CHAPTER III
EXPERIMENTAL

3.1 Materials and Equipment

3.1.1 Instruments and Apparatus

1. Anaerobic sequencing batch reactor (ASBR)
2. Gas chromatograph, Perkin-Elmer, AutoSystem GC.
3. Gas chromatograph, Perichrom, PR2100
4. Shimadzu UV-VIS spectrometer 2550, Barawindsor Co., Ltd.
5. COD reactor, HACH.
6. Spectrophotometer, HACH D/R 2000.

3.1.2 Seed Sludge and Supplementary Nutrient for Bacterial Growth

3.1.2.1. Seed sludge

Seed sludge was collected from the wastewater treatment plant of Boonrawd Brewery Co., Ltd., Bangkok, Thailand. The pH, temperature, and total suspended solids (TSS) of the seed sludge were 6.5, 24°C, and 35,622 mg L^{-1}.

Figure 3.1 Seed sludge collected from the wastewater treatment plant of Boonrawd Brewery Co., Ltd.

The seed sludge was heat-treated by boiling for 15 min to inactivate hydrogen-consuming bacteria and to harvest anaerobic spore-forming bacteria (Lay *et al.*, 1999, Hawkes *et al.*, 2002).

3.1.2.2. Feedstock

- D-glucose anhydrous ($C_6H_{12}O_6$), analytical reagent grade, AJAX Finechem, Australia.

3.1.2.3. Supplementary nutrient for bacterial growth

- Ammonium hydrogen carbonate (NH_4HCO_3), analytical reagent grade, AJAX Finechem Pty Ltd, Australia.
- Di-potassium hydrogen orthophosphate (K_2HPO_4), analytical reagent grade, AJAX Finechem, Australia.
- Magnesium chloride ($MgCl_2 \cdot 6H_2O$), analytical reagent grade, AJAX Finechem, Australia.
- Iron (II) sulphate ($FeSO_4 \cdot 7H_2O$), laboratory reagent grade, AJAX Finechem, Australia.
- Copper (II) sulphate ($CuSO_4 \cdot 5H_2O$), analytical reagent grade, AJAX Finechem, Australia.
- Cobalt (II) chloride ($CoCl_2 \cdot 5H_2O$), analytical reagent grade, AJAX Finechem, Australia.
- Sodium hydrogen carbonate ($NaHCO_3$), analytical reagent grade, AJAX Finechem, Australia.
- Sulfuric acid (H_2SO_4) 98%, analytical reagent grade, Lab-scan, Thailand.
- Hydrochloric acid (HCL) 37%, analytical reagent grade, Lab-scan, Thailand.
- Sodium hydroxide (NaOH), analytical reagent grade, Lab-scan, Thailand.
- Phenolphthalein ($C_{20}H_{14}O_4$), analytical reagent grade, Labchem, Australia.

3.1.3 Apparatus of ASBR

3.1.3.1 Time-controlling system

The timers, OMRON model H3CR-F, were used to control the time of each operation steps: (1) feeding, (2) reacting, (3) settling, and (4) decanting.

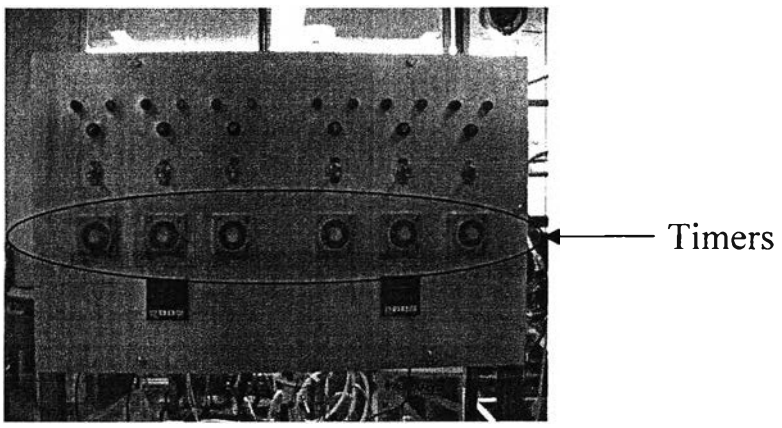


Figure 3.2 Time-controlling system.

3.1.3.2 Temperature-controlling system

This system comprising heater rod, thermocouple, and control box was used to control the system temperature. The system temperature was adjusted using heater to be around 37°C in mesophilic condition. This system was worked only during reacting period.

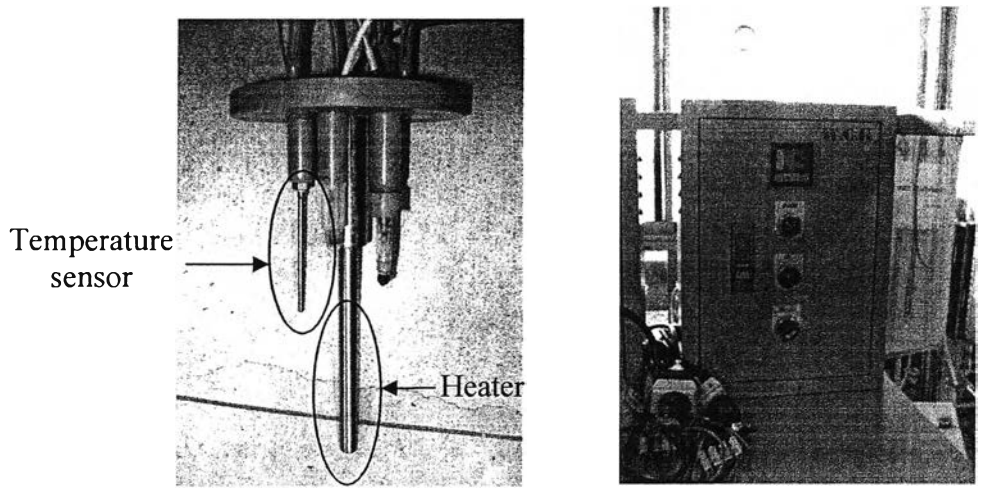


Figure 3.3 Temperature-controlling system installed at a cover of reactor.

3.1.3.3 pH-controlling and mixing systems

This system consisted of pH controller (Extech model 48PH2), pH electrode (Cole-Parmer Double-Junction Electrode), diaphragm pumps, and magnetic stirrer (40×20 mm, egg shape) for mixing. These equipments were used to investigate the effect of pH on biohydrogen production. The pH of the mixed solution was controlled automatically by feeding NaOH (1 M) and H_2SO_4 (1 M) solutions via diaphragm pumps. The liquid was homogeneously mixed using magnetic stirrer at 400 rpm.

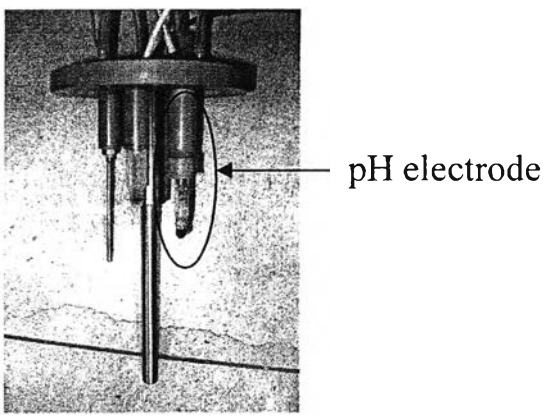


Figure 3.4 pH sensor installed at a cover of reactor.

3.1.3.4 Gas-measuring system

This system was composed of 2 flask filled with 1 M of HCl solution in order to prevent dissolution of the gas (Ueno *et al.*, 1996) and wet gas meter that was used to measure the volume of produced gas at room temperature.

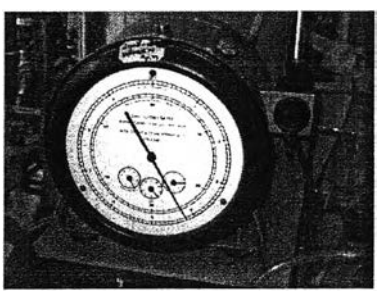


Figure 3.5 Wet gas meter.

3.2 Experimental Procedures

3.2.1 Feed Preparation

The main substrate solution consisted of organic and inorganic supplementary nutrients. For sustainable biohydrogen production, carbohydrates are the preferred organic carbon source for biohydrogen production. A glucose (or its isomer, hexose) is the fermentation substrate that has been mostly used to study in the laboratory because it is easily converted to hydrogen (Hawkes *et al.*, 2002). The influent was prepared by using D-glucose (AJAX Finechem) as a carbon source for wastewater and supplemented with following nutrients (Lin and Chang, 2004). Tap water was used as dilution water.

- NH_4HCO_3 5,240 mg/L
- K_2HPO_4 125 mg/ L
- $MgCl_2 \cdot 6H_2O$ 15 mg/ L
- $FeSO_4 \cdot 7H_2O$ 25 mg/ L
- $NaHCO_3$ 5 mg/ L
- $CoCl_2 \cdot 5H_2O$ 0.125 mg/ L
- $CuSO_4 \cdot 5H_2O$ 6,720 mg/ L

3.2.2 ASBR Operation

Two ASBR reactors were used in order to perform the biohydrogen production experiments. To inhibit the activity of photosynthetic bacteria, the system was operated without light illumination in 5 L PVC reactors. Each of them had an inner diameter of 13 cm and a height of 30 cm. The reactor was operated with working volume of 4 L. The schematic of the ASBR process is shown in Figure 3.6.

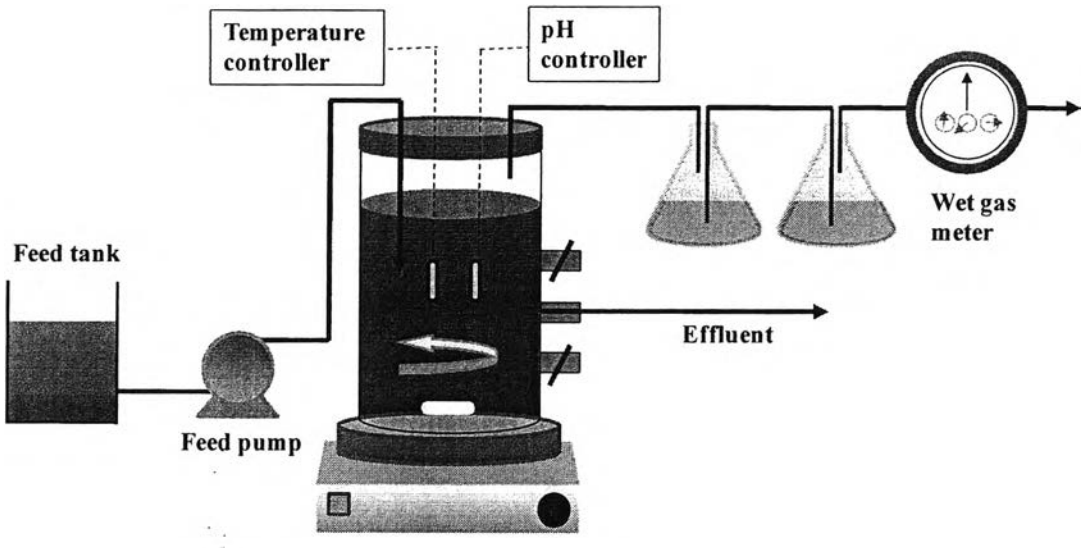


Figure 3.6 Schematic of the studied ASBR process.

ASBR operation consists of four steps: feeding, reacting, settling, and decanting. The operating conditions for the ASBR and time for each step are shown in Table 3.1. In the operation, time for each step was controlled by timers, which allow the feed pump to pump feedstock during feeding period. The short hydraulic retention time (HRT) was operated in order to prevent H_2 consumption by methanogenesis process (Hawkes *et al.*, 2002). Furthermore, the system temperature and pH were controlled using heater and pH-controlling system, respectively. Recirculation pumps were sometimes used for mixing purpose during reaction period.

The experiments were divided into 2 parts. The first part was conducted to examine the effect of COD loading rate varied from 10 to 50 with 10 kg m^{-3} d^{-1} increments under system with pH control at 5.5 and mesophilic temperature. The second part was conducted to investigate the effect of COD:N ratio on biohydrogen production under un-controlled pH and controlled pH at 5.5.

Table 3.1 Operating conditions for the ASBR

Operating Parameter	Value
HRT (h)	24
Influent volume (L/cycle)	1
Temperature (^{o}C)	37
Cycle time (min)	
Feed	20
React	180
Settle	140
Decant	20
Total	360

3.2.3 The Effects of COD Loading Rate under System with pH Control

Selection of a proper pH is crucial to enhance hydrogen production, due to the effects of pH on hydrogen-producing bacteria activity or metabolic pathways (Lay *et al.*, 2000). From the literature review, it was revealed that the optimum pH for production of hydrogen was observed in the pH range of 5 to 6 (Morimoto *et al.*, 2004). For hydrogen production from glucose by a mixed culture, the optimum pH for H_2 production was found at pH 5.5 (Fang *et al.*, 2002). The first batch of experiment was performed at COD loading rates of 10, 20, 30, 40, and 50 kg m^{-3} d^{-1}. The system pH was controlled at 5.5 by NaOH (1 M) and H_2SO_4 (1 M) solutions. The operational temperature was mesophilic condition of 37^{o}C, and each run lasted over 2 weeks to ensure that the system reached the steady state (composition of product gas nearly constant). Volume and compositions of produced gas, COD and pH of the effluent liquid, amount of glucose, and composition and amount of VFA were analyzed. Table 3.2 summarizes the conditions for investigating the effect of COD loading rate. Only those obtained under steady state conditions were reported.

Table 3.2 Conditions for investigating the effect of COD loading rate

Experiment	Condition 1	Condition 2	Condition 3	Condition 4	Condition 5
COD loading rate ($kg\ m^{-3}\ d^{-1}$)	10	20	30	40	40
Glucose ($g\ L^{-1}$)	9.375	18.75	28.125	37.5	46.875
Temperature (°C)	37	37	37	37	37
pH	5.5±0.05	5.5±0.05	5.5±0.05	5.5±0.05	5.5±0.05

3.2.4 The Effect of COD:N Ratio

The appropriate microbial supplementary nutrients as described above were added to a fed waste water in order to stimulate the microflora and enhance its degradation potential. The deficientcy in nitrogen, iron, and phosphorus sources in wastewater limits the bacterial growth and hydrogen production yield (Lay *et al.*, 2005). In this work, the effect of nitrogen concentration was investigated by adjusting the COD:N ratio of feedstock. Nitrogen source in feedstock mainly came from NH_4HCO_3, so the amount of NH_4HCO_3 in feed solution was varied. In case of COD:N ratio of 100:1.4, the amount of NH_4HCO_3 in feedstock was increased from 5,240 mg/dm^3 to 10,480 mg/dm^3 (2 times) and decreased from 5,240 mg/dm^3 to 2,620 mg/dm^3 (0.5 times) for COD:N ratio of 100:3.3. In each condition, the effect of operational pH was studied by operating in both systems without and with pH control. After reaching steady state for each batch of experiment, volume and compositions of produced gas, COD and pH of the effluent liquid, as well as VFA, were analyzed. Table 3.3 summarizes the conditions for investigating the effect of COD:N ratio.

Table 3.3 Conditions for investigating the effect of COD:N ratio

Experiment	Condition 1	Condition 2	Condition 3	Condition 4
COD loading rate ($kg\ m^{-3}\ d^{-1}$)	40	40	40	40
COD:N	100 : 1.4	100 : 1.4	100 : 3.3	100 : 3.3
Glucose ($g\ L^{-1}$)	37.5	37.5	37.5	37.5
NH_4HCO_3 ($g\ L^{-1}$)	10.48	10.48	41.92	41.92
Temperature (°C)	37	37	37	37
pH	Not controlled	5.5±0.05	Not controlled	5.5±0.05

3.3 Analytical Techniques

3.3.1 Steady-State Analysis

The steady-state condition of each experimental run was achieved when the properties of liquid product and produced gas, such as percentage of hydrogen, COD, and VFA concentration, were nearly constant (less than 15% variations).

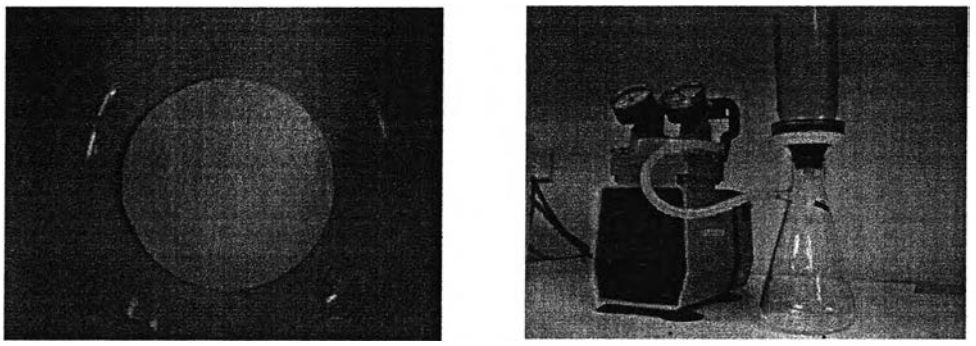
3.3.2 Total Suspended Solids (TSS) Analysis

3.3.2.1 Apparatus

- Desiccators
- Magnetic filter
- Drying oven
- Suction flask
- Glass-fiber filter disk (Pall-61631 A/E, 47 mm, 1 μm)

3.3.2.2 Procedure

A. Preparation of glass-fiber filter disk:



(a) (b)

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

- The disk with wrinkled side up was inserted in filtration apparatus, after that it was applied to vacuum and washed with three successive 20 cm^3 of distilled water.

- The glass-fiber filter disk was dried in an oven at 105°C for 1 h, left to be cooled in desiccators to balance temperature, and weighed.

B. Selection of filter and sample sizes:

- The sample volume was chosen to yield between 10 and 200 mg dried residue.

- If more than 10 min were required to complete filtration, filter size was increased or sample volume was decreased.

C. Sample analysis:

- The filtering apparatus and filter were prepared.

- The filter was wet with a small volume of distilled water to stick it to the apparatus.

- A sample was homogeneously mixed before test.

- A sample was pipetted onto the seated glass-fiber filter.

- The filter was washed with three successive 10 cm^3 of distilled water, and suction was continued for about 3 min after complete filtration.

- The filter was carefully removed from filtration apparatus and dried at least 1 h at 103 to 105°C in an oven, cooled in desiccators to balance temperature, and weighed.

- The cycle was repeated until the weight of sample nearly constant (less than 4% difference).

3.3.2.3 Calculation

$$\frac{\text{mg total suspended solid}}{\text{L}} = \frac{(\text{A} - \text{B}) \times 100}{\text{sample volume, } cm^3} \quad (3.1)$$

A = Weight of filter + dried residue [mg]

B = Weight of filter [mg]

3.3.3 Volatile Suspended Solids (VSS) Analysis

3.3.3.1 Apparatus

Apparatus listed in TSS including furnace was required.

3.3.3.2 Procedure

- The residue produced by TSS method was ignited in a furnace at a temperature of 500 ± 50°C.

- A furnace was heated up to 500°C for 1 h after inserting sample.

- The filter disk was left to partially cool in air until most of the heat was dissipated.

- The disk was transferred to desiccators, and weighed as soon as it was cooled to balance temperature.

3.3.3.3 Calculation

$$\frac{\text{mg total suspended solid}}{\text{L}} = \frac{(\text{A} - \text{B}) \times 100}{\text{sample volume, } cm^3} \quad (3.2)$$

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

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

3.3.4 COD Analysis (Closed Reflux, Colorimetric Method)

3.3.4.1 Apparatus

- Digestion vial (HACH) 16× 100 mm
- COD reactor (HACH)
- Spectrophotometer (HACH DR/2000).

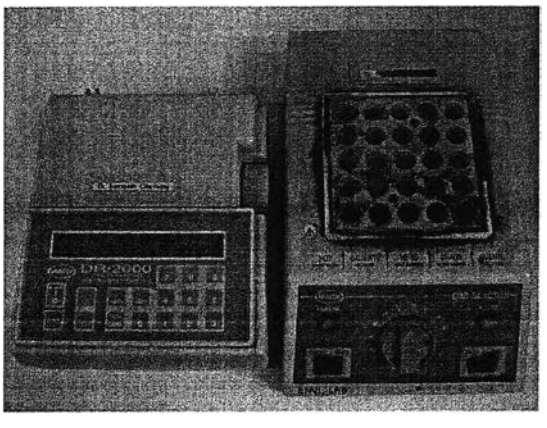


Figure 3.8 COD reactor and spectrophotometer.

3.3.4.2 Reagents

- *Digestion solution.* The following reagents were added into 500 ml distilled water: 10.216 g $K_2Cr_2O_7$ (primary standard grade) previously dried at 103°C for 2 h, 167 ml 98% H_2SO_4, and 33.3 g $HgSO_4$. 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 the ratio of 5.5 g Ag_2SO_4/kg H_2SO_4. The mixture was left to stand for 1 to 2 days to completely dissolve Ag_2SO_4.

3.3.4.3 Procedure

- Sample (dilute 100 times) of 2.5 ml was added to digestion vial.

- Digestion reagent of 1.5 ml was added to the vial. Afterwards, sulfuric acid reagent was slowly dropped for 3.5 ml into the vial.

- The vial was inverted several times to homogeneously mix the contents, and the vial was then placed in the preheated COD reactor.

- The vial was heated for 2 h, and then left for about 20 min to be cooled.

- The vial was placed into spectrophotometer for reading COD value at 600 nm by using method 440.

3.3.5 Total Nitrogen Analysis

3.3.5.1 Apparatus

- COD reactor (HACH)
- Spectrophotometer (HACH DR/2000)

3.3.5.2 Reagents

- HR total nitrogen hydroxide digestion vials
- Total nitrogen persulfate reagent powder pillows
- Total nitrogen reagent A, bisulfite powder pillows
- Total nitrogen reagent B, indicator powder pillows
- Total nitrogen reagent C vials, acid solution

3.3.5.3 Procedure

- The COD reactor was turned on and heated for 5 min.
- Reagent blank was prepared using a funnel by adding one total nitrogen persulfate reagent powder pillow to one HR total nitrogen hydroxide digestion vial.
- Sample was prepared as same as above method.
- 0.5 ml of organic-free water was added to the vial of reagent blank, and 0.5 ml of sample was added to the vial of sample. Then, the vial was capped and shaken vigorously for about 30 s.
- The vial was heated in COD reactor for 30 min. After that, the hot vials were removed from the reactor and allowed to cool to room temperature. (Important: remove the vials from COD reactor after exactly 30 min.)
- One total nitrogen reagent A powder pillow was added to the vial containing the digested blank and sample. The vial was capped and shaken for 15 s, then waiting for reaction period (3 min).

- One total nitrogen reagent B powder pillow was added to the vial containing the digested blank and sample. The vial was capped and shaken for 15 s, then waiting for reaction period (2 min).

- 2 ml of digested, treated sample, and reagent blank were added in each total nitrogen reagent C vial. The vial was capped and inverted slowly 10 times to mix, then waiting for reaction period (5 min).

- The vial was placed into spectrophotometer for reading COD value at 410 nm by using method 395.

3.3.6 Total Phosphorus Analysis

3.3.6.1 Apparatus

- COD reactor (HACH)
- Spectrophotometer (HACH DR/2000)

3.3.6.1 Reagents

- Total phosphorus test 'n tube vials
- Potassium persulfate powder pillows
- Sodium hydroxide solution, 1.54 N
- Molybdovanadate reagent
- Deionized water

3.3.6.2 Procedure

- The COD reactor was turned on and heated for 5 min.

- 5 ml of deionized water and 5 ml of sample were added to a total phosphorus test 'n tube vial (the blank) and total phosphorus test 'n tube vial (the sample), respectively.

- One potassium persulfate powder pillow for phosphonate was added to each vial using a funnel. The vial were tightly capped and shaken for dissolution.

- The vial was heated in COD reactor for 30 min. After that, the hot vials were removed from the reactor and allowed to cool to room temperature.

- 2 ml of 1.54 N sodium hydroxide was added to each vial. The vials were capped and inverted for mixing.

- A polyethylene dropper was used to add 0.5 ml of molybdovanadate reagent to the vials. The vials were capped and inverted for mixing, then waiting for reaction period (7 min).

- The vial was placed into spectrophotometer for reading COD value at 420 nm by using method 543.

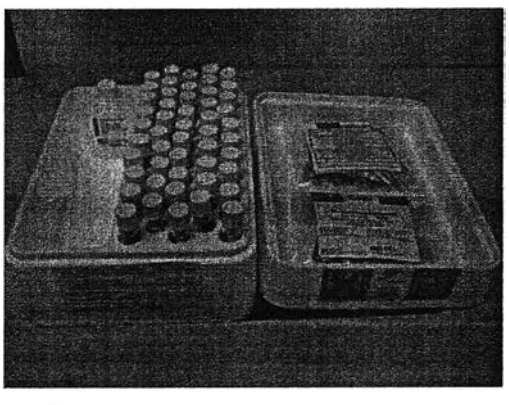


Figure 3.9 Total nitrogen and total phosphorus test kit.

3.3.7 Analysis of Produced Gases

3.3.7.1 Components of produced gases

The gas composition was 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.

3.3.7.2 Volume of produced gases

The volume of produced gas was measured at room temperature by the wet gas meter.

3.3.8 Component of Produced Liquids

The liquid composition was 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 splitless mode with helium at a pressure of 22 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 was 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 temperature of both injector and detector was 250°C.

3.3.9 The Amount of VFA

The amount of VFA was determined by distillation method. This technique recovers acids containing up to six carbon atoms and reports the results in term of acetic acid (Greenberg *et al.,* 1992).

3.3.9.1 Apparatus

- Distillation flask
- Condenser
- Heater

3.3.9.2 Reagents

- Sulfuric acid
- Standard sodium hydroxide solution, 0.5 M
- Phenofthalien indicator solution
- Acetic acid stock solution, 2,000 mg L^{-1}

3.3.9.3 Procedure

A. Recovery factor

The recovery factor (f) was determined for a given apparatus by taking 150 ml of acetic acid stock solution to distillation apparatus. Then, the sample was distilled. Finally, the recovery factor is calculated.

$$f = \frac{a}{b} \tag{3.3}$$

where

a = volatile acid concentration recovered in distillate [mg L^{-1}]

b = volatile acid concentration in standard solution used [mg L^{-1}]

B. Sample analysis

- 3 ml of H_2SO_4 was added to 150 ml of effluent solution in a beaker.

- The solution was mixed homogeneously.
- The mixed solution was placed to the distillation apparatus.
- The solution was continuously distilled.
- The first 5 ml of distillate was discarded.
- The 80 ml of distillate was collected.
- The 20 ml of distillate was titrated with 0.5 M NaOH using phenolpthalein as an indicator.

3.3.9.4 Calculation

$$\frac{\text{mg volatile acid as acetic acid}}{\text{L}} = \frac{\text{mL NaOH} \times \text{N} \times 60000}{\text{ml sample} \times \text{f}} \qquad (3.4)$$

where

N =Normality of NaOH solution

f = recovery factor

3.3.10 Glucose Quantification

3.3.10.1 Components

- Glucose (HK) Assay Reagent: the vial content was reconstituted with 20 ml of water before use.

3.3.10.2 Apparatus

- UV-VIS spectrometer (2550, Shimadzu)
- Quartz cuvette
- Pipettes capable of accurately dispensing 10 µl to 1 ml.

3.3.10.3 Procedure

A. Sample preparation:

- The sample was diluted with deionized water to 0.05-5 mg of glucose/ml (10x dilution for product).

B. Determination:

- The following solutions were prepared, as shown in Table 3.4, into the appropriately marked test tubes.

Table 3.4 Sample preparation for glucose quantification

Tube	Glucose assay reagent (ml)	Sample volume (ml)	Volume of deionized water (ml)
Sample Blank	-	2.0	1.0
Reagent Blank	1.0	-	2.0
Test	1.0	2.0	-

- The tubes were shaken and incubated for 15 min at room temperature, and then cuvette was filled with each sample for measuring absorbance.

- The absorbance at 340 nm was measured using deionized water as the reference.

3.3.8.4 Calculation

$$\frac{\text{mg glucose}}{\text{ml}} = \frac{(\Delta A)(TV)(F)(0.029)}{(SV)} \qquad (3.5)$$

$A_{\text{Total Blank}} = A_{\text{Sample Blank}} + A_{\text{Reagent Blank}}$

$\Delta A = A_{\text{Test}} - A_{\text{Total Blank}}$

TV = Total assay volume (ml)

SV = Sample volume (ml)

F = Dilution factor from sample preparation