The seed sludge was acclimated with various amounts of glucose depending on each operating condition.

3.2.2 ASBR Operation

ASBR operation was carried out through an automation system comprising four steps: feed, react, settle, and decant. It was controlled by timer for each step. Feed pump was controlled during feed period, and liquid level of 4 L was controlled using level control system. Furthermore, the system temperature was controlled using heater, and recirculation pump was used for mixing purpose during reaction period. Two series of experiments were consecutively conducted in 4 L PVC reactors. Series 1 was conducted to examine the effect of COD loading rate from 10 to 40 with 10 kg m^{-3} d^{-1} increments. Experiments of series 2 investigated the maximum COD loading rate at optimum pH from 40 to 50 with 10 kg m^{-3} d^{-1}. Table 3.1 summarizes the operating conditions for the ASBR.

Table 3.1 Operating conditions for the ASBR

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

3.2.3 The Effects of COD Loading Rate

The investigated operating COD loading rate were 10, 20, 30, and 40 kg m^{-3} d^{-1}. When the system reached the steady state for each operating condition, COD and pH of the influent, volume and compositions of produced gas, as well as VFA, were analyzed. T able 3.2 summarizes the conditions for investigating the effect of COD loading rate. After completing the COD loading rate experiments, the

optimum pH value was obtained. COD loading rate was calculated by following relationship:

$$\text{COD loading} = \frac{(\text{Feed COD}) \times (\text{Feed flowrate})}{\text{Working volume}} \qquad (4)$$

Table 3.2 Conditions for investigating the effect of COD loading rate

Experiment	Condition 1	Condition 2	Condition 3	Condition 4
COD loading rate (kg m^{-3} d^{-1})	10	20	30	40
Glucose (mg L^{-1})	9.375	18.75	28.125	37.5
Temperature (°C)	37	37	37	37
pH	Not controlled	Not controlled	Not controlled	Not controlled

3.2.4 The Maximum COD Loading at the Optimum pH

From the literature review, it was shown that the optimum pH for production of hydrogen was observed in the pH range of 5 to 6 (Morimoto *et al.*, 2004). The effect of pH on the continuous production of hydrogen by mixed culture was also investigated. The results showed the hydrogen yield reached the optimum at pH 5.5 (Fang *et al.*, 2002). The literature revealed that the optimum pH strongly affected the hydrogen production. Therefore, the optimum pH obtained in the previous part was used to study the effect of COD loading rate in this part. After obtaining the optimum pH, the pH of system was controlled using pH control system to maintain at the obtained value. COD loading rate was varied in order to find the maximum value that could be operated using the ASBR, and also, volume and compositions of produced gas, including VFA, were analyzed. Table 3.3 summarizes conditions for investigating the maximum COD loading rate at the optimum pH

Table 3.3 Conditions for investigating the maximum COD loading at the optimum pH

Experiment	Condition 1	Condition 2
COD loading rate ($kg\ m^{-3}\ d^{-1}$)	40	50
Glucose ($mg\ L^{-1}$)	37.5	46.875
Temperature (°C)	37	37
pH	5.5±0.05	5.5±0.05

3.3 Monitoring and Analysis

3.3.1 Steady-State Analysis

Steady-state conditions were considered to be established when the properties of product or produced gas, such as COD, VFA concentration, percent of hydrogen etc., were relatively unchanged (less than 15% variations). In each experiment, the reactor was operated for a period of around 7-14 days until reaching steady-state conditions.

3.3.2 COD Analysis (Closed Reflux, Colorimetric Method)

3.3.2.1 Apparatus

- Digestion vessel 16×100 mm
- COD reactor
- Spectrophotometer for reading COD value at 600 nm.

3.3.2.2 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 1000 ml.

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

3.3.2.3 Procedure

- Sample (dilute 100 times) of 2.5 ml was added to digestion vessel.
- Digestion reagent of 1.5 ml was added to the vessel. Afterwards, sulfuric acid was slowly dropped for 3.5 ml into the vessel.
- The vessel was gently inverted several times to homogeneously mix the contents, and the vessel was then placed in the preheated COD reactor.
- The vessel was heated for 2 h, and then left for about 20 min to be cooled.
- The vessel was placed into spectrophotometer for reading COD value at 600 nm.

3.3.3 Total Suspended Solids (TSS) Analysis

3.3.3.1 Apparatus

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

3.3.3.2 Procedure

A. Preparation of glass-fiber filter disk:

- The disk with wrinkle side up was inserted in filtration apparatus.
- The disk was applied to vacuum and washed with three successive 20 ml of distilled water.
- The glass-fiber filter disk was dried in an oven at 105°C for 1 h.
- The disk was 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 are required to complete filtration, increase filter size or decrease sample volume.

C. Sample analysis:

- The filtering apparatus and filter was assembled.

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

- A measured volume was pipetted onto the seated glass-fiber filter.

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

- The filter was carefully removed from filtration apparatus.

- The filter was 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.

3.3.3.3 Calculation

$$\frac{\text{mg total suspended solid}}{\text{L}} = \frac{(\text{A} - \text{B}) \times 1000}{\text{sample volume, ml}} \qquad (5)$$

A = Weight of filter + dried residue [mg]

B = Weight of filter [mg]

3.3.4 Volatile Suspended Solids (VSS) Analysis

3.3.4.1 Apparatus

Apparatus listed in TSS is required.

3.3.4.2 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 temperature after inserting sample.

- Usually, 15 to 20 min ignition is required for 200 mg residue.

- 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.

- The cycle was repeated.

3.3.4.3 Calculation

$$\frac{\text{mg valtile solid}}{\text{L}} = \frac{(\text{A} - \text{B}) \times 1000}{\text{sample volume, ml}} \qquad (6)$$

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

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

3.3.5 Analysis of Produced Gases

3.3.5.1 Components of Produced Gases

The gas composition was determined by a gas chromatograph (Model 5890 II, Hewlett Packard) using thermal conductivity detector (TCD) and a packed column (carboxene 1000) with helium as carrier gas.

3.3.5.2 Volume of Produced Gases

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

3.3.6 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).

3.3.6.1 Apparatus

- Distillation flask
- Condenser
- Heater

3.3.6.2 Reagents

- Sulfuric acid
- Standard sodium hydroxide solution, 0.5N

- Phenofthalien indicator solution
- Acetic acid stock solution, 2,000 mg L^{-1}

3.3.6.3 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 was calculated.

$$f = \frac{a}{b} \qquad (7)$$

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 0.5M NaOH using phenolpthalein as an indicator.

3.3.6.4 Calculation

$$\frac{\text{mg valitile acids as acetic acid}}{\text{L}} = \frac{\text{mL NaOH x N x 60,000}}{\text{mL sample x f}} \qquad (8)$$

where

N =Normality of NaOH solution

f = recovery factor

3.3.7 Glucose Quantification (Glucose (HK) Assay Kit)

3.3.7.1 Components

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

- Glucose Standard Solution

3.3.7.2 Apparatus

- Spectrophotometer suitable for measuring absorbance at 340 nm.

- Pipettes capable of accurately dispensing 10 µl to 1 ml.

3.3.7.3 Procedure

A. Sample preparation:

- The sample was diluted with deionized water to 0.05-5 mg of glucose/ml.

B. Determination:

- A volume of solution was pipetted corresponding to 0.5-50 µg of glucose. The assay was repeated, and the sample volume was varied if necessary, to give a ΔA_{340} between 0.03 and 1.6.

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

Table 3.4 Samples preparation for glucose quantification

Tube	Glucose assay reagent (ml)	Sample volume (ml)	Volume of deionized water (ml)
Sample Blank	-	2	1.0
Reagent Blank	1.0	-	2
Test	1.0	2	-

- The tubes were shacked and incubated for 15 min at room temperature.

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

3.3.7.4 Calculation

$$\frac{\text{mg glucose}}{\text{ml}} = \frac{(\Delta A)(TV)(F)(0.029)}{(SV)} \qquad (9)$$

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

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

TV = Total assay volume (ml)

SV = Sample volume (ml)

F = Dilution factor from sample preparation

3.4 ASBR Setup

In this research, ASBR was constructed in order to perform the biohydrogen production experiments. To inhibit the activity of photosynthetic bacteria, the system was operated without light illumination. The 5 L PVC reactors were conducted with working volume of 4 L. Each of them had an inner diameter of 13 cm and a height of 30 cm. The complete schematic for the ASBR process is illustrated in Figure 3.3, and the complete ASBR system is shown in Figure 3.4.

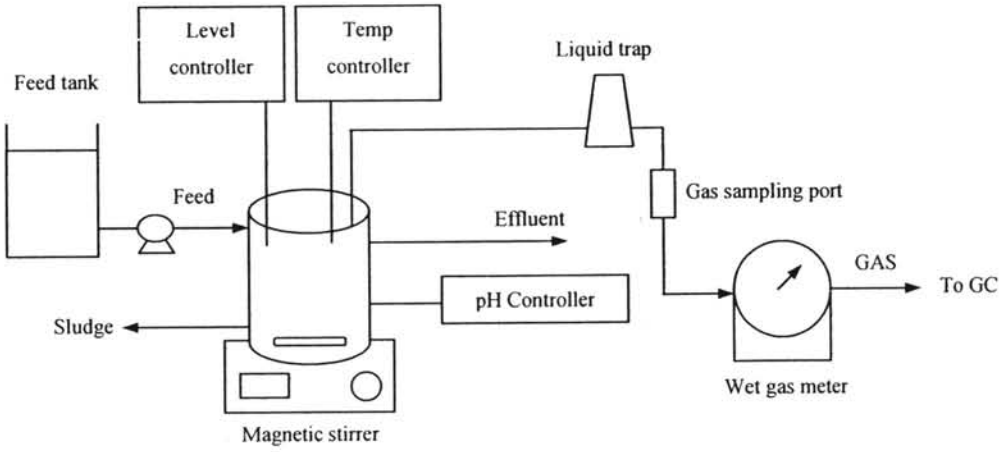

Figure 3.3 Schematic of the studied ASBR process.

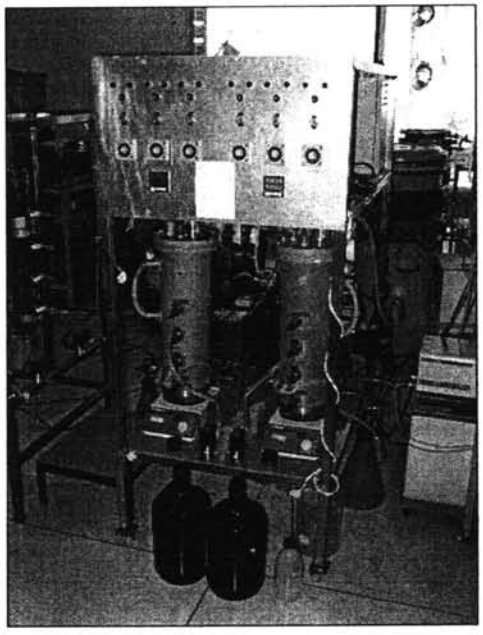

Figure 3.4 The complete ASBR system.

3.4.1 Time Control System

The operation of ASBR consists of four steps: (1) feed, (2) react, (3) settle, and (4) decant. For each step, it was controlled using the timers, OMRON model H3CR-F. The complete time control system is shown in Figure 3.5.

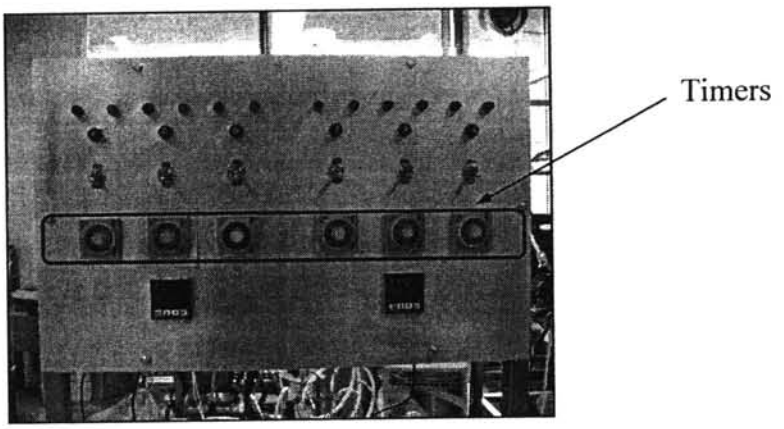

Figure 3.5 Timers, OMRON model H3CR-F, installed on control board.

3.4.2 Level Control System

The reactors were designed for working volume of about 4 L. During the feed period of ASBR operation, the level of liquid volume was fixed at around 4 L using level control system. The system comprises of level sensor and level control box that have connected to timer controlling the feed step. While timer can control the feed, level controller was used to ensure that liquid level would not exceed the required level. The level sensor is shown in Figure 3.6.

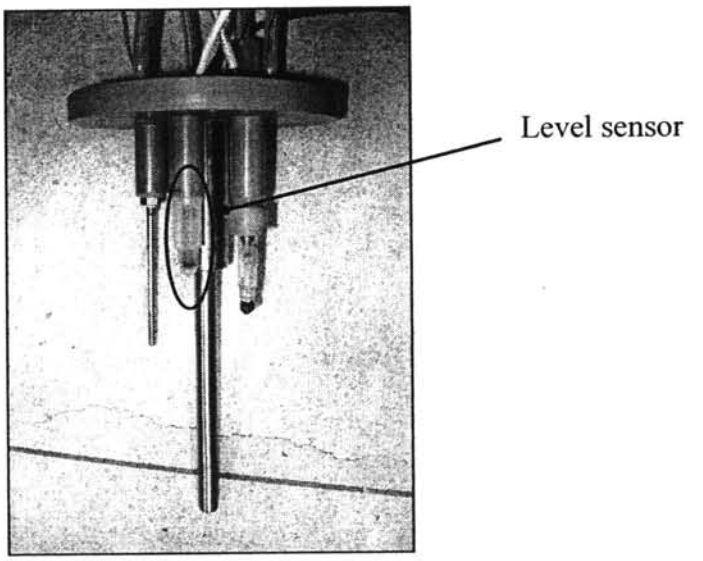

Figure 3.6 The level sensor installed at a cover of reactor.

3.4.3 Temperature Control System

This study was conducted to investigate the feasibility of biohydrogen production at mesophilic temperature range. The temperature control system consists of thermocouple type K used to measure system temperature and compare with set point at temperature controller box, as shown in Figure 3.7. The system temperature was adjusted using heater to be around 37°C. This system was used only during reaction period.

Figure 3.7 The temperature control box.

3.4.4 pH Control System

The pH control system consists of pH controller (Extech model 48PH2), as shown in Figure 3.8, pH electrode, as shown in Figure 3.9, pH pump control system, and mixer. The pH of the mixed solution was controlled automatically by feeding NaOH (1 M) and HCl (1 M) solutions via respective dosing pumps.

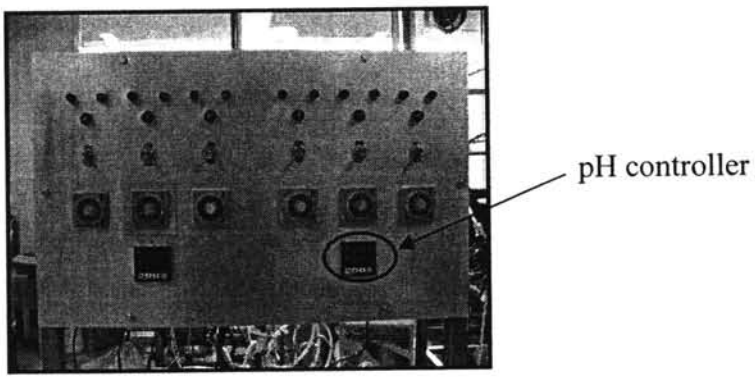

Figure 3.8 pH controllers (Extech model 48PH2).

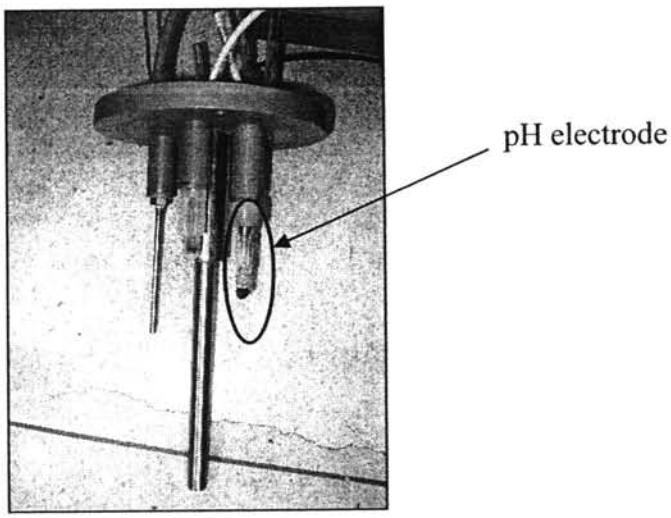

Figure 3.9 pH electrode.

3.4.5 Mixing System

In the reaction step of the cycle operation, the mixture initially contained in the reactor was completely mixed using magnetic stirrer. The fermentor was stirred at a constant stirring rate of 400 rpm to ensure homogeneous mixing and to facilitate rapid diffusion of hydrogen. However, the effect of mixing was not studied. The mixing system is shown in Figure 3.10.

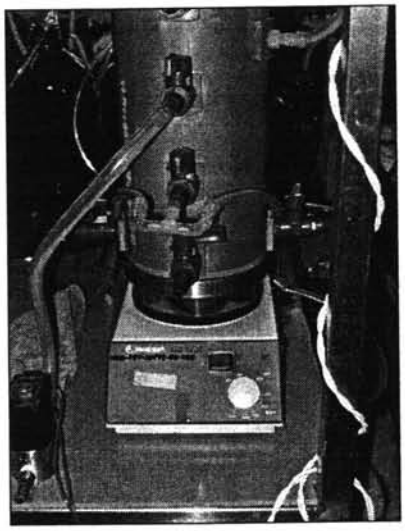

Figure 3.10 Mixing system (Magnetic stirrer).

3.4.6 Gas-Measuring System

The volume of evolved gas was measured at room temperature by the wet gas meter, as shown in Figure 3.11.

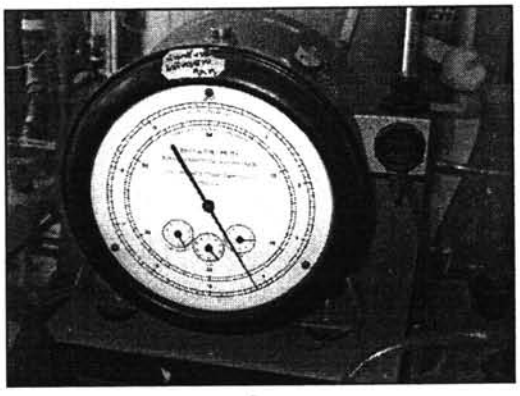

Figure 3.11 Gas-measuring system (wet gas meter).