Silicone rubber stopper

17) Three-way valve

18) Volumetric flask

19) Water bath

20) Water bath shaker

21) Surface area analysis (BET) (Autosorb-1, Quantachrome, US)

22) Positive charged microscope slide (HDA, China)

23) Cover slip (Menzel-Glaser, Germany)

24) Confocal laser scanning microscope (Olympus, FLUOVIEW FV10i, USA)

25) GenepHlow Gel/PCR Kit (Geneaid, Taiwan)

3.1.2 Chemicals

1) Acetic acid standard solution

2) NH_4HCO_3

3) Butyric acid standard solution

4) $CuSO_4.5H_2O$

5) $C_6H_{12}O_6$ (D-glucose)

6) H_2SO_4

7) H_2 gas standard (60% H_2)

8) $FeSO_4.7H_2O$

9) K_2HPO_4

10) $MnSO_4.6H_2O$

11) N_2 gas

12) $MgCl_2.H_2O$

13) KOH

14) $NaHCO_3$

15) $CoCl_2.5H_2O$

16) KNO_3

17) Propionic acid standard solution

3.1.3 Biological supporting materials (BM)

Coir

Corncob

Loofa sponge

Pine tree bark

Silkworm cocoon

Shell

Crab exoskeleton

3.2 Experimental procedures

3.2.1 Preparation seed sludge

The anaerobic sludge was gathered from the anaerobic digestion excrement treatment plants of Bureau of Environment and Health, Nonthaburi, Thailand. Then, it was screened by sieve filter with diameter 2.00 mm, and analyzed its characteristics; pH, Total Suspended Solid (TSS), Total solid (TS), Volatile Suspended Solid (VSS), Volatile Suspended Solid (VS), Total Kjeldahl Nitrogen (TKN), Chemical Oxygen Demand (COD), iron concentration; followed from APHA (2005). After that, it was heated at 90°C for 10 minutes to inhibit growth of hydrogen–consuming bacteria (Hawhes et al., 2002).

The seed sludge was cultured in nutrient solution slightly modified from Lin and Chang (2004). One litre of nutrient solution contained 10 g of $C_6H_{12}O_6$ (D–Glucose) as the carbon source, 5.240 g NH_4HCO_3, 6.720 g of $NaHCO_3$, 0.125 g K_2HPO_4, 0.100 g $MgCl_2 \cdot H_2O$, 15 mg $MnSO_4 \cdot 6H_2O$, 4.37 mg, and was diluted with distilled water. Before used, the seed sludge was heated at 90°C for 10 minutes to inactivate hydrogen-consuming bacteria.

3.2.2 Preparation substrate

Substrates wastewater was collected from various starch processing factories by grab sampling method. Collecting bottles were rinsed twice with the wastewater before used. Wastewater samples were stored in polyethylene bottle, kept in ice box, and suddenly transferred to refrigerator (operating temperature is 4°C) when arrived at laboratory. Sampled were kept in refrigerator until use. Before starting the operation, the substrate was analyzed its characteristics; pH, TSS, TS, VSS, VS, TKN, COD, and iron concentration (APHA, 2005).

3.2.3 Preparation and selection of biological supporting materials (BMs)

Studied BMs from plants were loofa sponge, coir, corncob, and pine tree bark. BMs from animals were silkworm cocoon, shell, and crab exoskeleton. Before using in the fermentation process, they were characterized and tested as following

3.2.3.1 Physical characterization

All BM were physically shortened into size of 0.5-1 cm and shaped into rectangular, parallelepiped or triangular. Average density of each type of BM were calculated from weight (g) divided by volume (mL). The volume of BM was calculated from its width x length x height.

3.2.3.2 Acid tolerance

Due to acidic products forming in the dark fermentation, BMs need to test for its acid tolerance. Acid tolerance was done by suspend BMs into acetic acid pH 4.0 for 7 days. Acetic acid is selected because it is primary by-production from dark fermentation. BM that could not maintain its structure or were slightly decomposed after the suspension was not selected for further study of biohydrogen production.

3.2.3.3 Cell immobilization ability

BMs were preliminary observed for cell immobilization ability by cultivation of seed sludge in nutrient solution (modified from Chang et al., 2002). 180 mL nutrient solution seeded with 92.8 mg VSS of seed sludge were cultivated in a batch with BM 5% (v/v). Cultivation was terminated when biogas stopped generating. The amount of immobilized biomass per gram of BM was determined by measuring

the difference in dry weights of BM before and after the immobilization and divide by gram of dry weight of added BM.

3.2.3.4 Hydrogen production efficiency

5% (v/v) of BM were inserted into batch experiment to investigate its hydrogen production efficiency from fermentation of the starch processing wastewater by mixed anaerobes. Highest yield in mL H_2/ g COD (both from plant and animal-based BM) were used in a next experiment.

3.2.3.5 Surface area analysis

From prior 3.2.3.1 to 3.2.3.5, BM which provided positive results and highest efficiency (both from plants and animals) were analyzed its surface area by gas adsorption method (Brunauer et al., 1938) at Institute for Scientific and Technological Research and Services, King Mongkut's University of Technology Thonburi, Thailand. The surface area was operated with Quantachrome Autosorb Automated Gas Sorption System (Autosorb-1, Quantachrome, US) using nitrogen as an analysis gas under 77.35 K. Outgassing temperature was 105.0 °C and outgassing time was 12 h, respectively.

3.2.4 Experiments

3.2.4.1 Batch reactor preparation

Every studies of the dark fermentation were done in batch reactors made of glass laboratory bottles. Each bottle was enveloped with black bag to make a dark condition. In order to avoid oxygen in anaerobic environment, the bottles were purge with nitrogen gas, enclosed with silicone rubber stopper and screw caps to prevent gas releasing. Then, they were placed in a water bath to control a working temperature, shaken with stirring rate 100 rounds per minute (rpm). Each bottle was connected with air bag to collect produced gas. Each condition was studied in triplicate.

3.2.4.2 Phase I - Examination of an optimum condition

Experiment in Phase I was ran with a working volume of 180 mL comprising 11 mL of seed sludge and 28 mL of nutrient solution. Optimal condition was considered environment which provided continuous hydrogen production and obtained highest hydrogen production yield.

Firstly, the anaerobic dark fermentation was operated at various temperature ranges (35±2 and 55±2°C) and various initial pH (4.0, 5.0, 6.0, 7.0, 8.0). The pH was adjusted by 6.0 M H_2SO_4 and 1.0 M KOH. Temperature and pH which provide continuously production of biohydrogen and obtain the highest biohydrogen yield were selected as an optimal initial pH and temperature.

Then various initial iron concentration was observed by operating the anaerobic dark fermentation under optimal initial pH and temperature to identify optimal initial ferrous ion concentration that provide continuous generation of biohydrogen and give highest biohydrogen yield. A studied initial ferrous ion concentration was 200, 400, 600, 800, 1000 mg Fe/L. Iron (II) sulphate ($FeSO_4$) was used as a source of iron.

3.2.4.3 Phase II - Comparison of biological supporting materials

BMs both from plants and animals which offer acid tolerance and the best cell immobilization were further used in biohydrogen production.

Various concentrations of BM (0, 5, 10, 15, 20 % (v/v)) were investigated with working volume was 180 mL, seed sludge 11 mL, and nutrient solution 28 mL in each batch. The fermentation condition was operated under optimal initial pH, temperature and initial iron concentration following from Phase I.

Concentration of BM which resulted in maximum hydrogen yield were selected to further study for microbial population and cell morphology on the BMs by bioinformatics analysis (Meier et al, 1999), 16S rRNA-based fluorescence in-situ hybridization (FISH) method (Chu et al., 2009), and scanning electron microscope (SEM) (Zampieri et al, 2006), respectively.

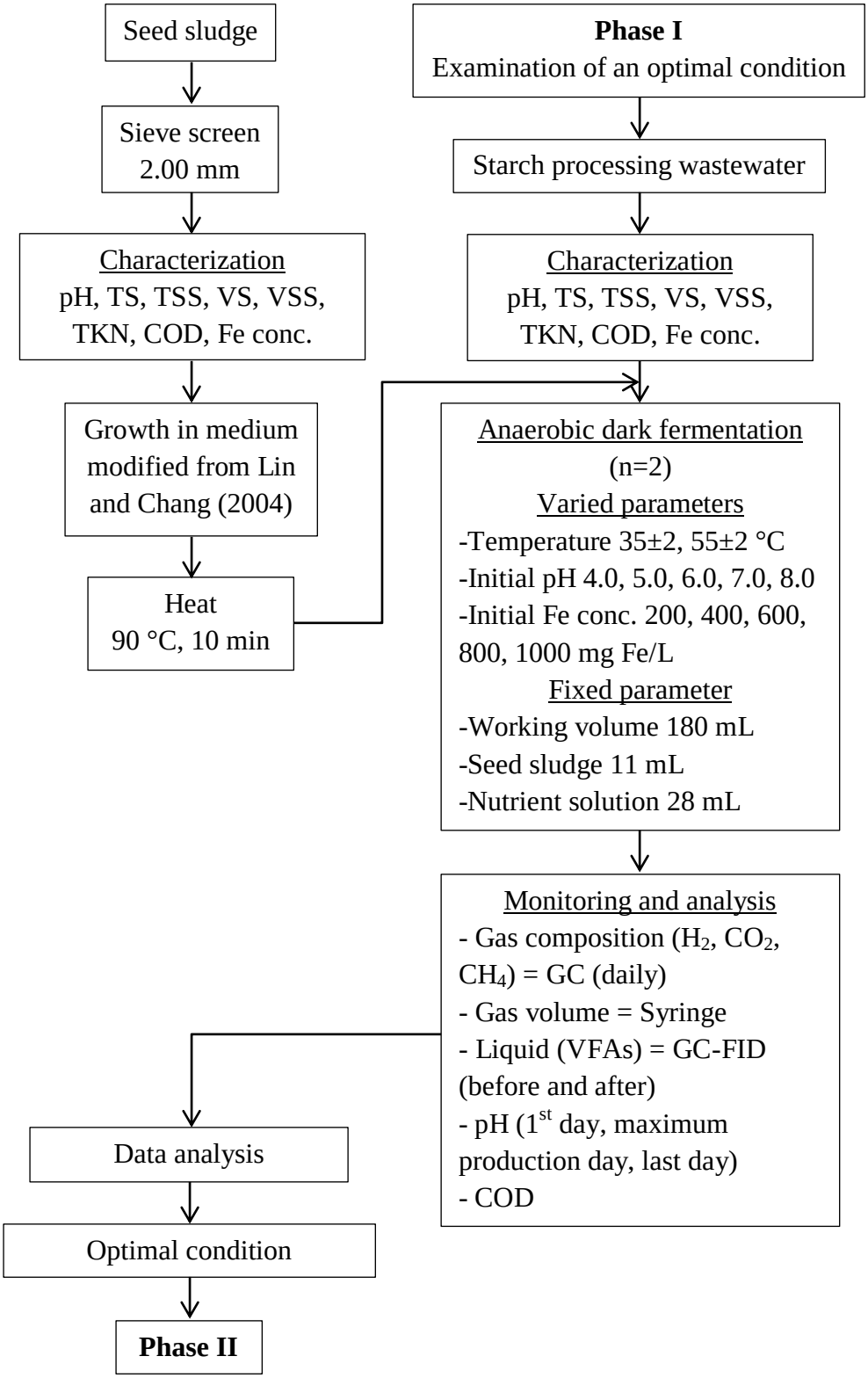

Figure 3.1 Experimental diagram

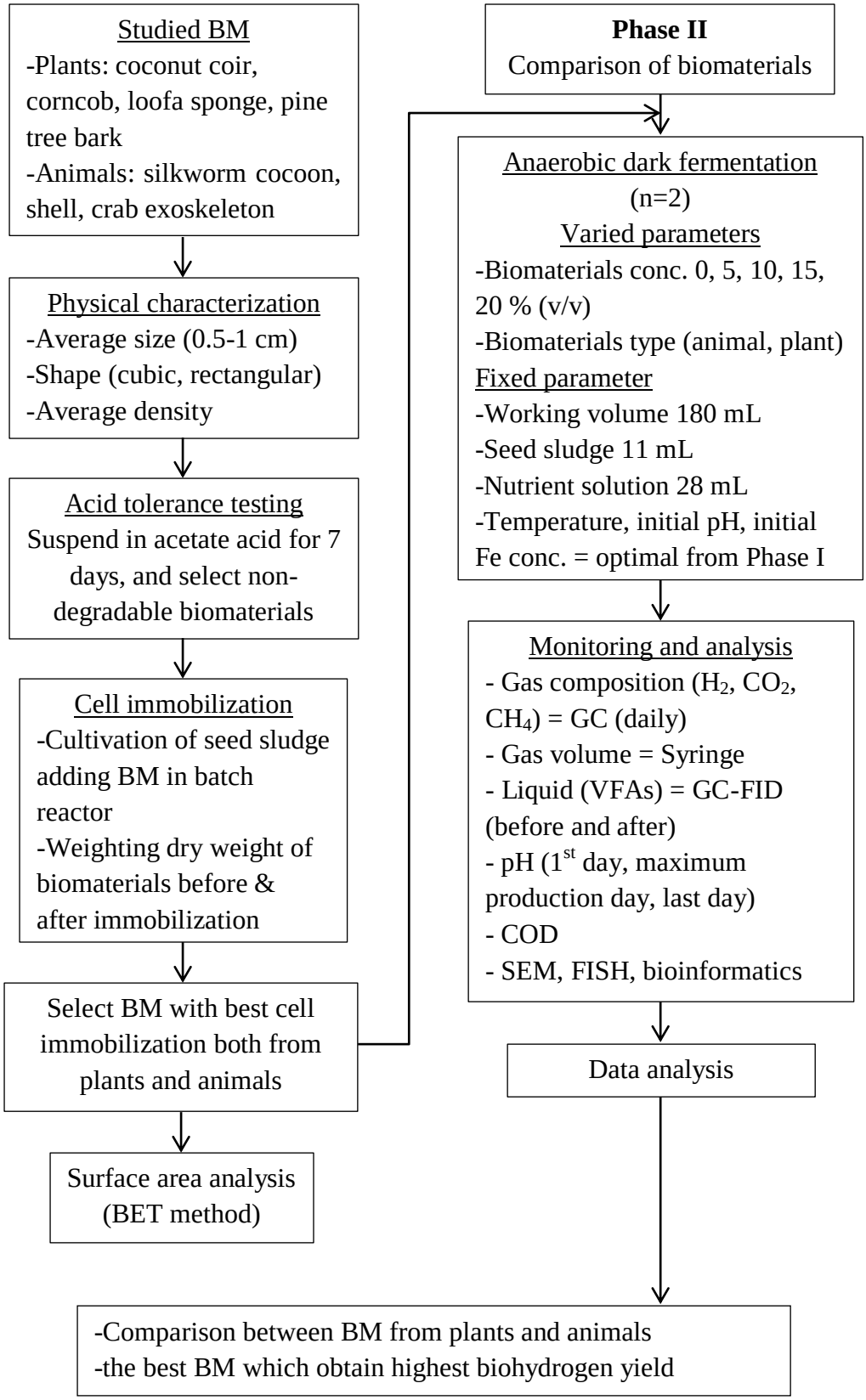

Figure 3.1 Experimental diagram (Cont.)

3.3 Monitoring and analysis

3.3.1 Gas products

Biogas collected in airbag was sampled to observed gas composition and volume. Especially after the fermentation initiation, biogas formation was watched at 2, 4, 8, 24 hours to find lag phase of the fermentation. When lag phase was discovered, biogas was collected every 24 hours.

Composition of produced biogas (H_2, CO_2, and CH_4) were daily monitored and analyzed by using gas chromatography (GC) (Varian STAR 3400, US), equipped with thermal conductivity detector. Chromatography column made of stainless-steel was packed (Alltech Molesieve 5A 80/100 10'x 1/8"). Carrier gases were Argon (Ar) for hydrogen and methane analysis, and helium for CO_2 analysis (Selembo et al., 2009). The temperatures of injector, detector and column were stably operated at 80 °C, 90 °C and 50 °C respectively.

Gas volume was daily measured using combination of 50 and 1000 mL glass syringe (Owen et al., 1979)

3.3.2 Liquid products

pH was monitored using pH strips on the first day, maximum production day, and last day of the fermentation.

Concentration of formed liquid products (acetic, propionic and butyric acids) was observed before and after the fermentation. The products were sampled for 5 mL using syringe, and centrifuged at 10,000 rpm for 2 minutes (min) to separated solid remaining in the sampled solution. Then, the concentration of liquid product was measured by gas chromatography – flame ionization detector (GC-FID), equiped with TG-WAXMS A column. The carrier gas was helium with flowing rate 3 mL/min. The operating temperature of injector and detector were 230 °C and 250 °C, respectively. The temperature of oven was 50 °C for 2 minutes then increasing to 230 °C by rate of 50 °C per minute. After that the temperature was controlled to stay at 230 °C for 3 minutes.

Chemical oxygen demand (COD) of wastewater was investigated after the fermentation according to APHA (2005) to identify performance in terms of wastewater treatment.

3.3.3 Cell morphology and microbial population

After the fermentation, concentration of BM which resulted in maximum hydrogen yield was further investigated cell morphology and microbial population.

The cell morphology and microbial community was determined using SEM (Zampieri et al. 2006), 16S rRNA FISH (Chu et al., 2009), bioinformatics analysis. The biological supporting materials (BMs) were collected from the day that obtained highest production of hydrogen, and were prepared by the following procedures.

3.3.3.1 SEM analysis

The BMs were pre-fixed with 2.5% gluteraldehyde for 24 hours and washed with 0.1 M phosphate buffer (pH 7.2-7.4) 3 times for 10 minutes each. After that, the fixed samples were post-fixed with 1-2% osmium tetraoxide (OsO_4) for 1-2 hours, and washed with distilled water 3 times for 10-30 minutes each. Then they were dehydrated with series of ethanol 20, 40, 60, 80, 95, 95, 95%, respectively, for 10-30 minutes each, and dried with critical point dryer for 30 minutes. The samples were coated with gold and monitored in SEM (JEOL, JSM-5600LV)

3.3.3.2 Specimen preparation for bioinformatics and FISH analysis

Microorganisms on BMs were collected by submerging BMs in 1x Phosphate buffer saline (PBS) and spinning using vortex to detach the cell on them. The detached cells were fixed with 4% formaldehyde in 1X PBS for 24 hours then washed 3 times with 1x PBS for 15 minutes each. After washing, 1:1 PBS:ethanol solution was added and the samples were stored at -20 $^{\circ}$ C for next usage.

3.3.3.3 Bioinformatics analysis

The specimens (from 3.3.3.2) were extracted for their DNA by following a PCR cleanup protocol in GenepHlow Gel/PCR Kit (Geneaid, Taiwan). 100 µL of specimen was transferred to a microcentrifuge tube. Added DF buffer 500 μ L to the tube and mixed by vortexing. Apply the sample mixture into the DF

Column. Centrifuged at 8,000 rpm for 30 seconds then discarded the flow-through. Add 500 µl of Wash Buffer in the DF Column and Centrifuged at 8000 rpm for 30 seconds. The flow-through mixture was discarded. In order to dry the column matrix, centrifuged again for 2 minutes at 14,000 and transferred the dried column into a new microcentrifuge tube. Added 50 µl of Elution Buffer and stand for 2 minutes until the buffer is absorbed by the matrix. The purified DNA was eluded by centrifuge for 2 minutes at 14,000 rpm.

The extracted DNA sample was processed and analyzed at BGI Co., Ltd (Taiwan). Firstly, the DNA sample was tested its quality then all the qualified DNA is used to construct a library. For amplification, the extracted DNA samples were amplified the 16s rRNA gene by Polymerase Chain Reaction (PCR) with the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') (Mizrahi-Man et al., 2013). The jagged ends of DNA fragment would be converted into blunt ends by using T4 DNA polymerase, Klenow Fragment and T4 Polynucleotide Kinase. Then add an 'A' base to each 3' end to make it easier to add adapters. After all that, too short fragments would be removed by Ampure beads. Only the qualified library can be used for sequencing. The bioinformatics analysis will be carried on with sequencing data.

The raw data were filtered to eliminate the adapter pollution and low quality to obtain clean reads. Then paired-end reads with overlap were merged to tags, and the tags were clustered to Operational Taxonomic Unit (OUT) at 97% sequence similarity by scripts of software USEARCH (v7.0.1090) (Edgar, 2013). Taxonomic ranks were assigned to OTU representative sequence using Ribosomal Database Project (RDP) Na,e Bayesian Classifier v.2.2. At last, alpha diversity, beta diversity and the different species screening were analyzed based on OTU and taxonomic ranks.

3.3.3.4 FISH analysis

Fixed samples (5 µL) were spotted on gelatin-coated fourteen-field glass slides and air dried at 37 ° C for 10 min. Then they were dehydrated with 50, 80, and 95% ethanol, respectively, for 3 minutes each, and air dried at room temperature. The probe pB196 (5'- CGG AAG ATT CCC TAC TGC -3') fluorescently labelled with hydrophilic sulfoindocyanine dye CY3 was used to target Firmicute

phylum (Meier et al., 1999). 50 µL of reaction buffer was added on each sample spot. The buffer contained the probe and hybridization buffer at ratio 1:9. The hybridization buffer consisted of 0.9 M NaCl, 0.01% SDS, and 20 mM Tris-Hcl (pH 7.2). A process was operated in a water-saturated equilibration chamber at 46°C for 60 minutes then washed with a washing buffer at 48°C for 60 minutes. The washing buffer contained 0.9 M NaCl, 0.01% SDS, and 20 mM Tris-Hcl (pH 7.2), and was removed by rinsing with distilled water. The cells were counter-stained with 300nM DAPI for 50 µL on each sample spot and leaved in humid chamber for 15 minutes. Washed a trace of the stain with distilled water and protected a de-colorization with anti-fade solution on each sample spot, then covered with a cover slide. Fluorescence image was detected with a confocal laser scanning microscope (Olympus, FLUOVIEW FV10i, US). For counting of bacteria, at least 4 slides for each sample, and at least 10 fields were counted for each slide. The average number of bacteria was taken.

3.4 Data analysis

Hydrogen gas production was calculated from headspace measurements and the total volume of biogas produced for each time interval according from equation presented by Logan (2004).

$$V_{H,i}=V_{H,i-1}+C_{H,i}\left(V_{G,i}-V_{G,i-1}\right)+V_H(C_{H,i}-C_{H,i-1}) \qquad \text{(Eq 3.1)}$$

While $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current (i) and previous (i-1) time interval, respectively, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen gas in the headspace of the bottle measured by means of gas chromatography in the current and next time intervals, and VH is the total volume of headspace in the reactor.

Cumulative hydrogen gas volume was calculated by using modified Gompertz equation (Lay et al., 1999)

$$H(t)= H_{max}\exp\left[-\exp\left\{\frac{R_{max}e}{H_{max}}\left(\lambda-t\right)+1\right\}\right]$$

(Eq 3.2)

While H(t) is cumulative hydrogen production (mL). H_{max} and R_{max} are maximum volume of hydrogen production potential (mL) and maximum hydrogen rate (mL/h), respectively. λ is lag phase (h), t stands for time (h), e equals to 2.71828.

Following statistical tools were used mean (X), standard deviation (SD) and percentage to explain (a) difference of the cumulative hydrogen production (ml) by time (day) at various pH, temperature and iron concentration (b) difference of the VFAs (mg/L) before and after the fermentation at various mentioned conditions (c) COD deduction before and after the fermentation (d) microbial population between BM with produced highest and lowest hydrogen production. Microsoft Excel 2010 supported for statistic calculation.