CHAPTER III

METHODOLOGY

3.1 Raw Material

The details of materials used in the experiments can be detailed as the following:

3.1.1 Fermentation substrates

Spent malt and waste yeast were provided with courtesy of Boonrawd Brewery Co., Ltd., Bangkok, Thailand. The details of substrate preparations are presented below:

3.1.2 Preparation of fermentation substrate

The spent malt obtained from the brewery as wet spent grain with the moisture content of 80%. It was brought to determine the total available extractable materials on dry weight basis. By using a mashing procedure, the ground spent grain sample was mashed in a water bath with stable at the temperature of 70 °C for 60 minutes. The % soluble material extracted was measured as glucose of 0.7% (w/v) on average basis, which is in equivalent to 7,000 mg/l of glucose. Therefore, in this study synthetic substrate contained glucose at the fixed concentration of 7,000 mg/l as it found in the spent malt extract throughout the entire experiments.

The spent yeast obtained consisting of thick and viscous cream-like slurry with 15 – 20% total solid. The yeast slurry was boiled for 20 minutes to terminate enzyme activity. After allowing to cool, the yeast slurry was centrifuged at 3,000 rpm for 10 minutes and the cream yeast was washed 3 times by water with the ratio of 1 : 3 (cream yeast : water). The suspension was then passed through a 150-mesh sieve. A washing step was necessary to remove ethanol and other inhibiting solvents from the brewery process prior to the experimental use. The cream yeast had moisture contents of about 80% and contained 16.70% of total solids of which the compositions based on dry weight include: crude protein 36.82%, ash 6.86% and fat 0.53% (Suphantharika, 1997). The other chemical characteristics were also analyzed of which the compositions include: TKN = 61,600 mg/l, TPO_4-P = 17,033 mg/l, Fe =

1.16 mg/l and S = 48 mg/l, respectively. The substrate was then stored at 0°C until use.

3.2 Seed sludge

The anaerobic mixed culture used in this study was taken from a full-scale UASB reactor treating brewery wastewater at Boonrawd Brewery Co., Ltd. The seed sludge was first washed 3 times with tap water and, sieved through a 150 mesh screen and then boiled for 30 minutes at 80 -85 °C to inactivate the methanogen and enrich hydrogen-producing microorganisms. This stock culture was maintained in a 30L CSTR (Figure D-1, APPENDIX D) fed with glucose at 30° C in fed-batch mode. The pH was controlled in the range of 4.7-5.5 by addition of NaOH+$NaHCO_3$. Methane gas was checked twice a month by using Gas Chromatography (GC-TCD) device.

3.3 Experimental apparatus and procedure

3.3.1 Feasibility study on heat treatment protocol

Two kinds of seed sludge taken from different sources: 1) from the UASB reactor of Boonrawd Brewery Co., Ltd. and 2) from the UASB reactor of Pepsi Co., Ltd. were tested as heated inoculum. The inoculums were heat according to Table 3-1. The test was undertaken by using 120 ml - serum bottle as a digester and 10 g/L glucose as substrate plus defined medium (modified from Mizuno *et al.*, 2000): NH_4Cl, 2600 mg; K_2HPO_4, 250 mg; $MgCl_2.H_2O$, 125 mg; $FeSO_4.7H_2O$, 5 mg; $CoCl_2.6H_2O$, 2.5 mg; $MnCl_2.4H_2O$, 2.5 mg; KI, 2.5 mg; $Na_2MoO_4.2H_2O$, 0.5 mg; H_3BO_4, 0.5 mg; $NiCl_2.6H_2O$, 0.5 mg; $ZnCl_2$, 0.5 mg, under mesophilic condition (~30°C). The medium pH was initially adjusted to 7.0 with buffer solution of NaOH + $NaHCO_3$. Gas was collected by water replacement technique (Figure 3-2) and analyzed by GC.

The heat treatment protocol used in this comparison tests were as follow

Table 3-1 Heat treatment protocol used in the comparison test

Inoculum	Heat treatment Protocal
1) Anaerobic digested sludge from	100 °C / 15, 20,30 min
Boonrawd Brewery Co., Ltd.	80 °C / 15, 20,30 min
2) Anaerobic digested sludge from	100 °C / 15, 20,30 min
Pepsi Co., Ltd.	80 °C / 15, 20,30 min

The best condition obtained from previous experiment was used for comparison the sludge from Boonrawd Brewery Co., Ltd. with the sludge taken from the 30L CSTR in the laboratory of Bauhaus- University, Weimar, Germany (the methodology and experimental result were attached as the research report in APPENDIX A).

3.3.2 Batch Experiments

There were 2 phases of batch experiments in this study. In phase I the experiment was conducted using serum bottle technique, whereas in phase II the experiment was carried out in a 1 l fermentor. Before reaching phase I, a preliminary test was conducted to initially examine the effects of glucose and brewery waste yeast on H_2 fermentation. Thus, combined factors of four different glucose concentrations (ranging 0 to 1500 mg), four different levels of waste yeast (0 to 2000 mg of COD), and four seed sludge concentrations (500 to 2,000 mg VSS) were investigated resulting a total of 18 experiment to observe H_2 production (see Table 4-1). The experiment in phase I was then conducted to identify optimal composition of glucose, waste yeast and seed sludge which give the highest H_2 production rate (R_{max}) by using serum bottle technique which modified by Owen *et al.*, 1979. (see Figure 3-1). The experiments, including those on preliminary test, are performed in 120 ml serum bottle, with effective volume of 100 ml, as a digester. Serum bottles are cleaned with

10% (v/v) HCl and dried before the experiments. The digesters with 5 different combined compositions of glucose, waste yeast and seed sludge were simultaneously operated under room temperature (mesophilic condition) of about 30°C (see Figure B-1, Appendix B). In this study, nutrient and mineral salt were not supplemented. This based on the hypothesis that waste brewer's yeast contains high nutritional values which is essential for H_2 forming microoganisms. The total volume of seed and substrate was 100 ml in 120 serum bottle. The volume of biogas was determined using volume displacement method as shown in Figure 3-2. Each experimental set-up was non-sterilized and carried out in triplicate.

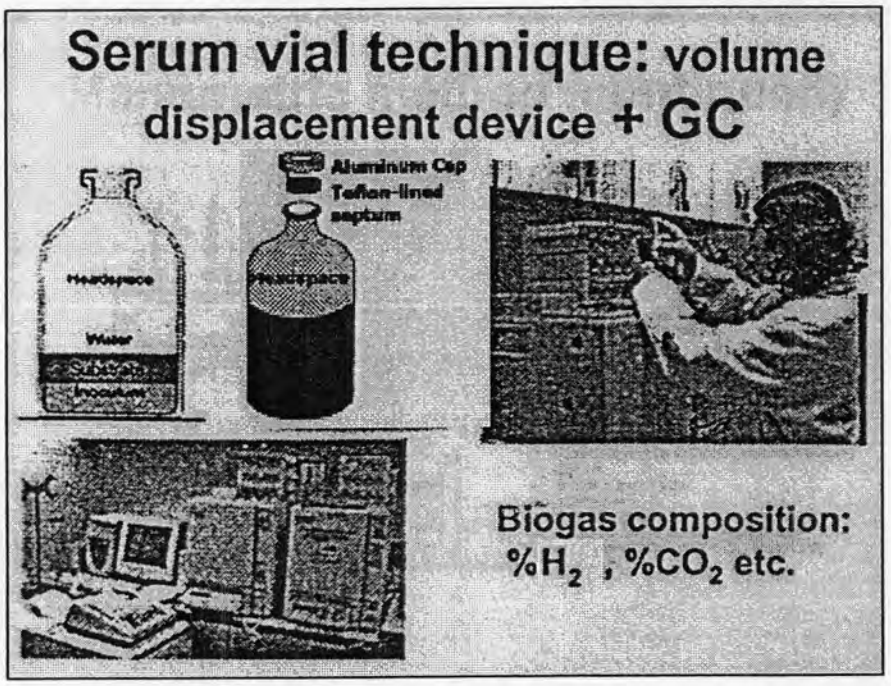

Figure 3-1 Serum bottle technique

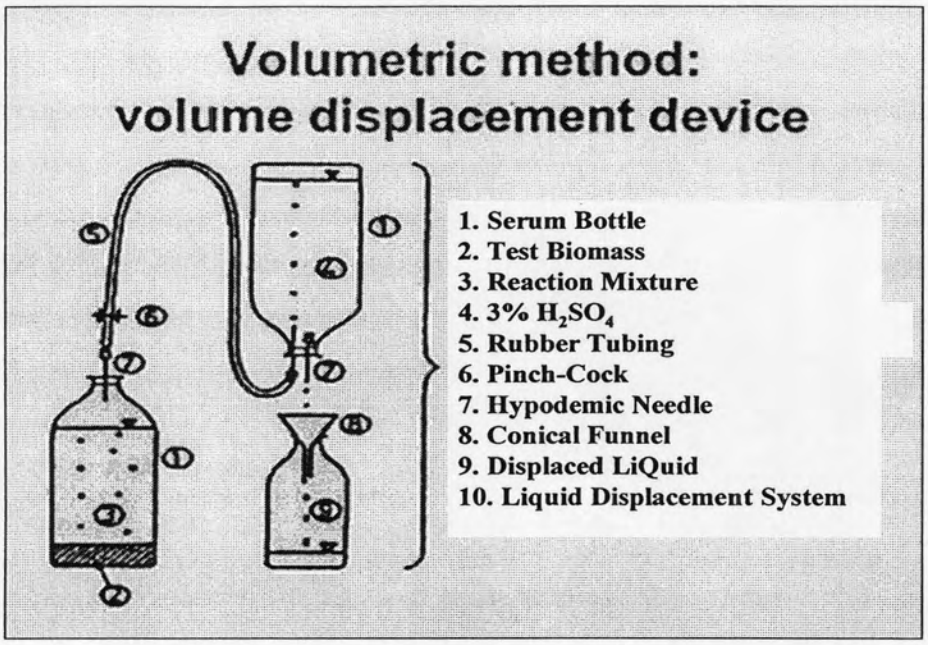

Figure 3-2 Volume displacement method used in the first batch study

In phase II, the experiment was scaled up to 1 l using CSTR, which equipped with automatic control devices to acquire experimental results on H_2 production. The aim of this experiment was to investigate the H_2 production from the batch culture between with and without brewery waste yeast, using the optimal results obtained from phase I. It also determined some kinetic parameters for further use as process design criteria in continuous H_2 study. This type of fermentor (Marubeni corp., Japan) equipped with an impeller and two baffles of the batch model as shown in Figure 3-2. Agitation rate in the fermentor was kept at 100 rounds per minute (rpm). The pH of mixed liquor was kept constantly at pH = 5.0 ± 0.2 by feeding buffer solution via a peristaltic pump. The fermentor was controlled at the temperature of 30°C. The amounts of biogas was measured using gas counter as shown in Figure 3-4 designed by Opaswatchai (1983). The volume of biogas production reported was calibrated to the standard conditions or STP at 0°C and 760 mmHg.

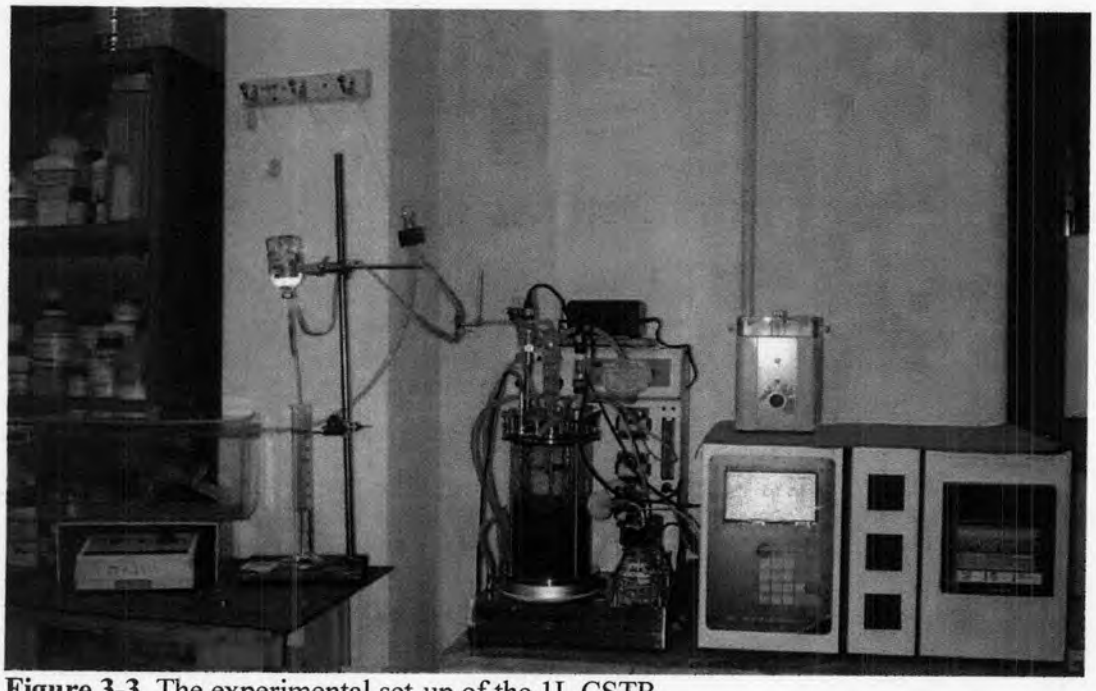

Figure 3-3 The experimental set-up of the 1L CSTR

The pH and temperature of 5.0 and 30°C respectively were used in this study and was selected on the basis of economy and feasibility for anaerobic treatment process. The culture pH of 5.0 was selected for use, considering from previous

investigations as the appropriate value for H_2 fermentation. This value is even slightly lower compared to those of 5.5 to 5.7 as reported by Kim *et al.*, 2004, Khanal *et al.*, 2004, Fang and Liu (2002), Lin and Chang (1999) and Ginkle and Logan (2005). In according to Metcalf and Eddy (2004), the reactor temperatures of 25 to 35°C are generally preferred to support more optimal biological reaction rate and to provide more stable anaerobic treatment, the increase temperature of wastewater. The temperatures from 20 to 30°C which as the low end of mesophilic temperature range was reasonable condition in respecting to the energy balance aspect.

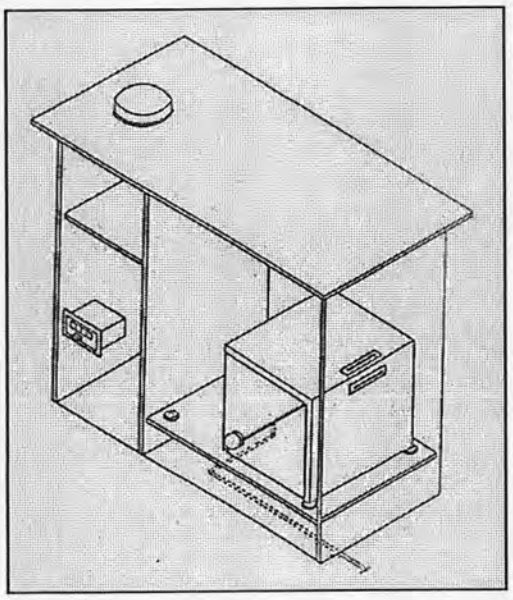

Figure 3-4 Gas counter designed by Opaswatchai (1983)

3.3.3 Continuous feeding study

The objective of this experiment was to investigate the continuous production of hydrogen in association with microbial community at various HRTs including 4, 8, 12 and 24 hours respectively using the CSTR. The reactor was operated for 10 days at each HRT to collect the results. Steady-state condition defined in the study was obtained when the variations of biogas were less than 10%. The continuous hydrogen experiment was conducted in an anaerobic CSTR of 3 l in capacity. The reactor was constructed with a 6 mm thick plexi-glass. The inner working volume of the reactor was 2.3 l with two liquid sampling ports, an effluent and a decanting port located on

the reactor wall. The reactor vent was through a gas pass to a 1 l Erlenmeyer flask with approximately 400 ml of water as gas trap where the biogas volume was then measured using gas counter. The agitation rate was kept at 100 rpm. The pH was kept constant at 5.0 by automatic titration (BL 981411 pH controller-HANNA-Italy) with a microtube pump MP3N (EYELA, Tokyo Rikakikai Co., Ltd) using 4 N NaOH mixed with 2 g/l of $NaHCO_3$ as shown in Figure 3-5 and Figure 3-6 respectively. The influent was controlled by peristaltic pump (Masterflex L/S, Cole-Parmer, USA). The reactor was operated under room temperature of about 30°C (See Figure D-1, APPENDIX B). The volume of biogas production reported was calibrated to Standard conditions or STP (0°C and 760 mmHg). Its operating parameters and feed characteristics of the continuous mode experiment are shown in Table 3-2.

Table 3-2 Reactor characteristics and operational parameters

Parameter	**Unit**	**Value**
Total reactor volume	l	2.3
Reactor compartment volume	l	1.296
Influent concentration	g glucose/l	7.0
Feeding Rate	l/d	2.3-13.8
Hydraulic retention time (HRT)	hour	4-24
Solids retention time (SRT)	day	10
pH	-	5.0 ± 0.2
Temperature	° C	~30

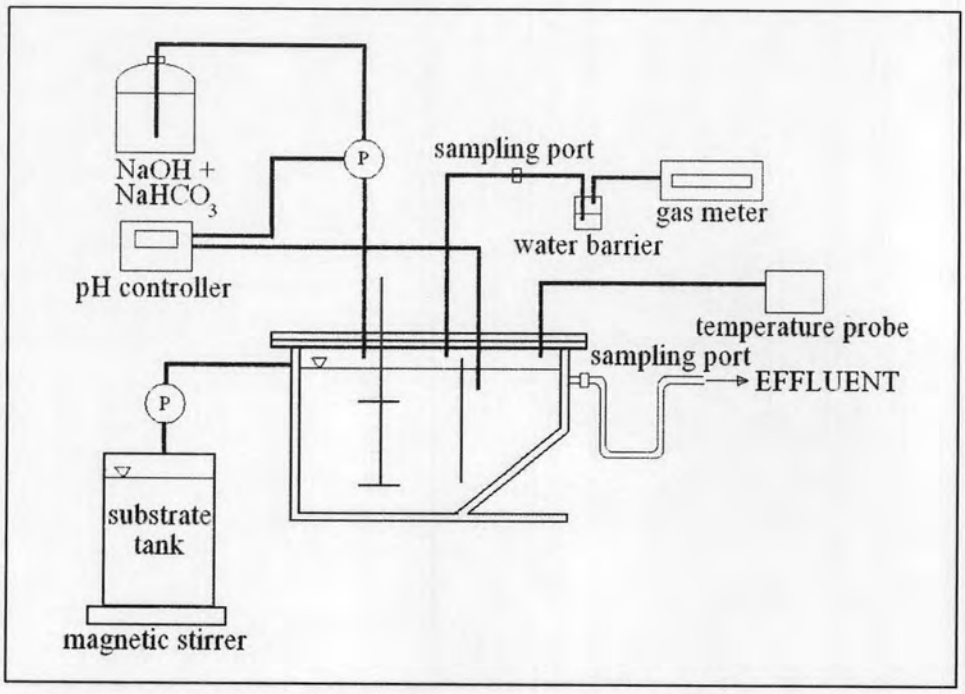

Figure 3-5 Schematic of the continuous stirred tank reactor

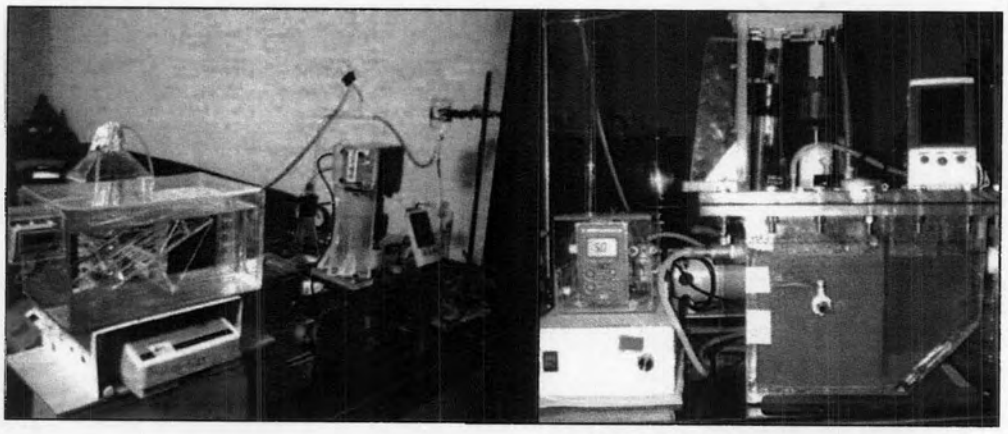

Figure 3-6 Photograph showing the CSTR in continuous feeding experiment

3.4 Analytical Methods

The percentage of hydrogen and methane in biogas were determined by a gas chromatography (Thermo Finnigan Trace GC) equipped with a thermal conductivity detector (TCD) and a 2-m stainless column packed with molecular sieve 5A with helium as a carrier gas. The operational temperature of the injection port, the oven and the detector were 120, 40, 150°C, respectively. The content of CO_2 were measured using a second GC-TCD (Shimadzu-8A) equipped with a 2-m stainless-steel column packed with Porapak T (50/80 mesh). The operational temperature of the injection port, the oven and the detector were 100, 70 and 100°C, respectively. Helium was used as a carrier gas. The concentration of the volatile fatty acids (VFAs) and ethanol were determined using a third GC (HP 6890) equipped with a flame ionization detector (FID) and a 30 m x 0.25 nm x 0.25 μm fused silica capillary column. The operational temperature of the injection port, the oven and the detector were 240, 170, 240°C, respectively. Nitrogen was used as a carrier gas. The supernatant of samples were pretreated with 0.45 μm membrane in disposable filter assembly before injection to GC-FID. The COD, alkalinity, MLVSS, TKN, TPO_4-P and pH were determined according to Standard Method (APHA, 1995). Glucose in the spent malt extract was determined by SCABA beer Analyzer equipped with a PAAR DMA-40 density meter while the glucose during the batch test interval was colorimetrically using an enzymatic color test (GOD-PAP Method). The concentration of brewery waste yeast reported was determined in terms of COD.

3.5 Experimental Design and Data Analysis

- Cumulative hydrogen production curves were obtained during the batch experiment and analyzed by using the modified Gompertz equation[2] as show below:

$$H(t) = H_{max} \exp\{-\exp[\frac{R*e}{H_{max}}(\lambda-t)+1]\} \quad (1)$$

Where H(t) is the cumulative hydrogen gas produced at time (t) (ml), H_{max} is the maximum of hydrogen production (ml), R is the maximum of hydrogen production rate (ml/h), λ is lag phase time (h) and e = 2.718281828. Hydrogen yield and specific hydrogen production rate were acquired by dividing the estimated H_{max} by mol glucose utilized and gram VSS of sludge, respectively. Equation 1 was fitted to the experimental data by minimizing the sum of square error (SSE) using the "Solver" function in Microsoft excel version XP.

- To examine the optimum fraction of the brewery residues, seed microorganism and the maximum of H_2 production rate, a full factorial central composite design (CCD) and response surface methodology (RSM) were used. Response surface methodology is an empirical statistical technique employed for multiple regression analysis using the data obtained from appropriate designed experiments to solve multivariate equation simultaneously. The graphical representations of these equations are called response surface, which can be used to describe the individual and cumulative effect of the test variables and their subsequent effect on the response (Montgomery, 2001). The range and the levels of variables employed in this study are listed in Table 3-3. The center value (zero level) chosen for experimental design were:- glucose = 750 mg/100 ml, waste yeast = 750 mgCOD/100 ml, and seed sludge = 1000 mg VSS/100 ml, respectively. In developing the regression equation, the test variables were coded according to the equation:

$$x_i = (X_i - X_i^*) / \Delta X_i$$

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[2] Gompertz equation was named to honor Benjamin Gompertz who employed this equation for calculation of mortality table at the early 1800s

Where x_i is the coded value of the i^{th} test variable, X_i is an uncoded volume of the i^{th} test variable, X_i^* is an uncoded value of the i^{th} test variable at the center point and ΔX_i is the step change value.

The batch test was run in triplicate resulting a total of 42 experiments were performed to a full factorial design for three independent variables each at five levels with 6 replicates of the center value. The values of maximum H_2 production rate (ml/ h) were obtained as the response of the design experiments (see Table 4-1).

A second order polynomial model (Equation 2) was used to predict the data set of the maximum hydrogen production rates.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j + \epsilon \qquad (2)$$

Where Y was the predicted response, x's were independent variables, β0 was the offset term, βi was linear coefficient, βii was squared coefficient and βij was the interaction coefficient. Design-Expert 6.0.10 was the software used for polynomial regression and response surface plots, respectively.

Table 3-3 Experimental range and levels of the independent variables

x_1, x_2 and x_3 are the coded value of the test values.

	Variables	Range and Levels				
		-1.682	-1	0	+1	+1.682
X_1	glucose (mg/100 ml)	329.5	500	750	1000	1170.5
X_2	waste yeast (mgCOD/100 ml)	0	0	750	1500	2011.5
X_3	Seed sludge (mg VSS/100 ml)	159	500	1000	1500	1841

- Method for Evaluation of Monod Coefficients: The integrated Monod equation (Equation 2-11) is useful in many applications for evaluation of bacterial transformation rate coefficients. Coefficients can be evaluated from progress curves of a few or even one batch experiment of reaction. However, the integrated Monod equation is somewhat cumbersome to use because it is a nonlinear implicit expression for substrate and organic concentration. Weighted least square is an approach that can be used to minimize differences between experimental data and model predictions when it is necessary to use an implicit expression in the model (Smith *et al.*, 1998). This study used a simple method for determination of the best-fit values for rate coefficients in the Monod equation and their uncertainties using weighted least squares analysis that described by Smith *et al.* (1998). The method is straightforward and is designed for easy implementation in a computer spreadsheet program such as Microsoft Excel. The substrate utilization rate coefficients, *k* and K*s*, were determined by fitting Equation 2-11 to substrate metabolism rate data using a computer spreadsheet and weighted nonlinear least-squares analysis.

$$SSWE = \sum_{i=1}^{n} (Wi(t_i^{obs} - t_i^{pred}))^2 \qquad (3)$$

Where SSWE is the sum of the squared weighted errors

Wi is an appropriate weighted factor.

t_i^{obs} is the time of I observation

t_i^{pred} is the value of t predicted by the model for the measured values of S, S_t^{obs}

Ideally, it would be desirable to minimize the differences between measured and predicted values of S ("△S") because the errors in the measurement of S are generally much larger than the errors in the measurement of t. However, for implicit equations such as equation 4, only the differences between predicted and observed values of t ("△t") can be calculated explicitly. The differences between predicted and observed values of S can be calculated explicitly. The differences between predicted and observed values of S can be estimated by multiplying △t by the local slope of the substrate disappearance curve, △M/△t. Therefore, Smith *et al.* (1996) proposed that the logical weighted factor is the local slope of the substrate disappearance curve:

$$w_i = \frac{\Delta S}{\Delta t} = \frac{S_{i-1}^{pred} - S_{i-1}^{pred}}{t_{i-1}^{pred} - t_{i-1}^{pred}}$$

Given this weighting factor, the quantify $w_i (t_{i-1}^{obs} - t_{i-1}^{pred})$ is:

$$w_i (t_{i-1}^{obs} - t_{i-1}^{pred}) = \frac{\Delta S}{\Delta t} (t_{i-1}^{obs} - t_{i-1}^{pred}) = S_{i-1}^{obs} - S_{i-1}^{pred} \qquad (4)$$

Which is the error in the prediction of S_{i-1}^{ob}. This approach provides an explicit approximate method to minimize ($S_{i-1}^{obs} - S_{i-1}^{pred}$) in lieu of an explicit expression for S as a function of t.

The model (Equation 2-11) was fitted to the data using the Solver under the Formula menu in Microsoft Excel to adjust the parameter estimates to minimize the SSWE. The best fit was obtained. The Solver can search quickly for a maximum, minimum, or specified value for any selected cell by varying the values of one or more other selected cell in a spreadsheet. The comparative study of the numerical model and the integrated Monod equation weighted least squares analysis by Smith (1996), showed the similar values of the rate coefficients were obtained. Based on the

significant overlap in the joint 95% confidence regions and the approximate 95% confidence intervals, there was not statistically significant difference in the estimates obtained by the two methods.

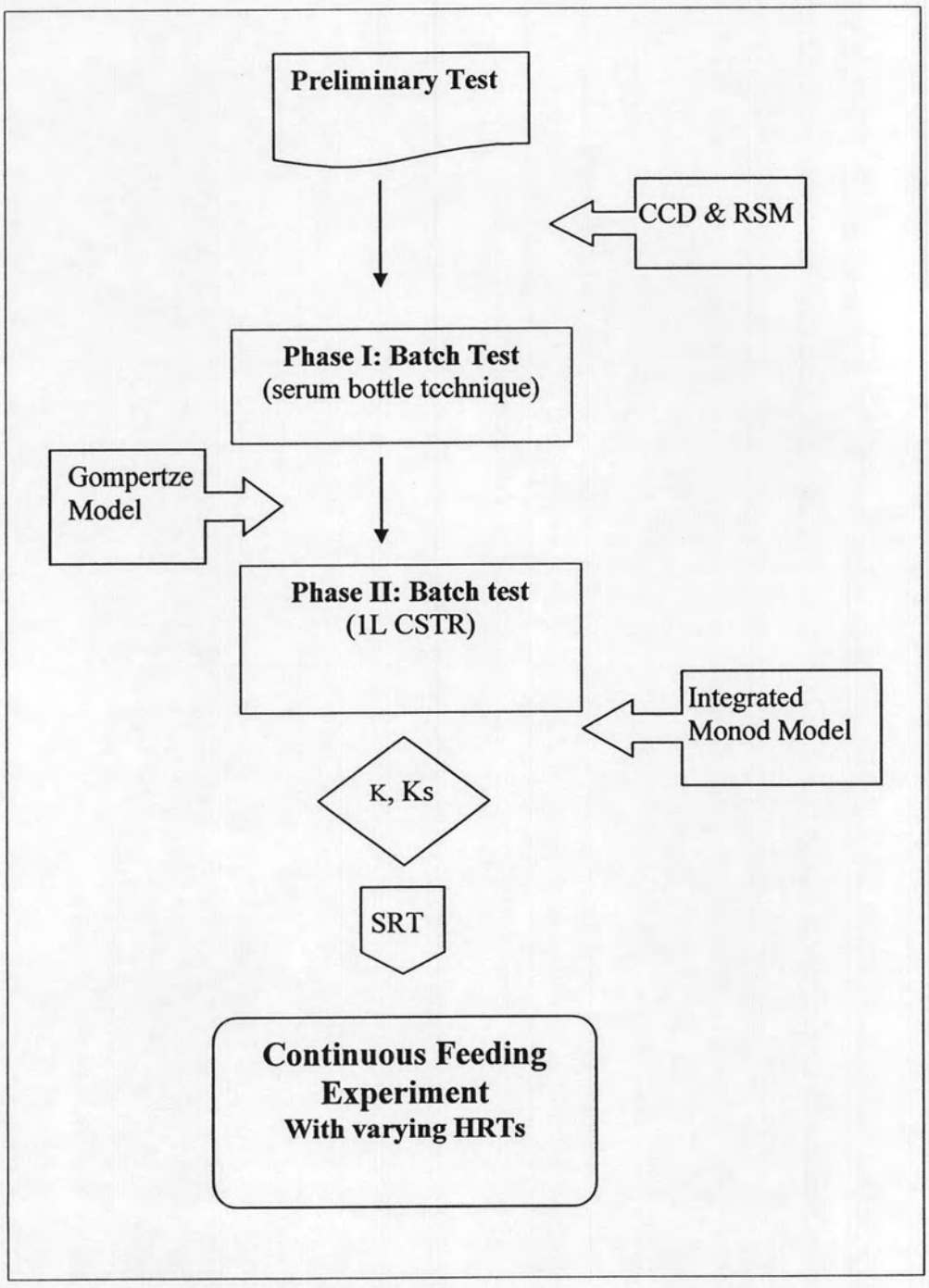

Figure 3-7 Flow diagram of methodology

3.6 PCR - DGGE Analysis

The V3 region of 16S rDNA gene from sludge sample taken was amplified by polymerase chain reaction (PCR) using PRBA338f (ACTCCTACGGGAGCAGCAG-3') and PRUN518r (5'-ATTACCGCGGCTGCTGG-3') primers (Ovreas *et al*, 1997) which correspond to position 338-357 and 518-534 in the E. coli 16S rDNA gene (GenBank accession no.j01695). Both primers complement with a conserved region of the domain bacteria. The GC clamp (5'-CGCCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGG-3') described in Muyzer *et al*, (1993) was attached to the 5' end of the forward primer PRBA338f. The PCR complied with 3 µl of extracted DNA suspension, 0.5 µM of each primer, 15 µl of Taq DNA polymerase Master Mix (Quigen, CA) and sterile water to the final volume of 30µl. The PCR condition was as follows: an initial denaturation step at 92°C for 2 minutes, followed by 25 cycles of a three-stage program with 1 minute at 92°C for denaturation, 0.3 minute at 55°C for annealing, and 1 minute at 72°C for extension, and a final extension step run for 6 minutes at 72°C. The PCR products were checked by gel electrophoresis with 1% agarose and visualized under UV illumination after ethidium bromide staining.

Denaturing gradient gel electrophoresis (DGGE) was perform using the D-code system (Bio-Rad). Acrylamide gel (7.5% w/v of 38:2 acrylamide-N, N'-methylene-bisacrylamide) was prepare with 20-70% denaturing gradient in which 100% is defined as 7M urea and 40% (v/v) formamide. Electrophoresis was performed at 60 °C for 6 h at a constant voltage of 130 V with TAE running buffer contained 40mM Tris-acetate and 2 mM Na_2-EDTA. Then, gen was stained with 100 µg ethidium bromide in 1 L deionized water for 15 minutes and photographed under UV illumination. To identify the species of selected DGGE bands, they were excised, extracted from the gel and used as the template for PCR re-amplification. The condition of PCR was as previously described expect the forward printer PRBA338f did not include the GC clamp.

3.6.1 Cloning, Sequencing, and Identification of Bacterial Species

16S rDNA amplicons were cloned into *Escherichia coll* JM109 using the pGEM-T easy plasmid vector system (Promega, Madison, Wis.) in accordance with

the manufacturer's instructions. The resulting transformants were plated on to luria-Bertani (LB) plates containing amlicillin, IPTG and X-Gal. White colonies were selected. To ensure that plasmid DNA isolated from White colonies is recombinant plasmid DNA, plasmid DNA were digested with restriction enzyme *Eco*RI at 37 °C overnight. The digested DNA was confirmed on agarose gel comparing to 200 base pairs ladder. Then , the ligation plasmid were cleaned using QIA Miniprep Kit (Qiagen,CA) and sequenced at Biogenomed Co., Ltd (Hongkong)

After acquiring DNA sequences, BLAST software (GenBank, www.ncbi.nlm.nih.gov/blast/BLAST.cgi) was used to determine its identity and nearest gene neighbor.

3.6.2 Analysis of DGGE images

The diversity and dynamic of the dominating microbial populations is observed from the appearance and disappearance of amplicons in the DGGE patern which indicates importance shifts in the microbial community structure. The intensity of an individual band is a semi quantitative measurement for the relative abundance of a specific bacteria population in the communities (Muyzer *et al.*, 1993).