CHAPTER III

METHODOLOGY

Cadmium (Cd), copper (Cu) and zinc (Zn) that are the three out of five heavy metals which frequently appear in biological sludges and industrial wastewater were used as the toxicants in the experiments. Bacterial assemblages from various locations were tested. The selected mixed-bacteria which had high ability to tolerate the conditions of sulfate (SO_4^{2-}) reduction and high heavy metal concentration functioned as seed under the most appropriate anaerobic condition. The SO_4^{2-} concentration was tested in order to avoid substrate competition and suppress MPB growth. At this step, the test was performed by various proportions of COD:SO_4^{2-} in order to find an optimum reactor conditions for simultaneous control of SO_4^{2-} and heavy metals in complex wastewater streams. Then the toxicity test was conducted. Influence of $SO_4^{2-}$and heavy metal(s) on the methanization of wastewater was observed. The potential interaction of metal(s) commingling with SO_4^{2-}were investigated. The predictive model to prevent system suffering from these toxicants was proposed.

The methodology of this research was divided into 3 main parts.

3.1 Microbial selection

3.2 An optimum reactor conditions for simultaneous control of SO_4^{2-} and heavy metals in complex wastewater streams.

3.3 Assessment of inhibitory effects of heavy metals to anaerobic microorganisms.

The flow diagrams are shown in Figure 3.1, 3.4, 3.5, 3.7 and 3.8. The details of each part are:

3.1 Phase 1 Microbial Selection

Bacterial assemblages (or communities) from five different locations, through natural selection, that they all may have a high ability to tolerate heavy metals to some degree were the mixed-bacterial culture to be studied in this chapter (Rationale was given in Section 2.2.4).

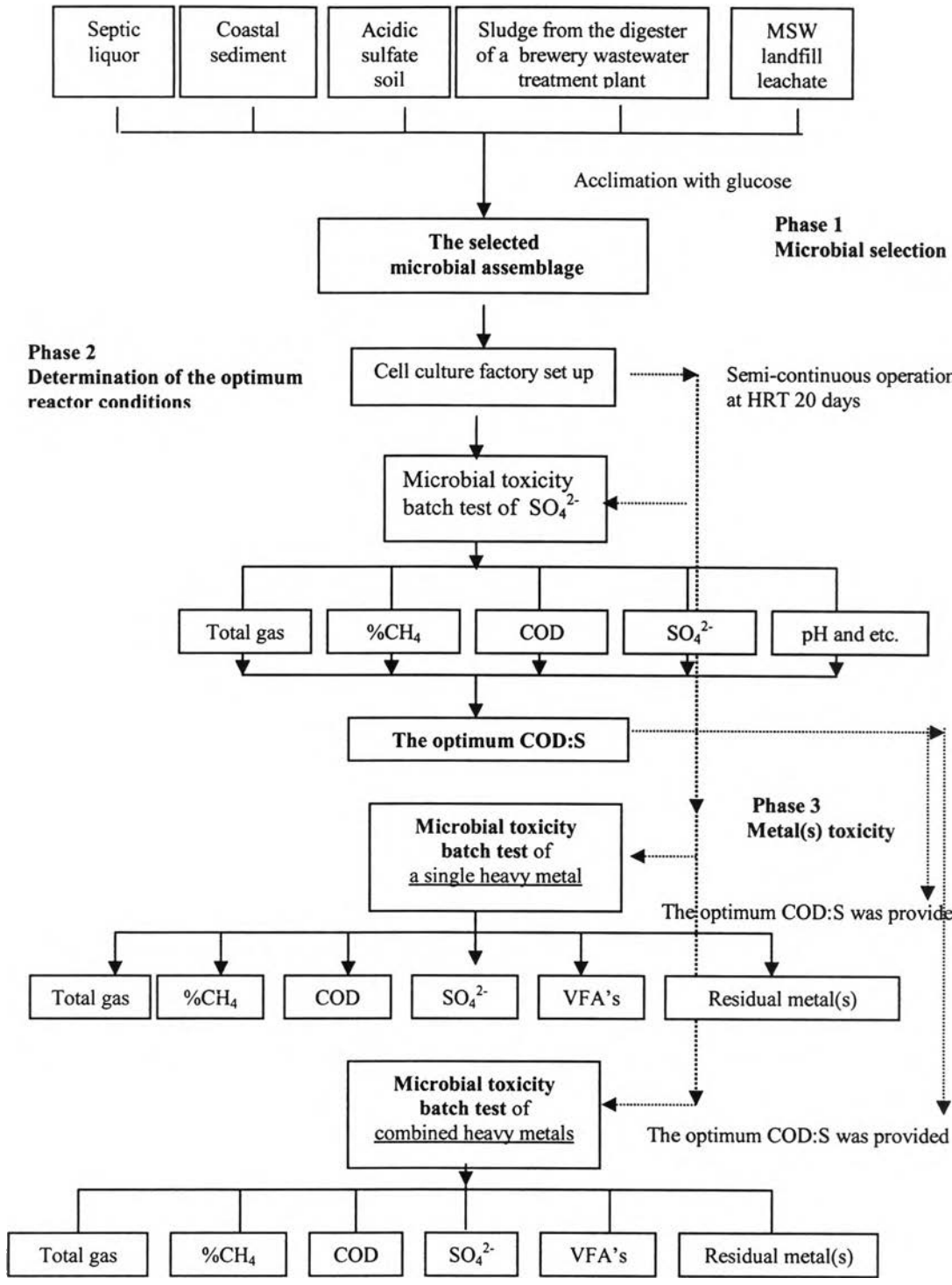

Figure 3.1 Flow diagram of the **overall experimental studies**.

The mixed liquor in a septic tank, sediment samples taken from a coastal area (Pattaya, Chonburi), sludge from the digester of a brewery wastewater treatment plant, acidic sulfate soil samples with due regard to temporal and spatial heterogeneities which was collected from the sites at Ongkarak (Nakornayok) where there is not any anthropogenic effect and a mixed-microbial culture from leachate (Nongkham landfill site, Bangkok) were selected to study.

3.1.1 Materials and Methods

In this study, a controlled environment was needed to ensure that the microorganisms had a proper medium in which to grow and that there could be comparability among reactors. Environmental conditions including pH, temperature regulation, nutrient and trace-element addition, initial loading factor (waste concentration/biomass in terms of mixed liquor volatile suspended solids or COD/MLVSS), anaerobic conditions, and proper mixing control were carefully controlled.

3.1.2. Bioreactors

For comparison of waste utilization, biomass and biogas production, five reactors were constructed from 6-liter capacity PET bottles. Each was equipped with two outlet ports, one for liquid sample withdrawal and the other for gas venting. The reactor was connected to a gas collection system, which was based on water displacement by the exiting gases (Figure 3.2). The five reactors were operated in batch mode.

For the studies on SO_4^{2-} reduction and the effect of heavy metal, the reactor was equipped with an outlet port for liquid sample withdrawal (Figure 3.3). The exiting gases vented through a rubber cap that was perforated and adjacent to a tube containing water. The ten reactors were operated in batch mode.

3.1.3 Feed Solution

A glucose composition consisting of 20,000 mgCOD/l and sufficient inorganics was used as the synthetic waste to maximize consistency for bacterial cell growth. The components followed those of Leighton and Forster (1997).

3.1.4 Start Up

The seed sludges obtained from the five natural locations (previously described) were initially characterized. Physical and chemical characteristics were measured including pH, conductivity, oxidation reduction potential (ORP), TDS, Cl^- level, alkalinity, NO_3^- and SO_4^{2-} concentrations, and organic matter or COD.

The biomass inoculum for each type of sample was started up in 2-liter PET bottles that were maintained at ambient temperature (around 31 ± 1^0C). They were shaken from time to time. The synthetic glucose-based substrate was gradually increased in concentration over a range from 2,000-10,000 mg/l for about 2 months. The biogas produced was observed in order to monitor the performance of the bacterial growth. Final mixed liquor volatile suspended solids (MLVSS) were determined in order to start the operation at the same content for each reactor.

3.1.5 Operation

The control conditions in the operation were: initial COD (20,000 mg/l), MLVSS (670 mg/l), initial pH (7), temperature (31 ± 1^0C), and working volume (5.5 liters).

For the comparison studies of the rate of waste utilization, biomass and biogas production, the five batch reactors (as shown in Figure 3.4) containing different kinds of mixed-microbial cultures (started at the same content of MLVSS) were fed with the glucose-based solution. The loading factor (COD/MLVSS) in each reactor was 30. The batch reactions were operated for 2 months. Biogas was measured every 3 to 6 hours. At selected times, liquid samples (5 ml) were withdrawn and analyzed for MLVSS, COD, and VFA's. COD removal efficiencies of all 5 reactors which contained no toxicants were reported.

For the comparison studies of SO_4^{2-} reduction and the effect of the presence of a heavy metal, a series of 10 reactors (as shown in Figure 3.5) was divided into two groups (A and B). They were fed with the glucose-based solution. Cu was the model metal used in this step. It was the most consistent heavy metal found in wastewaters (USEPA, 1981) and it had been recorded of high toxicity (Chiu-Yue Lin, 1992).

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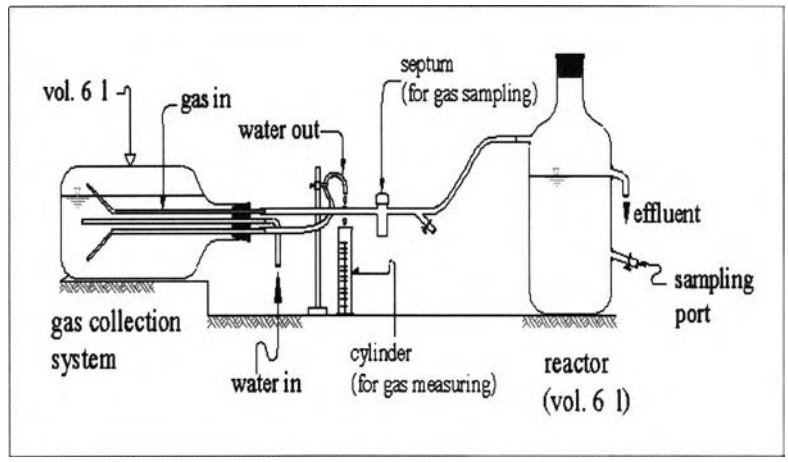


Figure 3.2 Bioreactor for the studies of waste utilization, biomass and biogas production.

Initial SO_4^{2-} in the form of ammonium salt at the concentration of 1,000 mg SO_4^{2-}/l was added to all reactors of Groups A and B. The COD:SO_4^{2-} ratio was 20:1 or the COD:S ratio was 60:1 in order that glucose not be limited. The SO_4^{2-} content in the solution was recorded. In addition, an initial Cu concentration of 10 mg/l was added to five reactors of Group B. The batches were operated for two months. At selected times, liquid samples (5 ml) were withdrawn and analyzed for SO_4^{2-}, MLVSS, and COD. For Group A, SO_4^{2-} reduction rates were measured over time. The specific rates of SO_4^{2-} reduction were determined. The values derived from each reactor were reported and shown. For Group B, The system recovery from Cu commingling with SO_4^{2-} was observed.

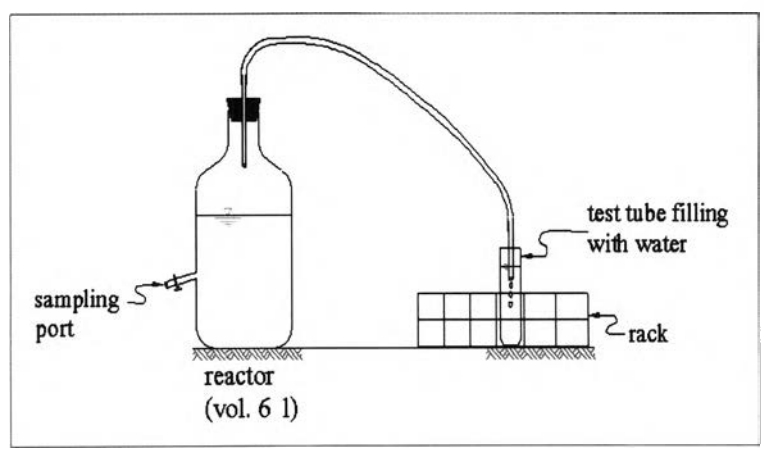


Figure 3.3 Bioreactor for the studies of sulfate reduction and the effect of heavy metal.

COD removal efficiencies of Group A that contained only SO_4^{2-} and those of group B that contained both SO_4^{2-} and Cu were determined in order to investigate SO_4^{2-}reduction and the effect of heavy metal in the sulfide detoxification and the metal remediation processes.

To compare the bacterial assemblages that show mutualistic interaction between SRB and MPB and to investigate the effect of heavy metals on anaerobic bacteria involved in metal remediation processes, COD removal efficiencies with mixtures that contain no toxicants, were compared with the two sets of samples, one that contained only SO_4^{2-}, and the other that contained both SO_4^{2-} and heavy metal. The results show the values that were obtained from all seed types.

Seed acclimation, start up, experiment set up, operation and gas collection were shown in Figure B1- B7.

The most appropriate bacterial assemblages which have the ability to grow by fermentation or by syntrophic association with methanogens and showed a positive effect of heavy metals on anaerobic reaction due to sulfide detoxification of the metal were isolated and identified. This mixed-bacterial culture was selected for further study in Phases 2 and 3.

Figures 3.4 and 3.5 show the flow diagram of the experimental study in Phases 1.

3.1.6 Isolation and Identification of Bacterial Culture

The selected mixed-bacteria were then isolated to identify the major type and species. Isolation of bacteria was performed by following that of Mori, et al. (2000). Identification of bacteria was performed by following that of Ohkuma and Kudo (1996). The 16S rRNA technic was carried out for Gene Analysis of the isolates (Appendix C).

The results from this section are presented in Chapter IV.

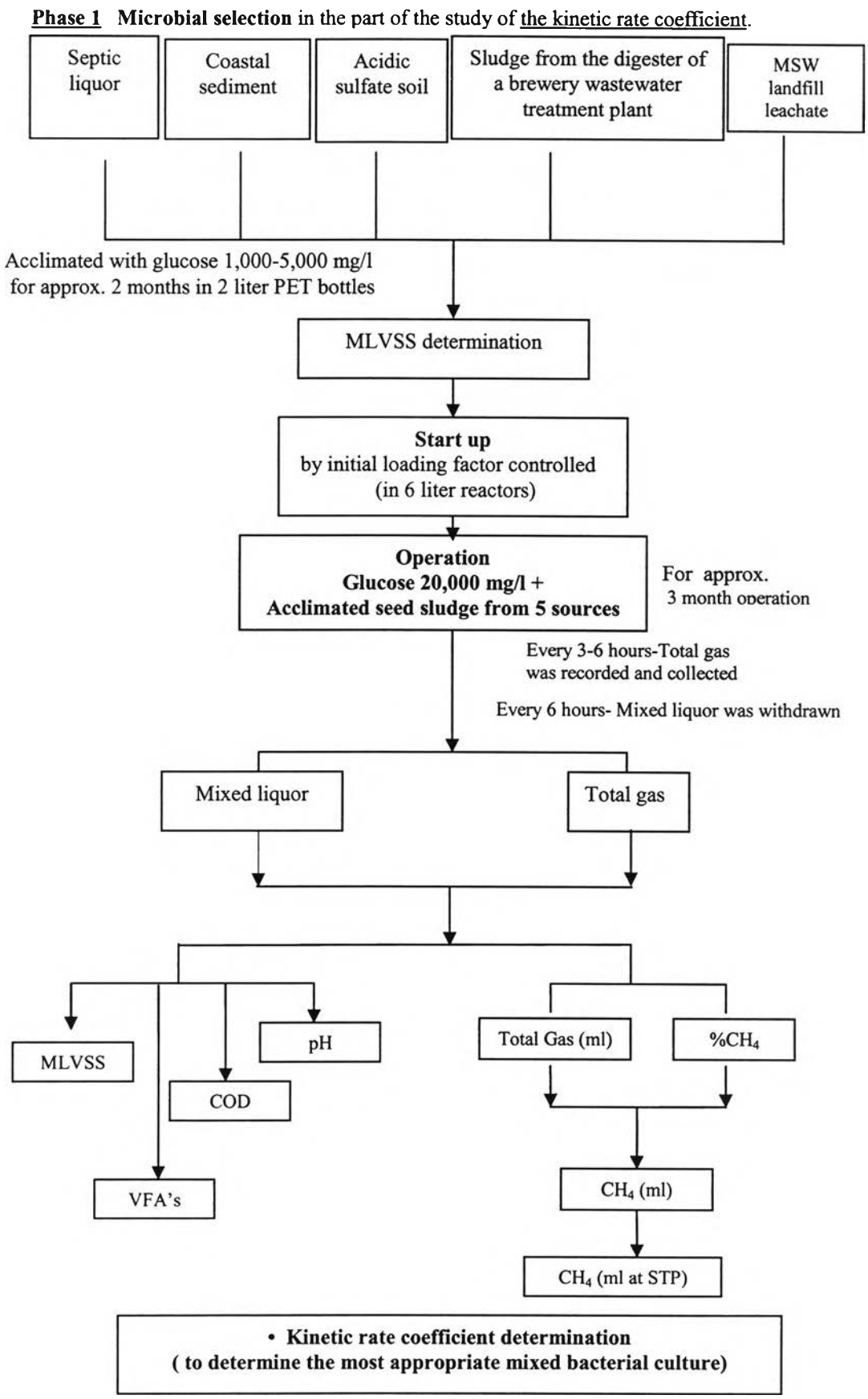


Figure 3.4 Flow diagram of the experimental study in **Phase 1**.

Phase 1- continued- Microbial selection in the part of sulfate reduction and system recovery by metal.

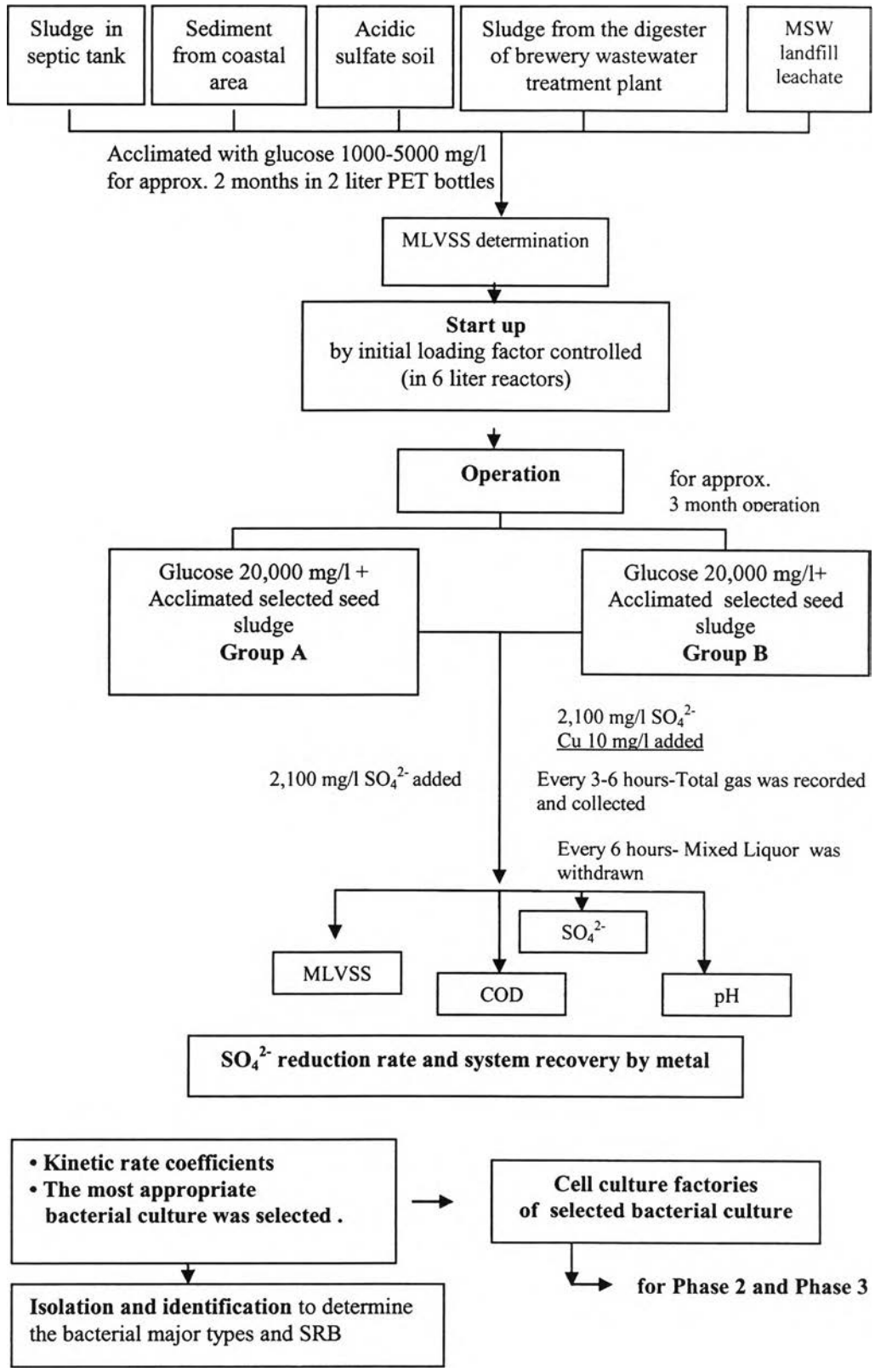


Figure 3.5 Flow diagram of the experimental study in **Phase 1 (under sulfate reducing condition).**

Table 3.1 The variable and parameters for the studies in **Phase1**.

Variables	Parameters
Control Variables	Initial COD and MLVSS (loading factor), initial pH=7, temperature (room condition), time of operation (for approx. 3 months, batch mode operation
Independent Variables	5 sources of mixed-bacterial culture
Dependent Variables	MLVSS, COD, CH_4, VFA's and pH

Table 3.1-continued- The variables and parameters for the studies in **Phase1**. (under sulfate reducing condition)

Variables	Parameters studied for Group A reactors
Control	Initial COD and MLVSS (loading factor), initial pH=7, temperature (room condition), time of operation (for approx. 3 months, batch mode operation
Group A Independent Variables	5 sources of mixed-bacterial culture and SO_4^{2-} 2,100 mg/l
Dependent Variables	COD, MLVSS, SO_4^{2-} and pH

Table 3.1-continued The variables and parameters for the studies in **Phase1**. (under sulfate reducing condition)

Variables	Parameters studied for Group B reactors
Control	Initial COD and MLVSS (loading factor), initial pH=7, temperature (room condition), time of operation (for approx. 3 months and batch mode
Group B Independent Variables	5 sources of mixed-bacterial culture, SO_4^{2-} 2,100 mg/l and Cu 10 mg/l
Dependent Variables	SO_4^{2-} , COD, MLVSS, and pH

3.2 Phase 2 An Optimum Reactor Conditions for Simultaneous Control of Sulfate and Heavy Metals in Complex Wastewater Streams

Experiments were performed in reaction bottles with 100 ml working volume by a batch test. These bottles, equipped with a separate gas collection system, were placed in a shaking water-bath with temperature controlled at 35±1 ^{0}C (Figure 3.6). A series of batch reactors were operated. The optimum performing organisms obtained from Phase 1 were used in this test. They were acclimated in 6 liter well mixing reactors for more than 2 months at room temperature (31±1 ^{0}C). The glucose synthetic waste used in the acclimation and the batch test also contained sufficient inorganics and their components applied from Leighton and Forster (1997) (Table 3.2). The hydraulic retention time (HRT) for the fill-and-draw operation type seed sludge digesters was 20 days. The synthetic waste concentration was 32,000 mg/l as COD and the initial concentration in the seed sludge was around 650 mg COD/l. SO_4^{2-} was added in the form of aqueous Na_2SO_4.

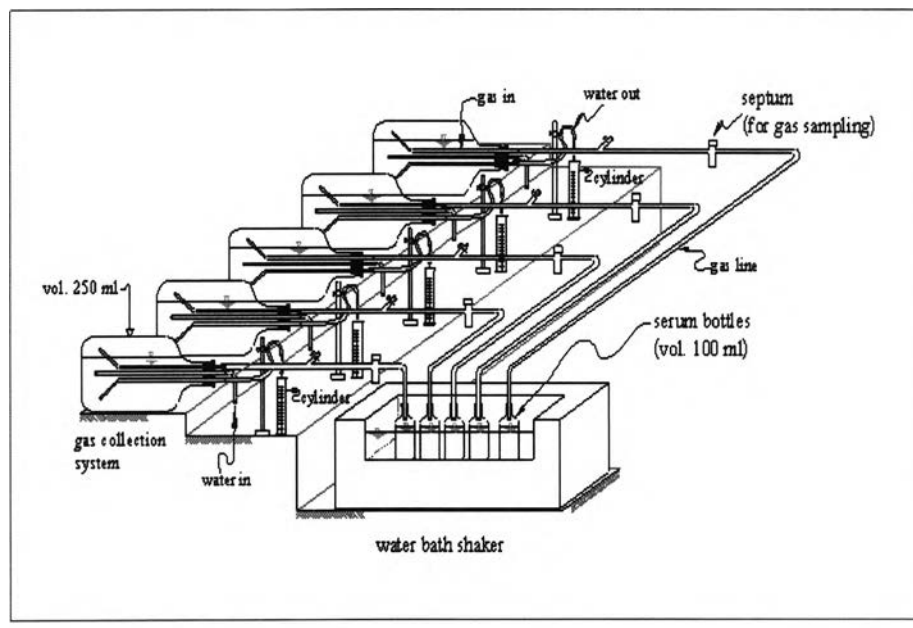


Figure 3.6 Experimental apparatus.

The reaction bottles were initially purged with N_2 and then seeded with 80 ml acclimated steady-state seed sludge. Proper amounts of 32,000 mgCOD/l synthetic waste and distilled water were added into the gassed reaction bottles to make the initial COD around 3,000 mg/l in order to mimic industrial wastewater. The real value

of COD from synthetic waste commingling with filtrated seed sludge was measured and recorded as influent COD. The initial biomass concentration was controlled to be around 10,000 mg/l. The initial loading factor (COD/MLVSS) in each bottle was equally controlled. The proper amount of the Na_2SO_4 solution had been added, giving rise to 0-2,500 mg SO_4^{2-}/l.

The real values of SO_4^{2-}content in the solution were measured and recorded. The total working volume of all the bottles was 100 ml. Optimum conditions were provided in this operation. Operational parameters of initial pH 7 and temperature of 35 ^{0}C were adjusted in order to facilitate anaerobic digestion. After preliminary operation of 5 minutes for minimizing the effects of environmental changes and gas phase difference, the experiment started. The contents were sampled every 6 to 12 hours with a syringe to determine MLVSS and SO_4^{2-}. Before sampling mixed liquor, for the purpose of measuring methane production, the total gas production was recorded and collected. The gas composition were then determined. The initial and final CODs were analyzed.

Photos of the experiments were shown in Appendix B.

Table 3.2 Glucose synthetic wastewater composition.

Constituent in 10 liters	Weight or Concentration
Glucose	300 g
Urea	45.76 g
$NaHCO_3$	128.16 g
KH_2PO_4	13.856 g
K_2HPO_4	10.656 g
$MgCl_2$	18.112 g
$FeCl_2.6H_2O$	0.1376 g
$NiSO_4.6H_2O$	0.1056 g
$MnCl_2.4H_2O$	0.1056 g
$ZnSO_4.7H_2O$	0.1056 g
H_3BO_3	0.0224 g
$COCl_2.6H_2O$	0.0112 g
H_3PO_4 12 MoO.24 H_2O	0.00832 g
$CuSO_4.5H_2O$	0.001056 g
COD of this glucose synthetic waste	32,000 mg/l

Phase 2 Study of the optimum reactor condition.

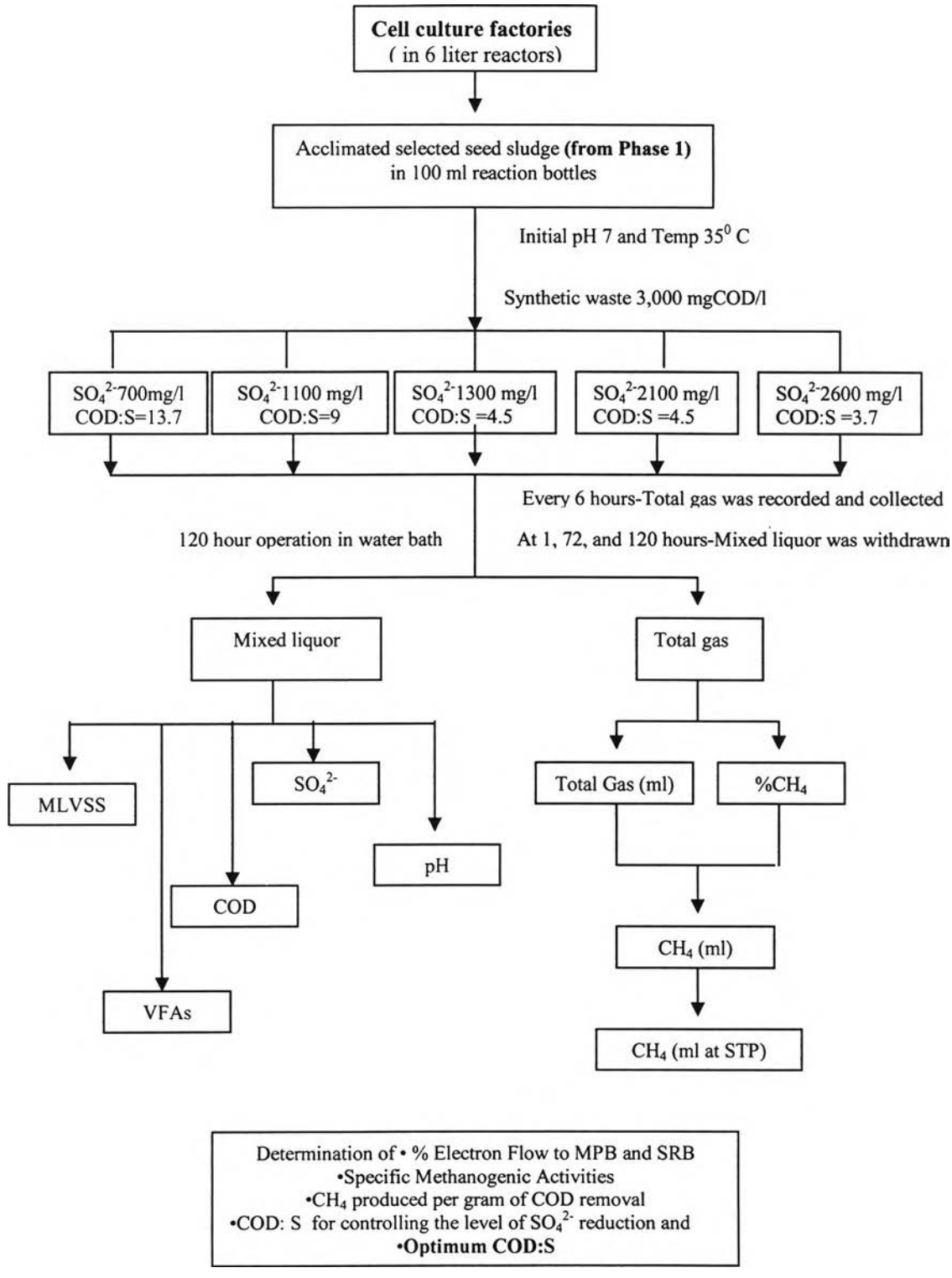


Figure 3.7 Flow diagram of the experimental study in **Phase 2**.

The mixed liquors sampled were filtered for MLVSS determination and the supernatants were taken for SO_4^{2-} and COD analysis. The gas compositions were determined by using a Shimadzu GC-14B gas chromatograph equipped with a thermal conductivity detector. SO_4^{2-} was measured according to the procedure of Standard Methods (APHA, 1992). Samples for COD analysis had been acidified and shaken for 2 hours to remove sulfide. Figure 3.7 shows the flow diagram of the experimental study and Table 3.3 summarizes the variables and parameters for the studies in Phase 2.

Table 3.3 The variables and parameters for the studies in **Phase 2**.

Variables	**Parameters**
Control	Selected mixed-bacteria, Initial COD and MLVSS, initial pH=7, temperature=35^{0}C, time of operation=120 hours and batch mode
Independent Variables	SO_4^{2-} concentration or COD:S (13.7-3.7)
Dependent Variables	SO_4^{2-}, CH_4, COD , pH and VFA's

Assays were run for a 120 hour period. The initial and final pH of the mixed liquors when the reaction bottle assay begins and finishes were recorded to ensure that pH would not cause any inhibition. The inhibition of methanogenesis and the competition between SRB and MPB were determined. Inhibition was quantified by determining the doses of the SO_4^{2-} and the COD:S ratios that caused major trouble or unfavorable conditions during the anaerobic digestion over a fixed period of time. The parameters used in measuring the effects of SO_4^{2-} and COD:S ratios were total gas, methane production and the effluent COD. The gas volumes were recorded at standard temperature (0^{0}C) and pressure (760 mmHg)(STP). The percentage of electron flow to SRB and MPB, and the specific methane activities (SMAs) were investigated to observe the competition between SRB and MPB, and the ability to produce methane, expressed as units of methane per one gram of biomass, respectively.

At this step, the optimum reactor conditions for control of SO_4^{2-} in wastewater were investigated. The optimum COD:S would be selected to set the optimum

condition. With optimum performing microorganisms, the task for assessment of inhibitory effects of heavy metals to anaerobic microorganisms could be continued.

The results from this section are presented in Chapter V.

3.3 Phase 3 Assessment of Inhibitory Effects of Heavy Metal(s) to Anaerobic Microorganisms

Toxicity batch bioassays were conducted to evaluate the effect of heavy metals on the activities of sulfate reducing bacteria and methane producing bacteria. Glucose was utilized as the electron donor for the process.

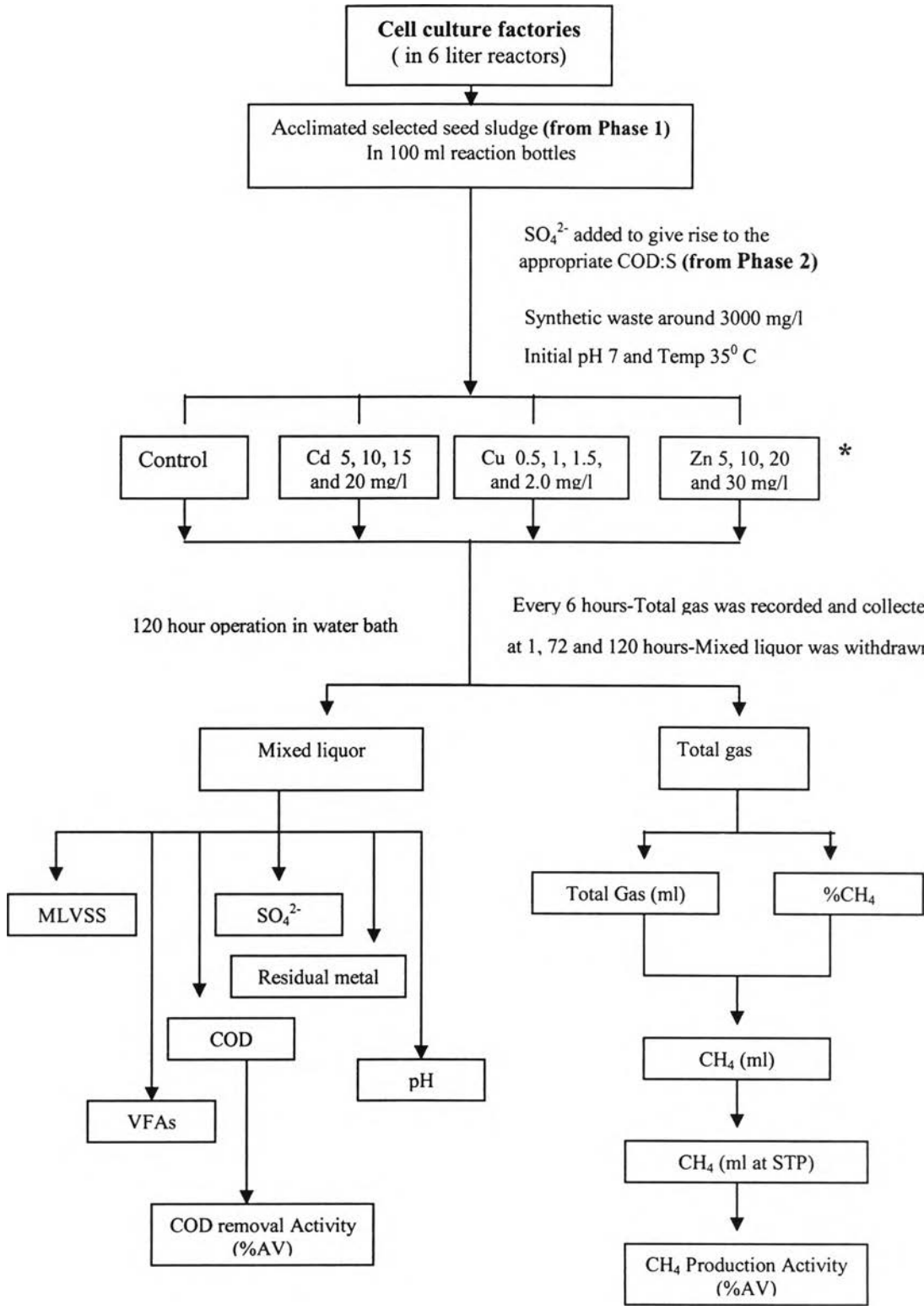
Cd, Cu and Zn were the model metals. They are the consistent heavy metals found in wastewaters (USEPA, 1981). Inhibitory concentrations were determined from the bioassays.

Addition of metal-resistant microorganisms, pH adjustment, and SO_4^{2-} anion additives that reduce metal bioavailability and promote methanogenesis are the approaches in this study.

Sulfate at an optimum amount (obtained from Phase 2) was added so that their availability in solution can fulfill the requirement for biomass activity. The SO_4^{2-} dosing utilized would be a value in the range that SO_4^{2-} reduction was controlled by the amount of SO_4^{2-}. This value corresponded to the COD:S ratio that promotes methanogenesis and avoids the competition between SRB and MPB in mixed microbial assemblages. This value agree well with the tested SO_4^{2-} based on the methane yield, and the percentage of $SO_4^{2-}$and COD removal of selected mixed-bacteria grown on glucose.

Experiments were also performed in reaction bottles with 100 ml working volume using a batch test (Figure 3.6). Glucose acclimated seed sludge was also used in this study. The glucose synthetic waste concentration was around 3,000 mg/l as COD. The real value would be recorded. SO_4^{2-} was added in the form of aqueous Na_2SO_4. Sulfate was added to bring the initial concentration to give rise to the optimum COD:S that obtained from Phase 2. The reaction was done in a well mixed reactor at 35 ^{0}C in a water bath. The glucose synthetic waste used in the test contained sufficient inorganics with a component composition that applied from Leighton and Forster (1997).

Phase 3 **Inhibitory effects of heavy metal(s) by microbial toxicity batch test.**



* **Combined metals** were replaced a single metal in other batch experiments.

Figure 3.8 Flow diagram of the experimental study in **Phase 3**.

The initial loading factor (COD/MLVSS) in each bottle was controlled to an equal level. The initial MLVSS was around 10,000 mg/l. The real value would be recorded. Figure 3.8 show the flow diagram of the experimental study in Phase 3.

Cd, Cu, and Zn were added to the reactors in a single soluble form and in a form of combined metals. The total gas production levels were recorded and collected at time 1, 3 ,6, 12, 24, 48, 72, 96, and 120 hours. The gas compositions were then determined. The mixed liquors were drawn with a syringe at time 1, 72, and 120 hours to determine MLVSS, SO_4^{2-} and metals. The mixed liquors sampled were filtered for MLVSS determination and the supernatants were taken for SO_4^{2-} and COD analysis. Gas compositions were determined by GC equipped with a thermal conductivity detector. The pH was recorded when the assays began and when they finished to indicate that pH was not a factor causing inhibition to the experiment. Table 3.4 summarizes the variables and parameters for the studies in Phase 3.

Table 3.4 The variables and parameters for the studies in **Phase 3**.

Variables	Parameters
Control	Selected mixed-bacterial , selected COD:S ratio, initial COD and MLVSS, initial pH =7, temperature=35^{0}C, time of operation=120 hours and batch mode
Independent Variables	Types and concentrations of soluble added metal (Cd, Cu and Zn) in both a single and combined metal form
Dependent Variables	COD, %CH_4, SO_4^{2-}, soluble metal concentration that left in the effluent , VFA's and pH

Assays were run for a 120 hour period. The parameters used in measuring the effects of the metallic ions were effluent COD and CH_4 production. Inhibition was quantified by determining the dose of the metals (added metals and soluble metals left in the effluent) that caused reduction in CH_4 production and reduction in COD removal over a 120 hour period compared with the performance of a fed control. An activity factor (AV) was used to indicate the extent of inhibition. The role of heavy metal on SO_4^{2-} reduction was observed.

The results from this section are presented in Chapter VI.

3.4 Analytical Methods

COD, SO_4^{2-}, metal(s), MLVSS, VFA's, $\%CH_4$ in biogas formed during testing, and pH were all measured using Standard Methods (APHA et al., 1992). Experimental data were obtained by performing the test at least duplication. The routine quality control was performed by the method blank for the equipment calibration. The calibration check standard was performed for the validation of a standard graph.

Biomass (MLVSS) was measured after filtration of samples through GF/C filter paper and was analyzed as described in Standard Methods (APHA et al., 1992).

Total biogas was measured by acidic water displacement. CH_4 analysis was determined by gas chromatography with a Shimadzu GC-14B using a WG-100 SS column, 1/4 O.D. x 1.8 m., flow rate of helium carrier 33 ml/minute. TCD 120 mA, and sample size 1ml.

VFA's: Acetic, propionic, and butyric acids were measured by gas chromatography (GC-FID, Hewlett Packard HP 6890 plus) equipped with Porapak P 100/120, flow rate of helium carrier 1.9 ml/minute. Oven 60 ^{0}C (hold 1 minute) ramp 20-120^{0}C (hold 2 minutes). Split ratio 200:1, and sample size 2 µl.

The COD was titrated by the potassium dichromate-ferrous ammonium sulfate method as described in Standard Methods (APHA et al. , 1992).

Sulfate was determined by the turbiditric method as described in Standard Methods (APHA et al., 1992).

Metals were analyzed through atomic absorption spectrophotometry (GBC, AVANTA).

Sulfide precipitate was analyzed through energy dispersive x-ray spectrometry (EDX).

Acclimated bacteria were collected from the cell culture flasks for examination of the bacterial morphology by the Gram stain technique. Differential detection of isolated genera of species was then performed on the 16S rRNA technic for gene analysis (Ohkuma and Kudo, 1996).

Table 3.5 contains the procedures utilized in the experimental studies.

Table 3.5 The procedures utilized in this study.

Parameter Studied	**Standard Methods Procedure (APHA et al., 1992)**
COD	5220 B
SO_4^{2-}	4500 SO_4^2 -E
Metal	3100
Solids	2540
MLVSS	2540E
Alkalinity	2320B
NO_3^-	4500 NO_3^- -B
Parameter Studied	**Method Procedure**
VFA	Gas Chromatography(GC-FID)
$\%CH_4$	Gas Chromatography(GC-TCD)
Bacterial Morphology	Gram strain technic
SRB Isolation	Mori et al. (2000)
Bacterial Identification	16S rRNA technic
pH	pH meter
Conductivity	Conductivity meter

3.5 References

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