CHAPTER IV

COMPARISON OF THE POTENTIAL EFFECTIVENESS OF SELECTED MICROBIAL ASSEMBLAGES IN ANAEROBIC PROCESS OF WASTEWATER CONTAINING HIGH SULFATE AND HEAVY METAL

4.1 Introduction

Anaerobic process and the resulting formation of methane (CH_4) arise from the co- operative action of many micro-organisms. Anaerobic digestion has been considered as a highly integrated process because of the close collaboration existing between all the members of bacterial population. Synergy can occur between species. A mixed culture is much more efficient in degrading waste than each strain alone. There were many studies concerning the mutualism in anaerobic bacteria, the relationship between different bacterial groups and the characteristics of many bacterial strains. However, the study of the outcome of the bacterial work will show the potential of the mixed bacterial groups from various sources. Improvement of the process can be obtained by a better knowledge of the performance of the mixed bacterial populations. The performance of the mixed bacterial populations was investigated in this study.

4.2 Purposes of this Chapter

This study investigated possible sources of microorganisms that show mutual interactions between sulfate reducing bacteria (SRB) and methane producing bacteria (MPB). The goal of this investigation was to compare the biological treatment potential of microorganism assemblages selected from various areas that currently have significant levels of both sulfate and heavy metals and to identify the microbial assemblage that shows the best performance in terms of COD removal, sulfate reduction, and tolerance of heavy metals, along with simultaneous CH_4 production.

The research focus in this study is to (i) investigate the bacterial assemblages that show mutual interaction between SRB and MPB, and (ii) investigate the effect of heavy metals on anaerobic bacteria involved in metal remediation processes.

4.3 Materials and Methods

Experiment follows the step specified in Figures 3.1 and 3.4. Mixed-microbial assemblages which were obtained from five different locations, i.e., the mixed liquor in a septic tank, sediment samples from a coastal area, sludge from the digester of a brewery wastewater treatment plant, acidic sulfate soil samples and a mixed-microbial culture from landfill leachate were used as seed. Detailed procedure for the start-up, operation and the experimental method are provided in Chapter 3 (Section 3.1). In the study of the specific rate of waste utilization, biogas and biomass production, and SO_4^{2-} reduction, the experiments were conducted in a batch mode inoculated with a parent acclimated culture that was developed in a semi-continuous-flow reactor. During the bioreaction time, the evolution of COD, MLVSS, total gas and CH_4 was followed. The outcome of the bacterial work that showed the potential of the mixed bacterial groups from various sources were examined. The microorganism comparison studies were divided into four main parts.

- Seed characterization and bacterial morphology.
- Measurement of the specific rate of waste utilization , biogas and biomass production.
- Measurement of the specific rate of sulfate reduction under sulfate reducing condition
- Evaluation of the effect of sulfate and heavy metal in bioreactors.

The major types of bacteria and the SRB in the selected mixed culture were isolated and then identified by the 16S rRNA technic.

4.4 Results and discussion

Different mixed-microbial cultures from various sources did not show the same characteristics, morphology or behavior. The morphology of acclimated bacteria was shown (Figure 4.1). The waste utilization, biomass production, the amount of total biogas and CH_4 generation, the sulfate reduction and the effect of heavy metal in reactors by various seed sources were measured. The kinetics of the potential capability of mixed cultures were ascertained. The specific rates of five assemblages from different locations in terms of waste utilization, biomass and biogas production

were compared. The ability of these assemblages in the reduction of SO_4^{2-} was studied.

4.4.1 Seed Characterization and Bacterial Morphology

The characteristics of the seed sludge from soil, sediment and liquor form, were summarized in Table 4.1.

Bacteria from different sources had different morphology (Figures 4.1(A)-(E)). Investigations on the species composition of the bacteria have shown that all consist mainly of Gram-negative species. Small amounts of Gram-positive species have been found. Because of the multiplicity of species in the mixed-bacteria, the system can adapt well to seasonal changes in temperatures, the influence of toxic substances, pH values deviating from neutral, salt concentrations, and change in the composition of the wastewater. The dominant morphology of bacteria from the mixed liquor in a septic tank and that from the digester of the brewery wastewater treatment plant were cocci. However that of bacteria from the coastal sediment at Pattaya (Chonburi) and a mixed-microbial culture from the leachate at Nongkham landfill site (Bangkok) were bacilli shaped. While bacteria found in acidic sulfate soil at Ongkarak (Nakornayok) were a conglomeration of undefined-shape. Thus, these results suggest that the mixed-bacterial assemblages may play a different significant role in the degradation of organic waste, the production of biomass and biogas, and the reduction of sulfate.

4.4.2 Measurement of the Specific Rate of Waste Utilization, Biomass and Biogas Production and the Assessment of COD Removal Efficiencies.

Regarding to the waste evolution, COD decreases continuously with bioreaction time. Figure 4.2 (Table D-1) shows the time course of COD from those different sources of bacteria. The waste removal is affected by the mixed-bacteria from different sources. Overall reductions in the range 38-63% are obtained.

Table 4.1 Composition of the seed samples in form of soil and sediment.

Seed Samples in the Form of Soil and Sediment	% Moisture	SO_4^{2-} (mg / kg Dry Weight))	NO_3^- (mg / kg Dry Weight)	Organic Matter (% Dry Weight)	Alkalinity (mg / kg Dry Weight as $CaCO_3$)	Cl^- (mg / kg Dry Weight)	TDS (mg / kg Dry Weight)	pH 1:1	ORP 1:1 (mV)	Conductivity 1:1 (μS / cm)
Coastal Sediment	10.98	1217	8.36	0.24	69.00	44283	2505	8.39	-55.1	4720
Acidic Sulfate Soil	23.58	1297	3.31	0.82	ND	1643	1398	2.58	220	2149

ND : Not detectable

Table 4.1 -continued- Composition of the seed samples in form of liquor.

Seed Samples in the Form of Liquid	SO_4^{2-} (mg / l)	NO_3^- (mg /l)	COD (mg /l)	Alkalinity (mg /l as $CaCO_3$)	Cl^- (mg /l)	TDS (mg /l)	pH	ORP (mV)	Conductivity (μS / cm)
Septic Liquor	145	0.913	607	453	764	640	8.36	-41.6	1282
Brewery Wastewater Treatment Plant	7.76	0.319	64256	1318	246	1220	7.93	-16.7	2440
Leachate	852	7.340	800	3492	86725	6755	8.26	-36.1	13500

54

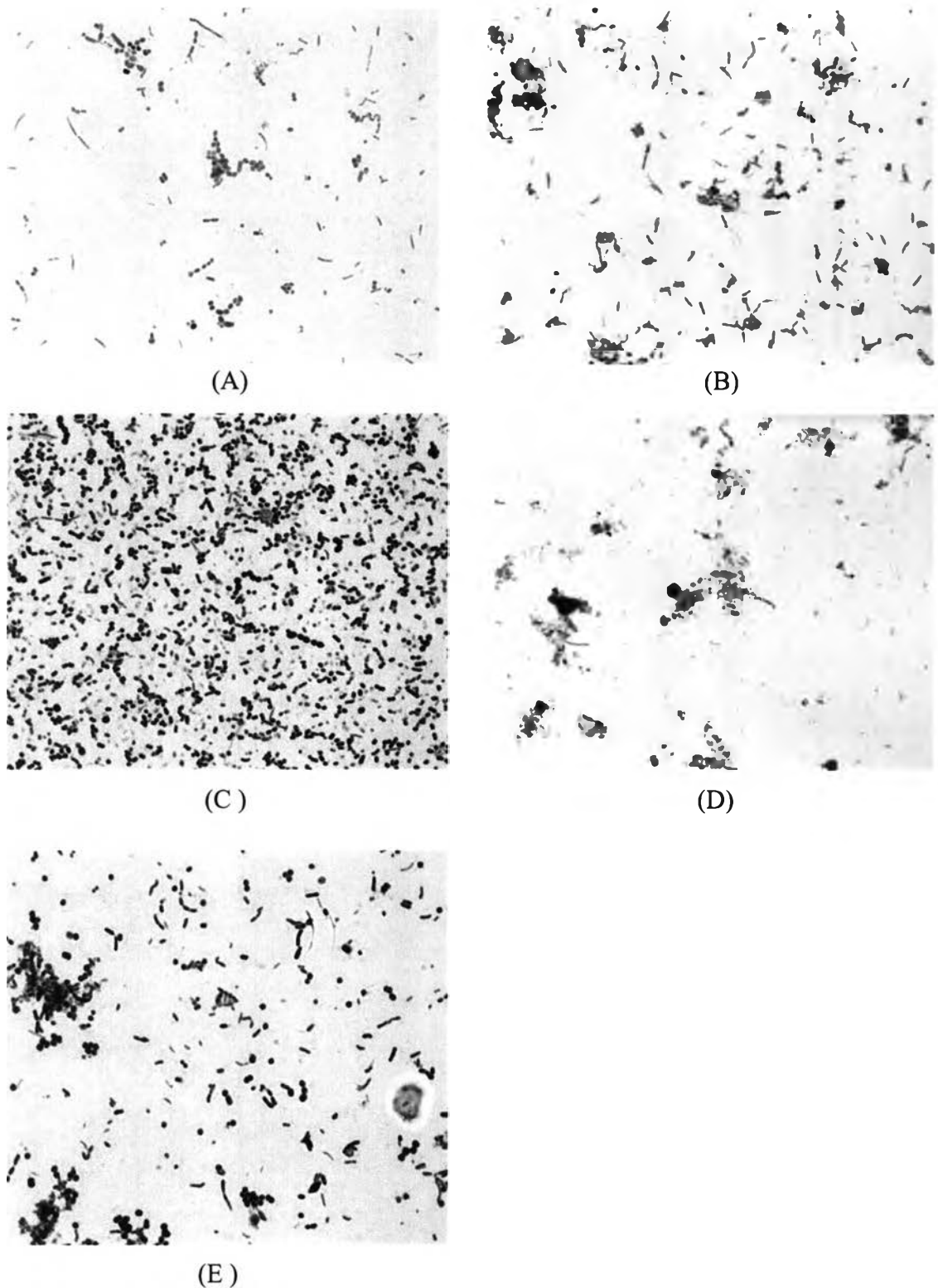
(A)

(B)

(C)

(D)

(E)

Figure 4.1 The morphology of acclimated seed sludge. (A), (B), (C), (D) and (E)
are the sources of seed sludge from septic tank, coastal area (500 m from
the shore), brewery wastewater treatment plant, acidic sulfate soil, and
leachate, respectively.

The COD removal results showed that the performances of the acclimated bacteria selected from various areas were different in digesting the organic waste. The average percentage of COD removal by the bacterial mixed-cultures from the septic tank, coastal area, brewery wastewater treatment plant, acidic sulfate soil, and leachate was 50, 63, 57, 38, and 42, respectively (Figure 4.3 Table D-1).
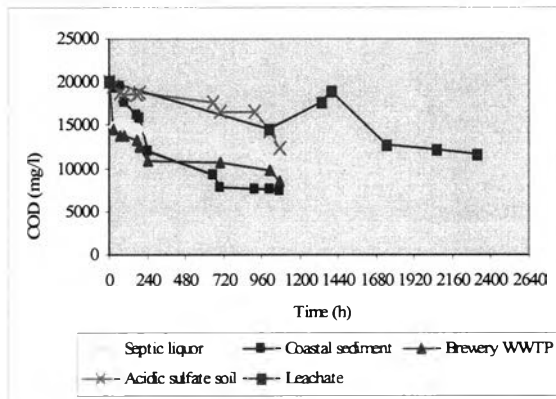
Figure 4.2 The time course of COD from the reactors that contained bacteria from various seed sources.
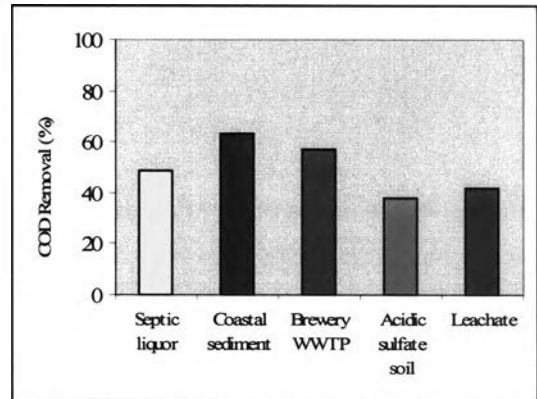
Figure 4.3 The average percentage of COD removal from the reactors that contained bacteria from various seed sources.

Regarding to the biomass, (Table D-2), its evolution follows the typical growth-cycle phases for batch cultivation: after an acclimation period (lag phase), the population of biomass was well adjusted to its new environment. Then, the microorganisms multiplied rapidly and an important increase in biomass concentration was observed (exponential growth phase), until a maximum size of population was reached (stationary phase). Finally, a decline in the biomass took place (death phase of microorganisms). Figure 4.4 shows the evolution of the biomass during bioreaction time in an experiment taken as an example inoculated with mixed-bacteria from septic liquor, with similar trends being observed for bacteria from different sources (Figure not shown and data in Table D-2).

However, in this study the processes were comprised of interrelated, mixed biological populations, (in particular acidogens and methanogens) resulting in the system having its own growth curve based upon overall combination of the growth of the individual bacterial types. The position and shape of a particular growth curve in the system, on a time scale, depended on the food or metabolites (VFA' s) available in the controlled environment.
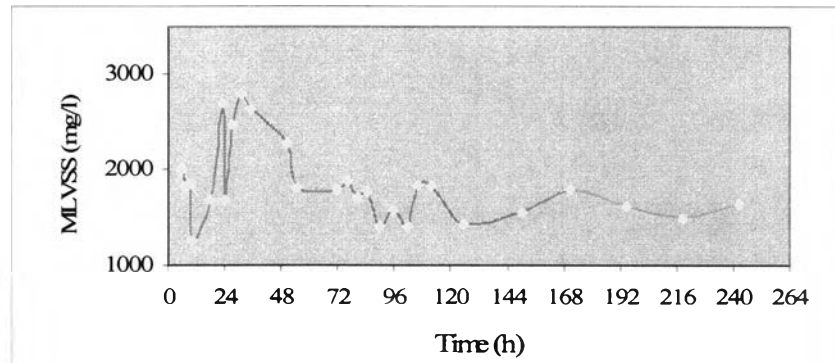
Figure 4.4 Evolution of biomass concentration in the anaerobic biodegradation of glucose from mixed-bacteria from a septic tank.

4.4.2.1 Waste Utilization

The value of the specific waste utilization rate, K, for the kinetic equations depends primarily on the specific wastewater of interest, because the species of organic waste vary for different wastewaters and, in particular, for industrial wastewaters and municipal wastewaters containing significant amounts of industrial wastes. However, in this study only glucose was used as substrate, thus, the overall rate constant, K, varied for different groups of microbial species in an acclimated bacteria.

In this study the specific waste utilization rate, K, was determined. The COD and MLVSS concentrations at various reaction times were shown in Table D-3.

From an industrial point of view, the most interesting period in the growth cycle of a batch cultivation is the exponential growth phase, when the population of biomass is perfectly acclimated to the substrate. In this period, the rate of production of biomass (X) is well described by the kinetic equation.

The rate of waste utilization by microbes can be represented by the Michaelis-Menten equation or by the specific rate of waste utilization equation. If a batch reactor is inoculated and values of cell mass and waste concentrations versus time of reaction are obtained, the data may be analyzed by either of the two approaches (Chapter 2 Section 2.1.1.3) to give the kinetic constants required for design. The inoculum must come from an acclimated parent culture developed in continuous-flow reactor in order for meaningful data to be obtained.

The specific rate of waste utilization equation is based on two limiting cases of the Michaelis-Menten equation. In the first case, the waste concentration is relatively large and the Michaelis-Menten equation is reduced to a pseudo-zero order reaction. In the second case, the waste concentration is relatively small and the Michaelis-Menten equation is reduced to a pseudo-first-order reaction. If the specific rate of waste utilization is a pseudo-zero order reaction, the rate equation is

$$\frac{-dS/dt}{\overline{X}} = K \qquad (4.1)$$

Where

$\dfrac{-dS/dt}{\overline{X}}$ = specific rate of waste utilization, mass/(mass microbes)(time)

dS/dt = rate of waste utilization, mass/(volume)(time)

K = specific waste utilization rate, $time^{-1}$

The negative sign indicates that the substrate is decreasing with respect to time. Rearranging equation(4.1) for integration gives

$$\int_{S_o}^{S_t} dS = -K\overline{X} \int_o^t dt \qquad (4.2)$$

Where

K = rate constant, specific waste utilization rate, $time^{-1}$

$\overline{X}$ = average cell mass concentration during the biochemical reaction-that is,
$X = (X_o + X_t)/2$, where X_o and X_t are the cell mass concentrations at the respective times $t = 0$ and $t = t$, mass/volume

Integration of equation (4.2) yields

$$S_t - S_o = -K\overline{X}t \qquad (4.3)$$

or

$$S_t = S_o - K\overline{X}t \qquad (4.4)$$

Equation (4.4) is of the form $y = mX + b$; thus plotting S_t on the y-axis versus X t on the x-axis on arithmetical paper will result in a straight line if the reaction if the reaction is pseudo-zero order, and the slope will be equal $-K$.

If the specific rate of waste utilization is a pseudo-first order reaction, the rate equation is

$$\frac{-dS/dt}{\overline{X}} = KS \qquad (4.5)$$

Where

K is the rate constant, or specific waste utilization rate, volume/ (mass microbes)(time). Rearranging for integration gives

$$\int_{S_o}^{S_t} \frac{dS}{S} = -K\overline{X}\int_{o}^{t} dt \qquad (4.6)$$

Integration results in

$$Ln\ S\ ]_{S_o}^{S_t} = -K\overline{X}\ t \qquad (4.7)$$

Equation (4.7) may be simplified to

$$Ln\ S_t = Ln\ S_o - K\overline{X}t \qquad (4.8)$$

Equation (4.8) is of the form $y = mx+b$; thus plotting S_t on the y-axis versus $\overline{X}$ t on the x-axis on semilog paper (or plotting Ln S_t on Y-axis versus $\overline{X}$ t on arithmetrical paper) will results in a straight line if the reaction is psudo-first order, and the line slope will equal $-K$.

Since in general wastewaters there are numerous waste present, the rate constant, K, for the kinetic equations and the constants for the Michaelis-Menten equation represent overall average values.

The parameter used for waste in the previous kinetic equations must be in terms of biodegradable material since the waste represents the food substance or substrates for the microbes. It could be the 5-day BOD, the biodegradable fraction of the COD, the biodegradable fraction of the TOC, or the biodegradable fraction of any other organic parameter used. In this study, the biodegradable COD was used.

For this evaluation, the terms Ln S_t, Ln S_o, and K$\overline{X}$ t were calculated by fitting the experimental data (S, t, and $\overline{X}$) to a polynomic expression by least-square regression analysis, deriving this expression with respect to the time and multiplying by biomass concentration ($\overline{X}$) at any time. According to the procedure, the experimental data were in Table D-3 and Figures 4.5(A)-(E) that show the plot for

experiments. It can be seen that, points lie satisfactorily around a straight line, which confirms that the selected model is adequate to correlate the anaerobic biodegradation of this waste to mixed-bacteria from all of the sources taken in this study.

Hence, the waste utilization rate is well described by a pseudo-first order kinetic equation ($S_o >> S_1$). The slope K, was determined. The numerical waste utilization rate (K) obtained from mixed bacteria from five different sources were compared.
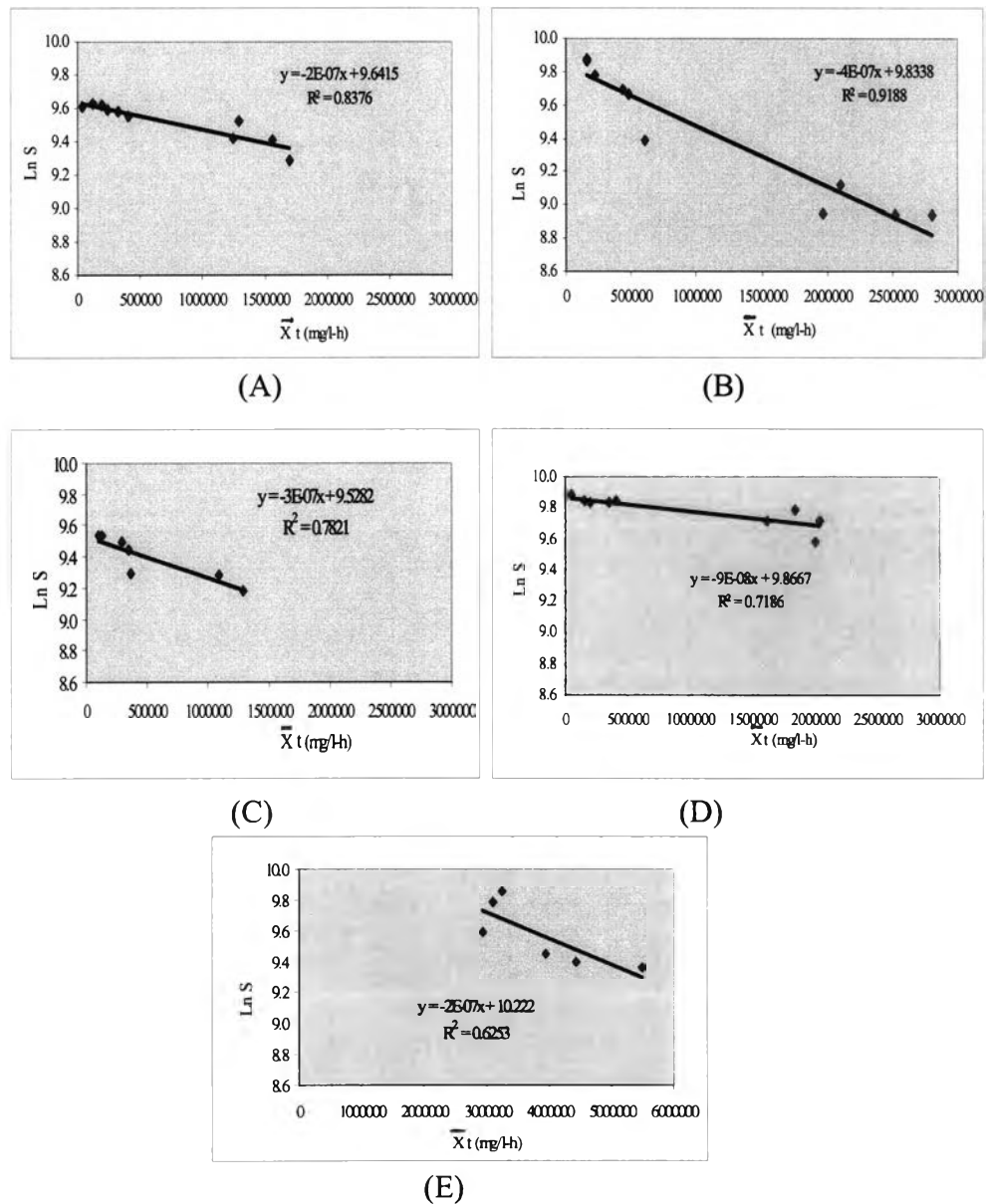

(A)

(B)

(C)

(D)

(E)

Figure 4.5 Graphical determination of the specific waste utilization rate, K, of mixed-bacteria from various sources. (A), (B), (C), (D) and (E) show the numerical values of acclimated seed sludge from septic liquor, coastal sediment, brewery wastewater treatment plant, acidic sulfate soil, and leachate, respectively.

Each mixed-microbial assemblage has its own particular rate of waste utilization. After least-square regression analysis, the slopes are determined, which provide values of 2×10^{-7} (septic liquor), 4×10^{-7} (coastal sediment), 3×10^{-7} (brewery wastewater treatment plant), 9×10^{-8} (acidic sulfate soil), and 2×10^{-7} (leachate) (liter/mg-hour). There is a variation in the rate constant of bacteria from different sources. The large value for K suggests that bacteria have more easily waste bioutilized. The variation in K is even for a certain type of bacteria. The lower K values are from bacteria relatively difficult to waste bioutilized. The results show that bacteria from the coastal sediment followed by bacteria from the brewery wastewater treatment plant are the top and the second top most bacteria that are easily biodegraded.

The numerical values of the specific waste utilization rate that was determined from different mixed-bacteria from various sources were summarized in Table 4.2.

4.4.2.2 Biomass Production

Another kinetic parameter is related to biomass evolution during growth phase of a batch cultivation: the cellular yield coefficient $Y_{X/S}$. This parameter defined as the ratio of biomass produced and waste utilized, and can be expressed by the equation:

$$Y_{X/S} = \frac{dX}{dS}$$

$$Y_{X/S} = \frac{\Delta X}{\Delta S}$$

The cell growth, substrate utilization and effects of endogenous metabolism are considered. In a batch culture, if substrate for growth was present in only limited amounts, it would be depleted first and growth would cease. A portion of the substrate is converted to new cells and a portion is oxidized to organic end products. Because the quantity of new cells produced has been observed to be reproducible for a given substrate, the following relationship has been developed between the rate of substrate utilization and the rate of growth.

$$r_g = -Y r_{su} \qquad (4.9)$$

$r_g = dX/dt$ = rate of bacterial growth, mass/unit volume . time

Y = maximum yield coefficient, mg/mg (defined as the ratio of the mass of cells formed to mass of substrate consumed, measured during any finite period of logarithmic growth)

r_{su} = dS/dt = substrate utilization rate, mass/unit volume . time

In bacterial systems used foe wastewater treatment, the distribution of cell ages is such that not all the cells in the system are in the log growth phase. Consequently, the expression for the rate growth must be corrected to account for the energy required cell maintenance. Other factors, such as death and predation, must also be considered. Usually, these factors are lumped together, and it is assumed that the decrease in cell mass caused by them is proportional to the concentration of organisms present. This decrease is often identified in the literature as the endogenous decay. The endogenous decay term can be formulated as follows:

$$r_d \text{ (endogenous decay)} = -k_d X \qquad (4.10)$$

where

k_d = endogenous decay coefficient, $time^{-1}$

X = concentration of cells, mass/unit volume

When Equation (4.10) is combined with Equation (4.9)

$$r'_g \quad = \quad -Y r_{su} - k_d X \qquad (4.11)$$

r'_g = net rate of bacterial growth, mass/unit volume . time

$$dX/dt \quad = \quad -Y(dS/dt) - k_d X \qquad (4.12)$$

$$(X-Xo)/\Delta t \quad = \quad -Y(S-So/ \Delta t) - k_d X \qquad (4.13)$$

Divided by (X-Xo)

$$1/\Delta t \quad = \quad Y(-S+So)/ (X-Xo)\Delta t - k_d X /(X-Xo) \qquad (4.14)$$

$$1/\Delta t \quad = \quad Y (So -S)/(X-Xo)\Delta t - k_d (X)/(X-Xo)$$

Assuming that Xo is much less than X. Thus,

$$1/\Delta t \quad = \quad Y (So -S)/(X-Xo)\Delta t - k_d \qquad (4.15)$$

According to equation (4.15), a plot of $1/\Delta t$ values versus $(So-S)/(X-Xo)\Delta t$ values in the experiments must lead to a straight line whose slope and intercept will be will be Y (or $Y_{X/S}$) and k_d, respectively.

Computation table to determine the coefficients $Y_{X/S}$ and k_d was set in Table D-4 and the plot was as shown in Figures 4.6 (A-E).
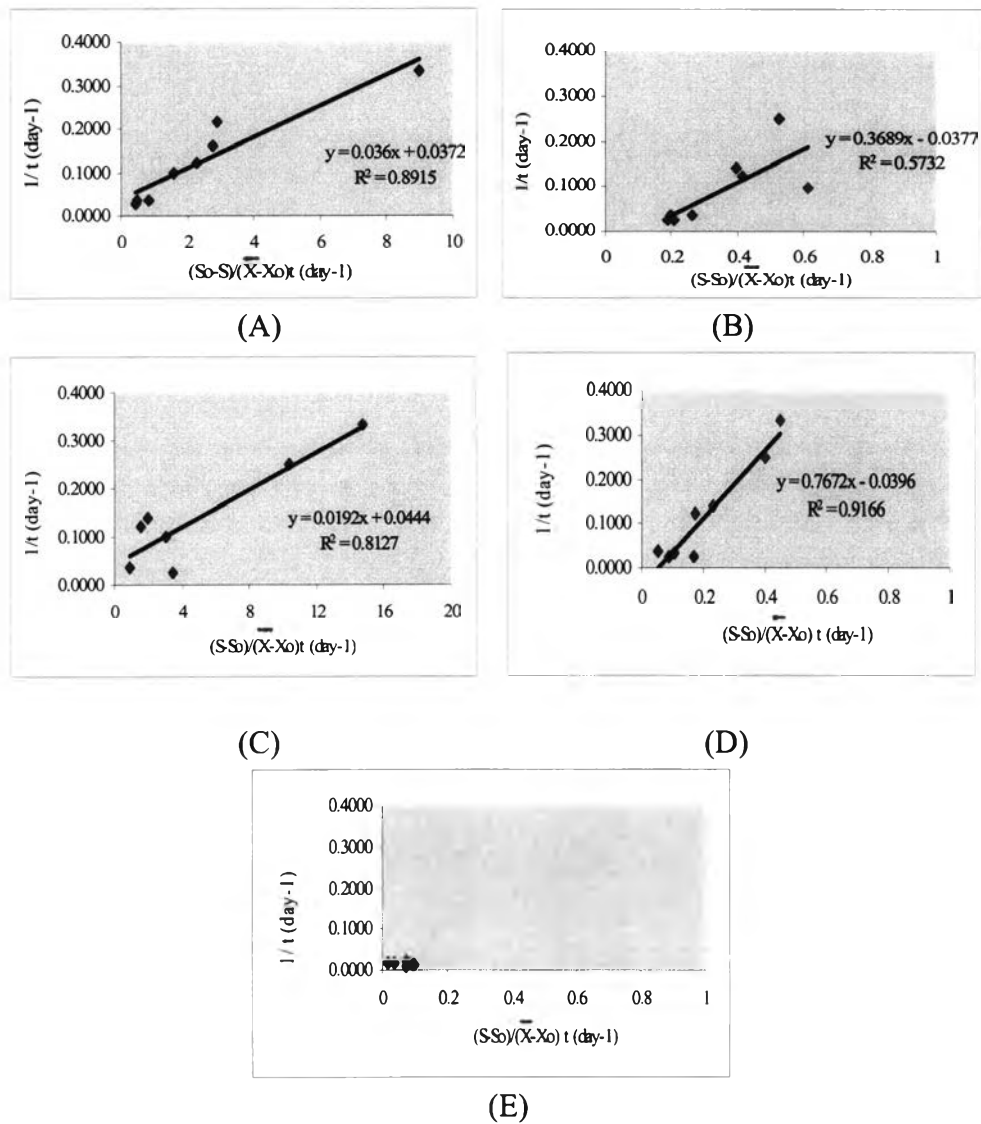
Figure 4.6 Graphical determination of the cellular yield coefficient, $Y_{X/S}$, of mixed-bacteria from various sources. (A), (B), (C), (D) and (E) show the numerical values of acclimated seed sludge from septic liquor, coastal sediment, brewery wastewater treatment plant, acidic sulfate soil, and leachate, respectively.

Figures 4.6 (A)-(E) show the plot of data obtained from the experiments. Points lie satisfactorily around a straight line with positive slope, $Y_{X/S}$, and an intercept, k_d, confirms the validity of Equation (4.15). After least-square regression analysis, a slope of 0.036 (septic liquor), 0.3689 (coastal sediment), 0.0192 (brewery WWTP), 0.7672 (acidic sulfate soil), and not detected (leachate) (expressed in the unit of mg cell created/mg substrate utilized) are obtained. These values are close to

with others proposed in the literature for the overall combined anaerobic biodegradation, i.e. 0.05-0.10, of different wastewaters (Tchobanogous et al., 2003).

An intercept of 0.0372 (septic liquor), -0.0377 (coastal sediment), 0.0444 (brewery WWTP), -0.0396 (acidic sulfate soil), and not detected (leachate) (expressed in the unit of day^{-1}) are obtained. The values proposed in the literature are -0.03 to - 0.04 day^{-1} (Tchobanogous et al., 2003).

As for the reactor that contained mixed bacterial culture from leachate (Figure 4.6 (E)), there were not enough data to determine the cellular yield coefficient $(Y_{X/S})$ and the endogenous decay coefficient (k_d). This culture need more time in acclimation.

The cellular yield coefficient, $Y_{X/S}$, from the reactor that contained mixed-bacteria from acidic sulfate soil is the highest value. The high value for $Y_{X/S}$ suggests a very high amount for biomass production. $Y_{X/S}$ is the rate at which cell growth will take place when completely controlled by the rate of substrate flux toward the cell. Bacteria from acidic sulfate soil produce the least yield in the form of biogas (4.4.2.3), hence most of the waste utilized would become the yield in the form of biomass. Mixed culture consists of different bacterial types that performed different function. This $Y_{X/S}$ expresses the overall value of mixed-bacteria whose role is different, i.e. acid production, CH_4 production and SO_4^{2-} reduction, and so on. Thus, the cellular yield coefficient is not the only parameter which consent the bacterial mutualism to function in the way that show the advantage of the anaerobic process in CH_4 production, COD utilization, and SO_4^{2-} reduction.

Biomass yield coefficient minimization is a major requirement in processes for the biotreatment of sewage and industrial wastewater. High biomass from substrate (waste) yield coefficients result in excessive production of a major by product, i.e., sludge (Geoffrey, 1997). In this study, bacteria from a brewery wastewater treatment plant yield the lowest value compared with all cultures from five sources.

Feitkenhauer et al. (2001) studied the growth kinetics of the thermophilic *Bacillus thermoleovorans* sp. A2 on phenol as sole carbon and found that the yield coefficient, $Y_{X/S}$, of 0.8-1 gram cell dry weight/ gram phenol. They reported the cell yields, $Y_{X/S}$, of mesophilic bacteria were in the range 0.4 - 0.52 gram cell dry weight/ gram phenol. In this present study, the experiments revealed the higher values of

the cellular yield coefficients. This is because the experiments and determination were based on glucose removal as a model substrate and the microorganisms were the mixed-microbial assemblage which performed mutualistic function in waste utilization.

From this experiment, the $Y_{X/S}$ could not be determined from bacteria originate from leachate. It can be explained that the rate of production of biomass is well described by the kinetic equation when the population of biomass is perfectly acclimated to the substrate. Bacteria from leachate need more time to acclimate to reduce the lag time. This reflected the time consumed in the start-up for system operation.

Another parameter that was interested is the endogenous decay coefficient, k_d. This value is also of importance. Mixed-bacterial culture from a brewery wastewater treatment plant shows the highly tolerated performance in the system toward the lowest numerical value obtained.

Very few studies concerning the growth of microorganisms take any account of microbial death (Geoffrey, 1997). Microorganisms are not immortal (Mason et al., 1986). The fundamental problem that is encountered in differentiating between living and dead microorganisms is that for a microbe to be identified as live in conventional test procedures, it is also necessary for the microbe to be viable. However, in investigation where efforts have been made to identify dead microbes as a component state in growing cultures, numbers have been shown to be vanishingly small, prompting the hypothesis that death occurs as a result of lysis, such that under typical growth conditions death and lysis are coincident events.

The numerical values of cellular yield coefficient ($Y_{X/S}$) and endogenous decay coefficient (k_d) that were obtained from different mixed-bacteria from various sources were summarized in Table 4.2.

4.4.2.3 Biogas Production

Regarding to biogas evolution, total gas occurred after 2 days of operation. Figure 4.7 (Table D-5) shows the time course of the total gas production from bacteria from fives different sources. The rate of total gas production was determined and shown in Figure 4.8 (Table D-5). The percentage of CH_4 was gradually increased after

24 hours of operation. The percentage of CH_4 profile and the amount of CH_4 were shown in Figures 4.9 and 4.10 (Tables D-6 and D-7), respectively. As for leachate, it was noticed that the time consumed to start producing CH_4 gas (the lag time) was more than 20 times higher than that of the bacteria from any other sources (Figure 4.10). This reflected the time that was consumed in the start-up for the whole system operation, not of particular microorganisms. This culture need more time in acclimation.
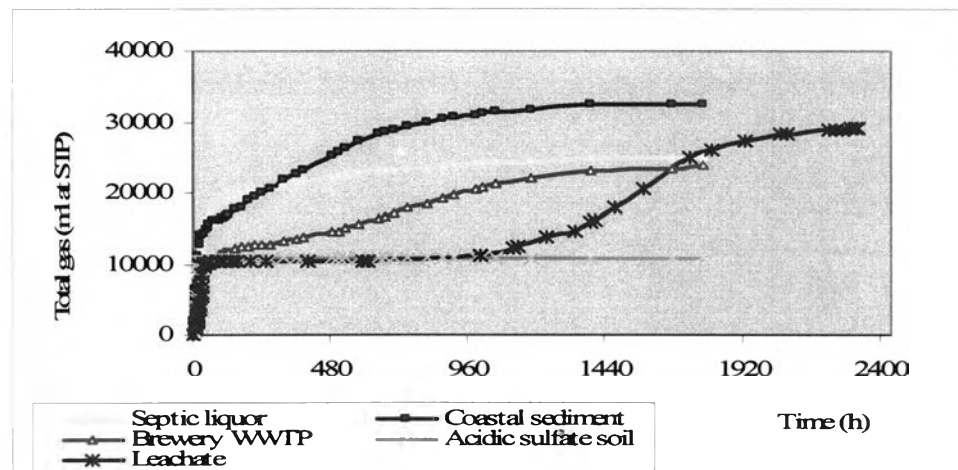

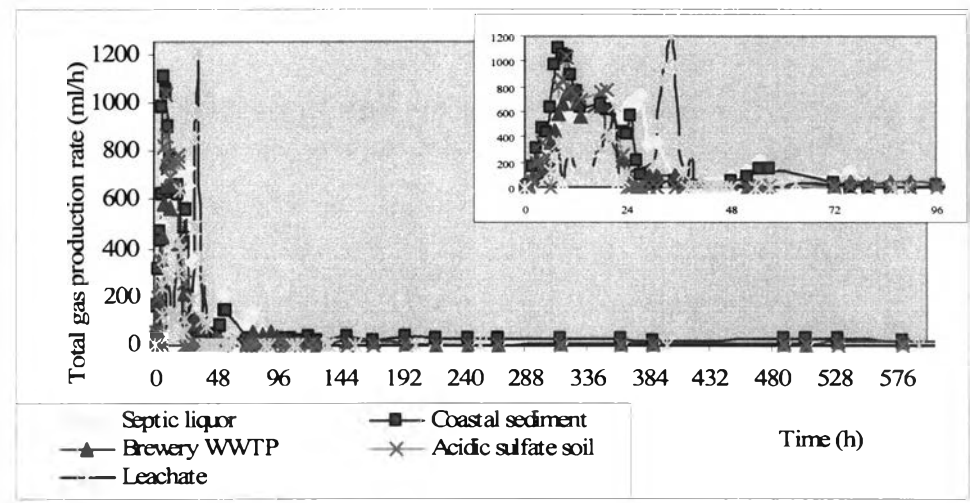**Figure 4.7** The time course of total gas production at STP.

Figure 4.8 The total gas production rate. The phases separation, acidogenic and methanogenic phase, were shown.
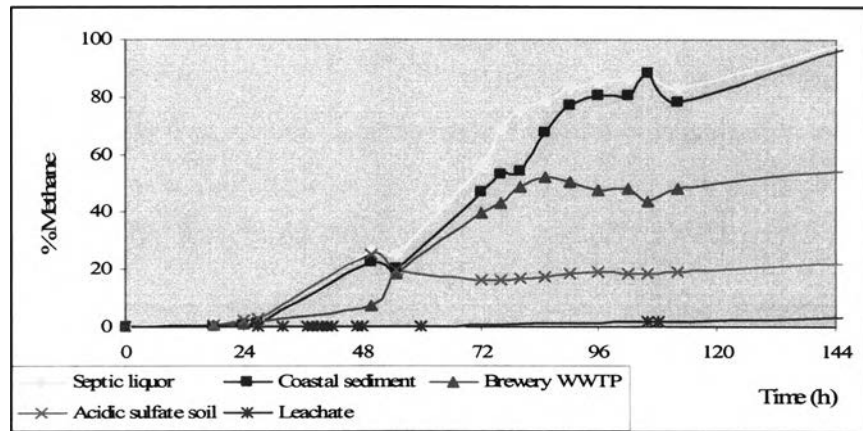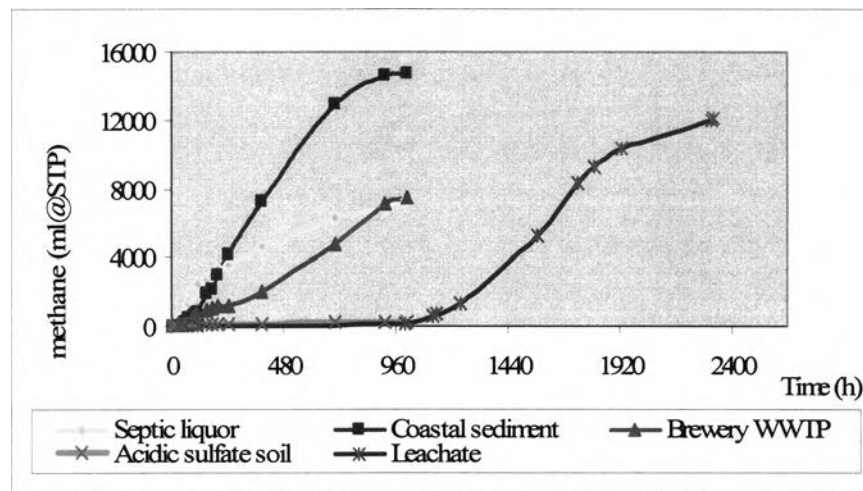
Figure 4.9 The methane production profile.

Figure 4.10 The time course of methane production.

Note: The total gas that was recorded at room temperature (31.5^{0}C) was converted to the amount of total gas at STP (Appendix A).

The CH_4 production profile in Figure 4.9 clearly differentiate acidogenic and methanogenic phases. During an experiment, the evolution of the waste concentration and biomass were also followed. Then the specific CH_4 production rate were determined.

The Michaelis-Menten equation also gives the relationship between the rate of fermentation product production, dP/dt. The rate of product production may also be expressed as the specific rate of product production, (dP/dt)/X, where X is the cell mass concentration (Aiba et al., 1965).

$$\frac{dP/dt}{X} = K_p \qquad (4.16)$$

Where

K_p is the specific CH_4 production rate, volume/(mass microbes)(time). Rearranging for integration gives

$$\int_{P=o}^{P=P} dP = K_p \int_{o}^{t} \overline{X} \, dt \qquad (4.17)$$

($\overline{X}$ is used in the determination.)

Integration results in

$$P = K_p \, \overline{X} \, t \qquad (4.18)$$

Equation (4.18) is simplified and is of the form y = mx; thus plotting P on the y-axis versus $\overline{X}$t on the x-axis results in a straight line and the line slope equals K_p, which is the specific CH_4 production rate. Computation table to determine the coefficients K_P was set in Table D-8 and the plot was shown in Figures 4.11 (A)-(E).

The experimentally determined data pairs (P, $\overline{X}$t) were used to plot for determining the specific CH_4 production rate, K_P. After least-square regression analysis, a slope of 0.0033 (septic liquor), 0.0059 (coastal sediment), 0.0072 (brewery WWTP), 0.00008 (acidic sulfate soil), and 0.0097 (leachate) (expressed in the unit of ml at STP/(mg/l of biomass)-hour) are obtained.

We can represent product production as a conversion of a number of waste, dP/dS. Described formalism is useful as a first step in biotechnological studies aimed at planning and optimization of microbial growth. It gives estimate how much waste should be supplied to reactor to obtain required amount of target product. where dP is the increase in product production consequent on utilization of the amount dS of waste, and dP and dS are respective infinitely small increments. It should be noticed that rigorous definition of $Y_{P/S}$ as derivative dP/dS stems from the fact that $Y_{P/S}$ can vary in time, the negative sign being introduced because of P and S vary in opposite senses. It expresses explicitly the waste utilization for product: how much mass units of particular waste should be consumed to produce one unit volume of CH_4. Computation table to determine the coefficients $Y_{P/S}$ was set in Table D-9 and the plot was shown in Figures 4.12 (A)-(E).
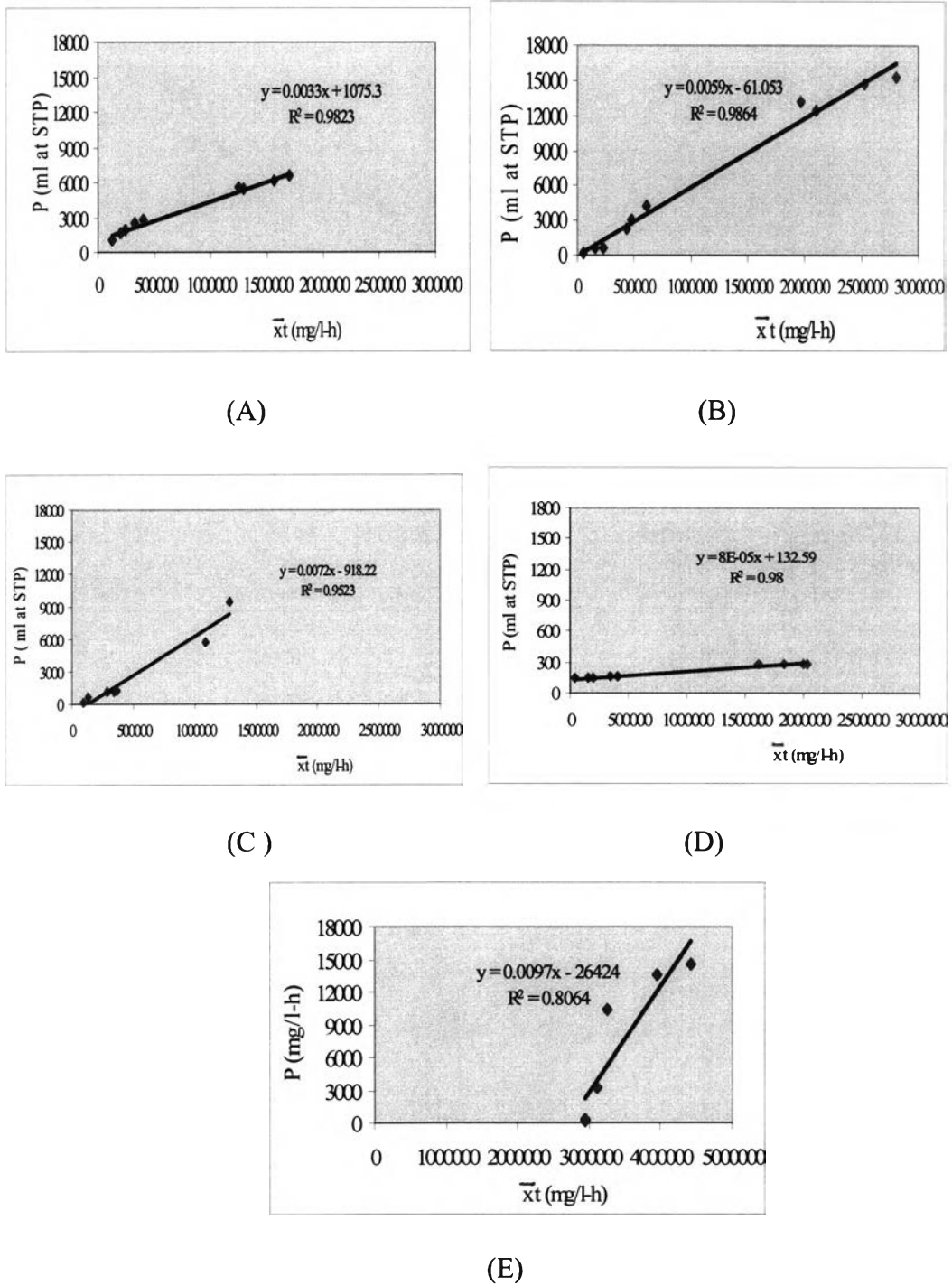
(A)

(B)

(C)

(D)

(E)

Figure 4.11 Graphical determination of the specific methane production rate , K_p, of mixed-bacteria from various sources. (A), (B), (C), (D) and (E) show the numerical values of acclimated seed sludge from septic liquor, coastal sediment, brewery wastewater treatment plant, acidic sulfate soil, and leachate, respectively.

Figure 4.12 Graphical determination of the methane production yield coefficient, $Y_{P/S}$, of mixed-bacteria from various sources. Accumulated methane and COD removed were used in the determination. (A), (B), (C), (D) and (E) show the value of acclimated seed sludge from septic liquor, coastal sediment, brewery wastewater treatment plant, acidic sulfate soil, and leachate, respectively.
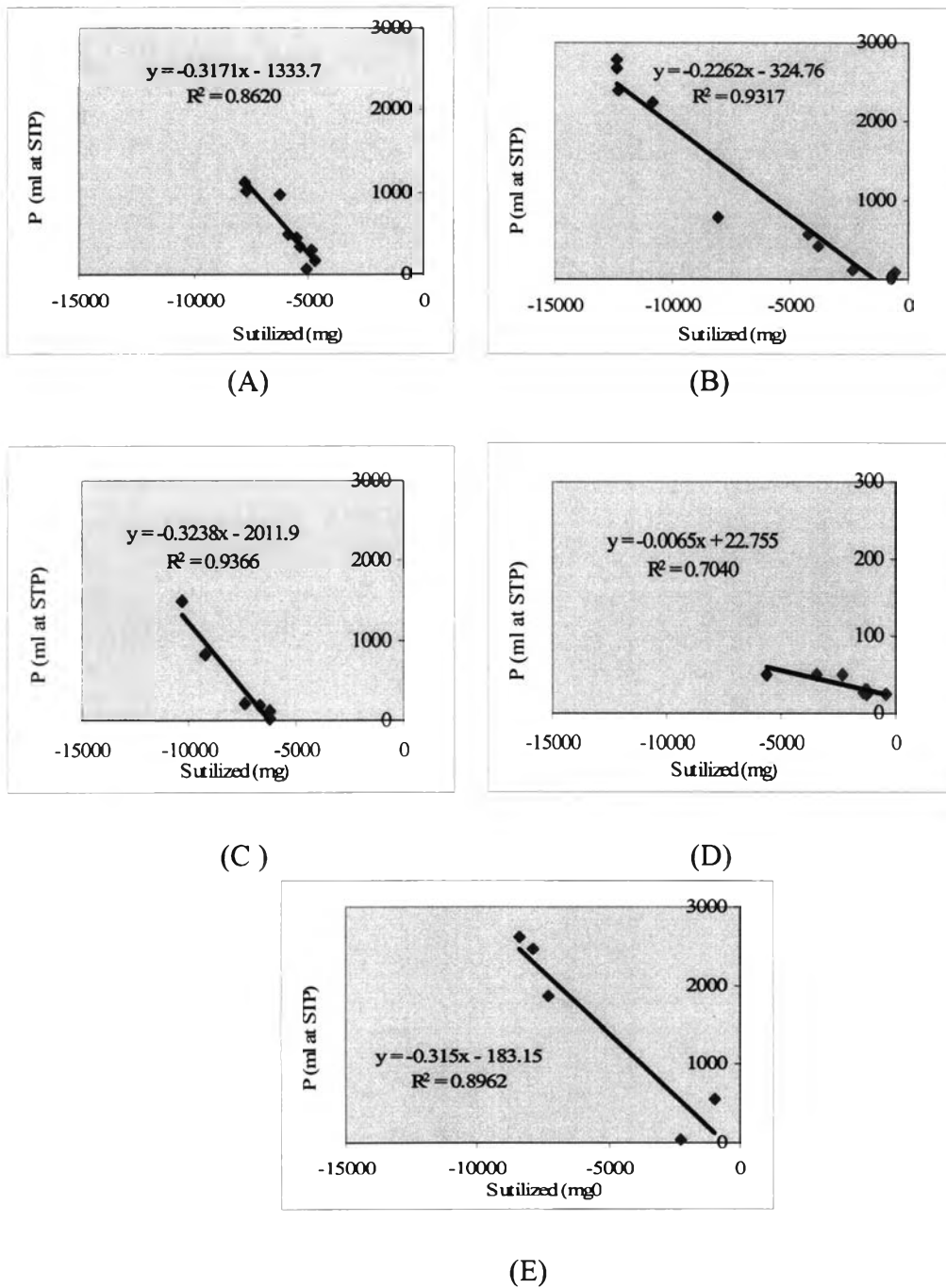
A plot of the cumulative volume of CH_4 against the S utilized ,$(S_0\text{-}S_t)$, is a straight line of slope coinciding with the CH_4 production yield coefficient ($Y_{P/S}$). The CH_4 production yield coefficient, $Y_{P/S}$, defined as the ratio of CH_4 produced in the experiment to the waste utilized. From this study, a slope of 317 (septic liquor), 226 (coastal sediment), 324 (brewery WWTP), 65 (acidic sulfate soil), and 315 (leachate) expressed in ml (at STP) per gram of biodegradable waste utilized. Estimates of CH_4 production yield coefficient, $Y_{P/S}$, were comparable for all types of bacteria, which exhibited a different yield. These data suggest that, bacteria from a brewery wastewater treatment plant exhibited the highest value of CH_4 production yield coefficient, $Y_{P/S}$. This seems to be support the results from the specific CH_4 production rate, K_P (Figure 4.11(C)). The highest possible proportion of CH_4 was produced from a gram of biodegradable waste. This shows the highest performance of this mixed-bacterial culture to degrade environmental pollutant and yield the highest amount of CH_4. The theoretical amount of CH_4 of 350 ml at STP per a gram of glucose utilized is reported (Tchobanoglous and Burton, 1991). Table 4.2 summarized the numerical values of CH_4 production yield coefficient, $Y_{P/S}$, obtained from this study.

4.4.2.4 VFA Degradation

Figures 4.13 (A), (B), (C), and (D) showed the time course of VFA's in the system which contained mixed-bacterial cultures from different sources. Data were recorded in Table D-10. In acidogenesis, glucose was degraded and served as electron donor. The principal products of fermentation were butyric, propionic and acetic acid. The butyric and propionic acid would be fermented further to produce H_2, CO_2, and acetic acid. The time for acetic acid formation was around 2 days (Figure 4.14 and Table D-11). Butyric and propionic acid were the metabolites at much lower rates. The increase of butyric and propionic acid was due to glucose fermentation. The decrease of acetic acid (Figure 4.14) was related to the tendency of CH_4 formation (Figure 4.10). The sludges did not show the same behavior. The bacteria originating from the septic liquor, the coastal sediment and the brewery wastewater treatment plant reduced a large part of the initial COD (Figure 4.3) without acetate accumulation (Figures 4.13 and 4.14) and produced a high amount of CH_4 (Figure 4.10), while the

sludge from acidic sulfate soil showed a poor CH_4 production and high acetate accumulation (Figure 4.13 (D)).
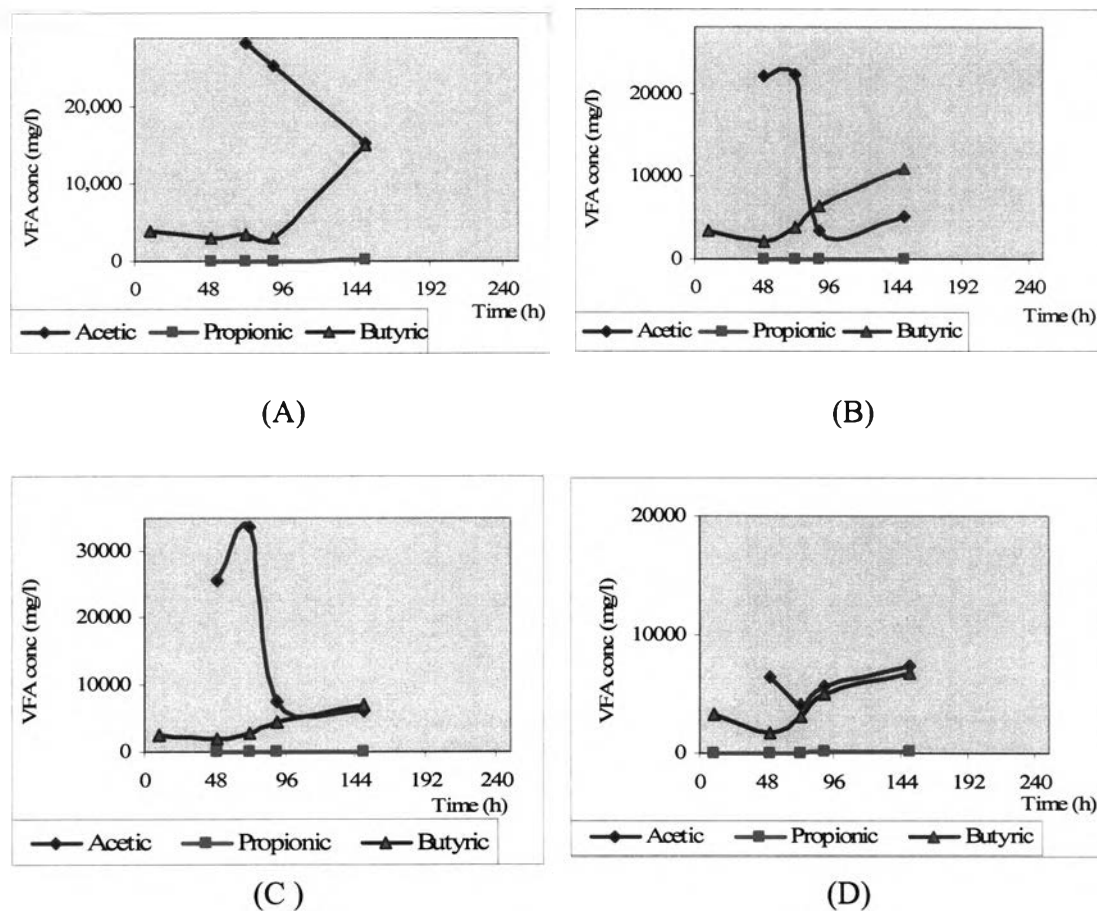

(A)

(B)

(C)

(D)

Figure 4.13 The time course of VFA degradation from reactors that contained mixed-bacteria from various sources. (A) septic liquor, (B) coastal sediment, (C) brewery wastewater treatment plant, and (D) acidic sulfate soil.

Among five different assemblages, the bacteria from the acidic sulfate soil had the lowest COD removal efficiency and CH_4 production (Figures 4.3 and 4.10). The VFA's, especially acetic acid, were found to be accumulated in the reactor (Figure 4.13(D)). When accumulation of VFA's was taken place, this preparation appeared to inhibit more in the growth of methanogens because the corresponding CH_4 production decreased. On the other hand, the bacteria of the coastal sediment and the brewery wastewater treatment plant performed satisfactorily in terms of conversion of initial COD to acetic. Acetic acid produced from the coastal sediment assemblages and the brewery wastewater treatment plant assemblages was readily used as substrate, while

the content of propionic and butyric acid remained low. The present investigation confirms the importance of the sludges consisting of different populations that are able to degrade more effectively the volatile fatty acids produced in the acidogenic phase of anaerobic digestion. The number and type of bacterial populations enriched in the process is very important for rapid and complete conversion of intermediate acid products derived from anaerobic metabolism.
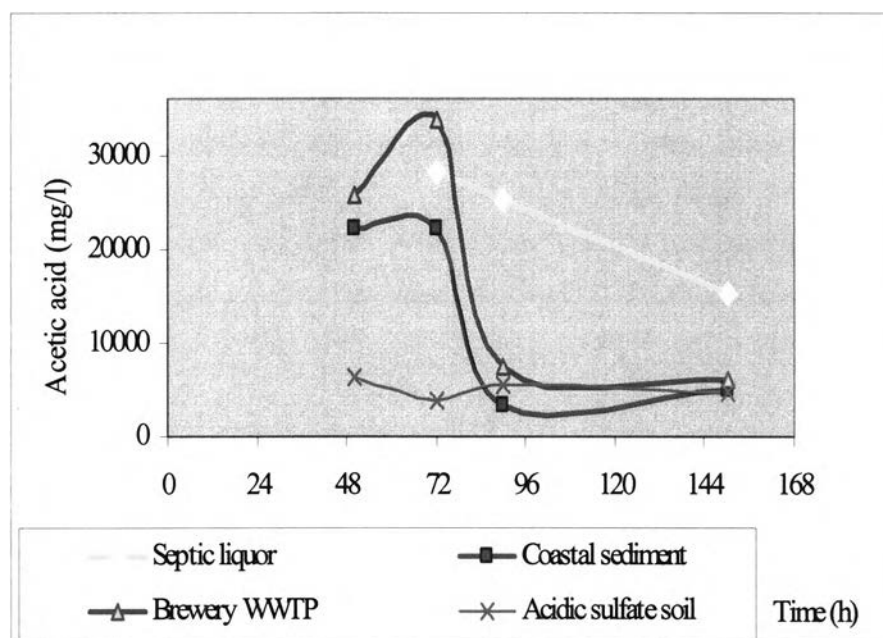

Figure 4.14 The time course of acetic acid in reactors that contained mixed-bacteria from various sources.

As observed in Figure 4.14, the acetic acid, a major substrate for methanogenesis, decreased continuously with the degradation time in accordance with the progression of CH_4 production (Figure 4.10). This result strongly suggested that acetate in the solution was also mostly used as a substrate for methanogenesis. The decrease in acetic acid concentration and the increase in propionic as well as butyric concentration occurred after 2 days.

4.4.3 Measurement of the Specific Rate of Sulfate Reduction Under Sulfate Reducing Condition and the Evaluation of the Effect of Sulfate Reduction and Heavy Metal in Bioreactors

For the comparison studies of the specific sulfate reduction rate and the effect of the presence of heavy metal, the two groups of reactors, A and B, were fed with the glucose-based solution. Sulfate at the concentration of 2,100 mg SO_4^{2-}/l was measured to all 10 reactors of Groups A and B. In addition, an initial Cu (the model metal used) at the concentration of 10 mg/l was added to five reactors of group B. (Section 3.1.5). For group B, The system recovery from Cu commingling with SO_4^{2-} was observed.

The occurrence of sulfate reduction in anaerobic bioreactors is in most cases unwanted. Sulfate reduction can be used to remove sulfate and heavy metals from wastewaters, but in these cases sulfate reduction has to be favored.

This part of the study addressed the role that sulfate reducing bacteria (SRB) played in the removal of Cu. In these processes, H_2S produced by the SRB was used to precipitate CuS. In the biosulfide process, the biological sulfate reduction occurred in the reactors from all of the five different sources of bacteria. Visual observation of the solids indicated a very fine black suspended precipitate appearing in all of the reactors that then had dense packing and good settling.
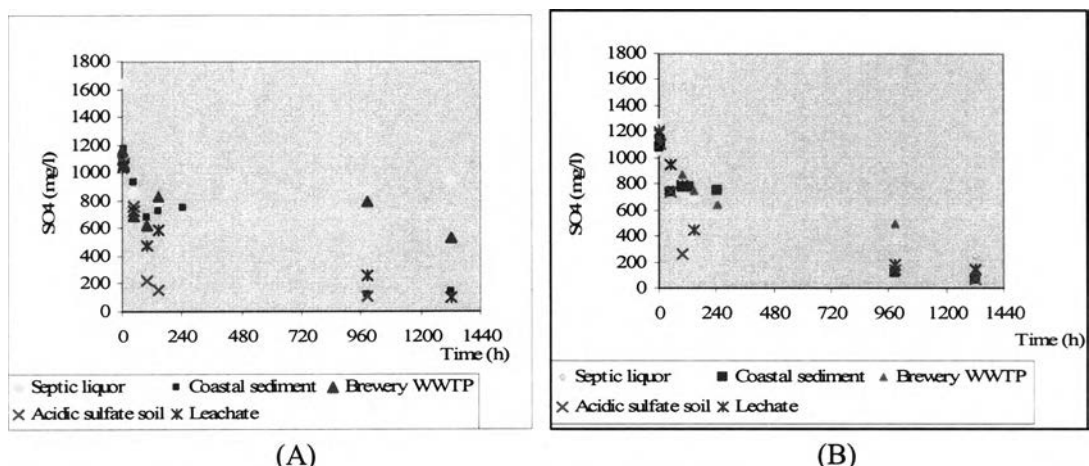

(A)   (B)

Figure 4.15 The time course of sulfate reduction. Group A reactors contain only sulfate. Group B reactors contain both sulfate and Cu.

During an experiment, the evolution of the concentration of SO_4^{2-} from all reactors was followed (Table D-12). Figures 4.15(A) and (B) show the evolution of SO_4^{2-} of the Group A and Group B reactors during bioreaction time in experiments. Sulfate reduction occurred in both the acidogenic and methanogenic phases since the beginning. Different seed types showed differences in the rate of sulfate reduction. This observation showed the different performances of SRB from different sources. Intriguingly, it was observed (from graph) that the amount of SO_4^{2-} reduction in the Group B reactors was higher than that of Group A. This implied the system recovery from Cu commingling with SO_4^{2-} for Group B reactors.

Figure 4.15(A) depicted that SO_4^{2-} was reduced continuously at the beginning but the reduction was disrupted. H_2S occurred in the system may cause the system unfavorable by inhibiting the SRB. When the system contains a heavy metal (Figure 4.15(B)), Cu support detoxification then it is harmless to microorganisms and their environment. Thus, sulfate was reduced continuously.

Here again, the specific SO_4^{2-} reduction rates were assumed to base on the Michaelis-Menten equation. The Ln SO_4^{2-} at bioreaction time was calculated and plotted versus time multiply by average biomass at any time, $\overline{X}$ t (similarity to Equation 4.8). Computation tables were set (Tables D-13 for Group A and Table D-14 for Group B reactors). The specific of SO_4^{2-} reduction rate of bacteria from the five sources with SO_4^{2-}(Group A) and with SO_4^{2-} commingling with Cu (Group B) were determined.

Bacteria from all sources can reduce SO_4^{2-} in anaerobic system. Figure 4.16(A) shows the plot to determine the specific SO_4^{2-} reduction rate of reactors that contained SO_4^{2-}. Points do not lie around a straight line. On the other hand the reactors that contained SO_4^{2-} and heavy metal (Cu=10 mg/l), points lie satisfactorily around a straight line confirming the validity of Equation (4.8). This deduces that Cu reacts with sulfide (the product from SO_4^{2-} reduction) in the system and precipitate as metal sulfide. H_2S was reduced and gave less toxic to SRB in the system. In the latter case, the SO_4^{2-} reduction results in a straight line because the reaction conforms to a pseudo-first order, and the line slope equals –K.
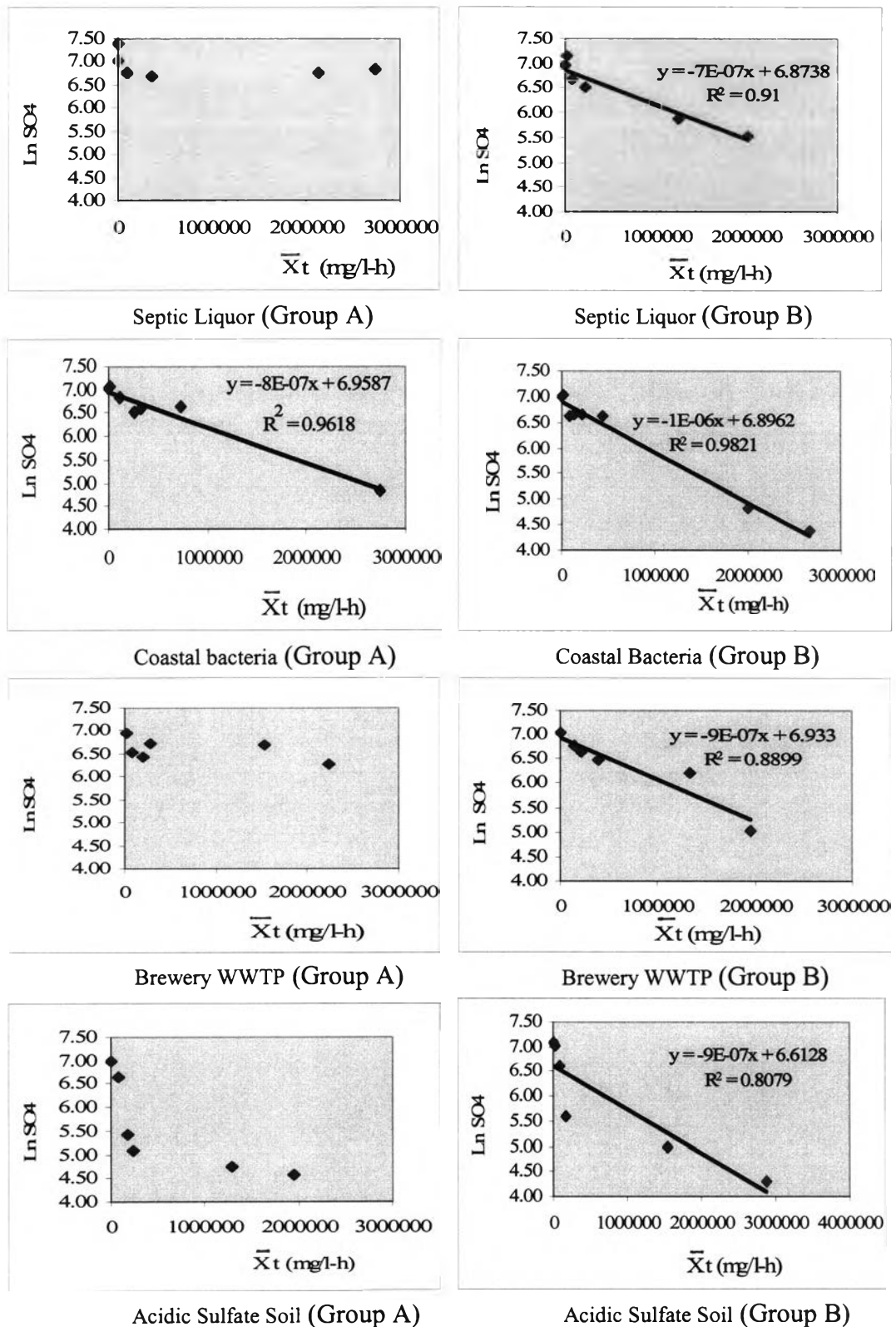
Figure 4.16 Graphical determination of the sulfate reduction rate of mixed-bacteria from various sources. **Group A** reactors contained only sulfate. **Group B** reactors contained both sulfate and Cu.

Leachate (Group A)   Leachate (Group B)

Figure 4.16 (-Continued-) Graphical determination of the sulfate reduction rate of mixed-bacteria from various sources. **Group A** reactors contained only sulfate. **Group B** reactors contained both sulfate and Cu.
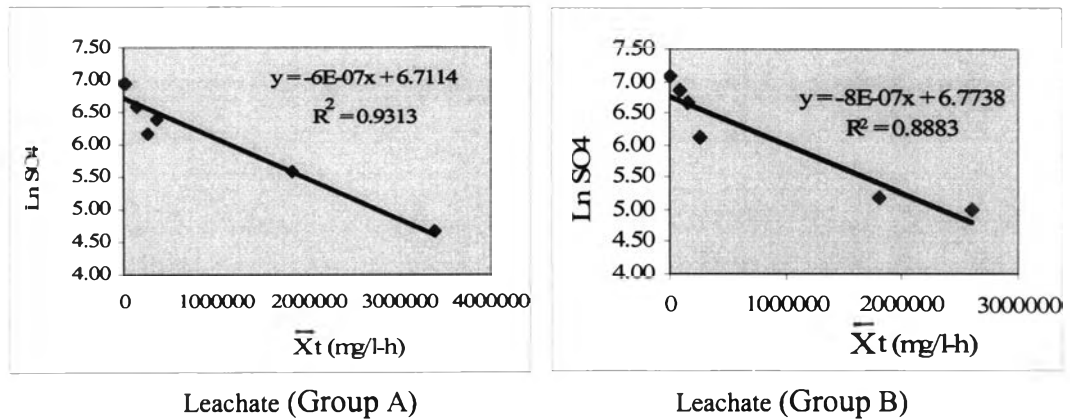
Cu showed the positive effect on the reduction of SO_4^{2-} especially for the bacterial assemblages from septic liquor, a brewery wastewater treatment plant and acidic sulfate soil due to sulfide detoxification of the metal. The specific SO_4^{2-} reduction rates for all types of bacteria in Group B reactors are 7×10^{-7}(septic liquor), 1×10^{-6}(coastal sediment), 9×10^{-7}(brewery WWTP), 9×10^{-7}(acidic sulfate soil) and 8×10^{-7}(leachate), expressed in the unit of liter/mg-hour. Without Cu, the plot of $LnSO_4$ versus $\overline{X}$ t does not result in a straight line (for bacteria from septic liquor, brewery WWTP and acidic sulfate soil). This implied that SRB in the system were inhibited. With Cu, SRB show higher performance in SO_4^{2-} reduction. The numerical values of specific SO_4^{2-} reduction rate obtained from Group B reactors (reactor that contained $SO_4^{2-}$and Cu) were summarized Table 4.2.

Table 4.2 summarizes the specific values, efficiencies in terms of COD removal and the dominant morphology of the acclimated seed sludge from the different sources.

The effect of SO_4^{2-} reduction and heavy metal in bioreactors was evaluated in terms of COD removal from the reactors. The percentage of COD removal of reactors without any toxicants (data from Section 4.4.2), with contained SO_4^{2-} 2,100 mg/l, and with contained both SO_4^{2-} 2,100 mg/l and Cu 10 mg/l were determined and shown in Figure 4.17(Tables D-15 and D-16).

Table 4.2 The kinetic values, efficiencies, and the dominant morphology of acclimated seed sludge from different sources.

Bacterial Sources	K (l/mg biomass-h)	$Y_{X/S}$, (mg biomass created/ mg substrate utilized)	k_d (mass cells/ total mass cells-day)	K_P (ml at STP/ (mg/l)h)	$Y_{P/S}$, * (ml CH$_4$ produced at STP / g substrate utilized)	%COD Removal	Bacterial Morphology	Specific SO$_4^{2-}$ Reduction Rate after providing heavy metal under SO$_4^{2-}$ reducing condition (l/mg biomass-h)
1. Septic Liquor	2×10^{-7}	0.0360	+0.0372	0.0029	317	50	Chain-forming cocci	7×10^{-7}
2. Coastal Sediment	4×10^{-7}	0.3689	-0.0377	0.0059	226	63	Short- rods	1×10^{-6}
3. Brewery Wastewater Treatment Plant	3×10^{-7}	0.0192	+0.0444	0.0072	324	57	Chain-forming cocci and bicocci	9×10^{-7}
4. Acidic Sulfate Soil	9×10^{-8}	0.7672	-0.0396	0.00008	65	38	Undefined-shape	9×10^{-7}
5. Leachate	2×10^{-7}	-	-	0.0097	315	42	Short- rods and bicocci	8×10^{-7}

* The theoretical amount of CH$_4$ of 350 ml at STP per gram of glucose utilized is reported (Tchobanoglous and Burton, 1991).

77

An advantageous process is related to the selection of an appropriate mixed-bacterial culture from the sources that best avoid inhibition of bacterial activity by Cu and H_2S and facilitate metal separation (Cu removal) and system recovery.
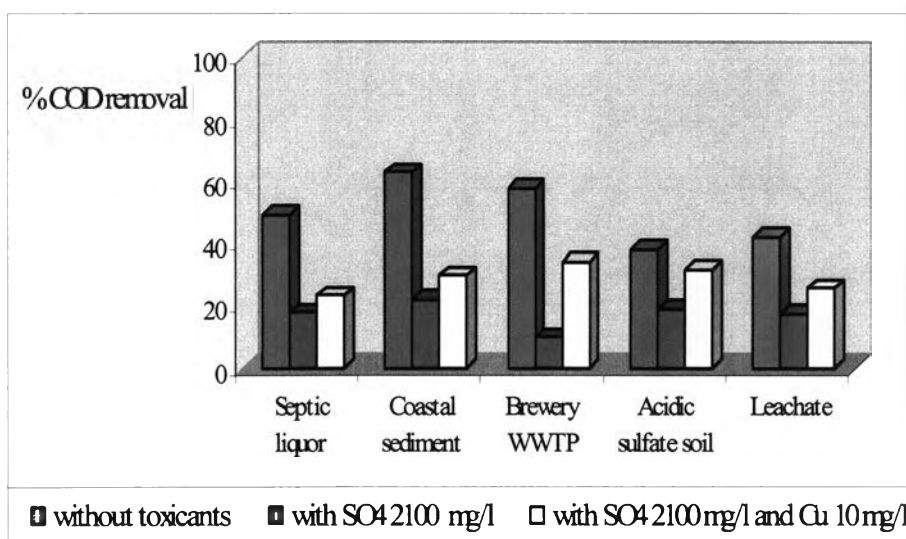

Figure 4.17 Effect of sulfate, and the combination of sulfate and Cu on COD removal in anaerobic process.

The system performance was different in terms of COD removal. In the cases without any toxicant addition, the system performance was quite high. With 2,100 mg SO_4^{2-}/l, the performances of all the reactors were much lower in terms of COD removal because biological sulfate reduction occurred, producing H_2S. With 2,100 mg SO_4^{2-}/l and 10 mg Cu/l, all reactors showed a higher performance in COD removal than in the case without Cu addition because CuS was precipitated, avoid inhibition of the bacteria in the system.

Among these reactors, the reactor with bacteria obtained from the brewery wastewater treatment plant showed the highest performance in the system recovery resulting from metal sulfide precipitation thereby avoiding system inhibition. These results suggested that bacteria from various sources provided different recoveries in terms of COD removal.

From the results obtained in this chapter, mixed-bacteria from the brewery wastewater treatment plant showed the highest mutualistic interaction between SRB and MPB. This mixed-culture provided the highest values in the specific CH_4 production rate (K_P), CH_4 production yield coefficient ($Y_{P/S}$), while

provided the lowest value in the endogenous decay rate (K_d) and the cellular yield coefficient ($Y_{X/S}$). This mixed-culture also provided the high value in the rate of COD utilization and that of SO_4^{2-} reduction. This mixed-culture showed the highest performance in recovery of the system to higher levels of COD removal as a result of metal sulfide precipitation.

We, therefore, considered the mixed bacterial culture from the brewery wastewater treatment plant as the high performance assemblages and selected this culture for the study of anaerobic bioreactors in simultaneous lowering of COD and sulfate with precipitation of metal sulfides, with methane production.

4.4.4 Isolation and Identification of Bacteria

The major types of bacteria and the species of SRB from the selected mixed culture were isolated and then identified. *Alicycliphilus* sp. R-24604, *Sporosarcina* sp. PIC-C28, and *Micrococcus luteus* are the three major types which were found in the mixed culture of bacteria in the brewery wastewater treatment plant. The % similarity of these three types was 100 (210/210), 98 (474/480), and 94 (451/476), respectively. The accession number is AM 084015, DQ 227790, and AB 079788, respectively. *Clostridium gangahwense* strain HY-42-06 is the SRB which was found in this culture. The % similarity is 99 (248/249) and the accession number is AY 90329. The electrophoregrams of identification were shown in Appendix C. The scanning electron micrograph of acclimated seed sludge was shown in Figure F-1 (Appendix F).

Table 4.3 The results of bacterial identification.

Isolate Number	Closets Sequence	% Similarity	Accession Number
1	*Micrococcus luteus*	451/476 (94%)	AB 079788
2	*Sporosarcina* sp. PIC-C28	474/480 (98%)	DQ 227790
3	*Alicycliphilus* sp. R-24604	210/210 (100%)	AM 084015
4 (SRB)	*Clostridium gangahwense* strain HY-42-06	248/249 (99%)	AY 903294

4.5 Conclusion

A mixed-bacterial culture from the brewery wastewater treatment plant was proved to be feasible, reliable, and effective in system recovery from metal and SO_4^{2-} as well as CH_4 production and waste reduction. Bacteria from different sources exhibited different rate of waste degradation, CH_4 production and the yield values. The three lists of the overall process performance data obtained from bacteria originated from a brewery WWTP were as followed. This bacterial process resulted in the highest specific CH_4 production rate of 0.0072 ml/(mg/l of biomass)-hour and the highest CH_4 production yield coefficient of 324 ml(at STP)/gram of COD removed. While the endogenous decay coefficient (k_d) was not found and the cellular yield coefficient ($Y_{X/S}$) was the lowest and exhibited at 0.2246 mg biomass created/mg waste utilized, respectively. While SO_4^{2-} reduction rate still occurred with the rate 9×10^{-7} l/mg-hour. It was found that the process was relatively easy to operate and start-up and result in quite a large COD reduction (57%) and a short time (2 days) to start-up. As for the waste utilization rate, this mixed-culture showed the top second value of 3×10^{-7} l/mg-hour which was in the same order of magnitude followed bacteria from the coastal sediment (4×10^{-7} l/mg-hour). The present investigation confirms the importance of the selection of the best populations that are 1) able to degrade effectively the VFA's produced in the acidogenic phase of anaerobic process 2) metal-resistant 3) able to degrade SO_4^{2-} in acidogenic phase for preventing sulfate reducing bacteria (SRB) and methane producing bacteria (MPB) by heavy metal sulfide precipitation 4) produce methane in high amount and 5) provide a short lag time. The conclusion was drawn and divided into 3 parts.

In the system without any toxicants: Bacterial assemblages originating from the septic tank, the coastal sediment and the brewery wastewater treatment plant reduced a large part of the initial COD without acetate accumulation and produced the high amount of methane, while the bacteria from the acidic sulfate soil showed a poor methane production. Bacteria from a brewery wastewater treatment plant gave the highest yield in terms of the highest specific CH_4 production rate as well as CH_4 production yield coefficient. In the system with high sulfate: Bacteria from the coastal sediment gave the highest specific sulfate reduction rate followed by the both cultures originating from the brewery waste water treatment plant and from acidic sulfate soil. In the system containing sulfate and heavy metal: Bacteria from the

brewery wastewater treatment plant showed the highest potency in facilitating recovery of system in terms of COD removal by metal sulfide precipitation thereby avoiding system inhibition.

Sludge from the brewery wastewater treatment plant was found to be the most appropriate bacterial assemblages due to its ability to show mutualistic interaction between SRB and MPB and useful for application in anaerobic process of waste containing both high sulfate and heavy metals. The exceptional properties in kinetic coefficients make this culture an excellent candidate for technical application.

The major types of this mixed-bacterial culture was *Sporosarcina* sp. PIC-C 28, *Alicycliphilus* sp. R-24604, and *Micrococcus luteus*. The sulfate reducing bacterial type was *Clostridium ganghwense* strain HY-42-06.

4.5 References

Aiba, S., Hamphrey, A.E., and Millis, N.F. 1965. Biochemical engineering. New York: Academic Press.

Feitkenhauer, H., Schnicke, S., Muller, R., and Markl, K. 2001. Determination of the kinetic parameters of the phenol-degrading thermophile *Bacillus themoleovorans* sp. A2. Applied Microbiology and Biotechnology 57(5-6): 744-50.

Geoffrey, H. 1997. Microbial consortia for multiple pollutant biodegradation. Pure and Applied Chemistry, 69:2343-2356.

Mason, C.A., Hammer, G., and Bryers, J.D. 1986. The death and lysis of microorganisms in environmental processes. FEMS Microbiology Reviews 39: 373-401.

Merchuk, J. C. and Asenjot, J.A. 1995. The Monod equation and mass transfer. Biotechnology and Bioengineering 45: 91-94.

Reynolds, T.D., and Richards, P.A. 1996. Biological concept. Unit Operations and Process in Environmental Engineering pp. 32-35. Boston: PWS Publishing Company.

Tchobanoglous, G. and Burton, F.L. 1991. Revised from Metcalf & Eddy Inc. <u>Wastewater Engineering Treatment, Disposal, and Reuse.</u> pp. 1277-1279. Singapore: McGraw Hill Inc.

Tchobanogous, G., Burton, F.L., and Stensel, H.D. 2003. Revised from Metcalf & Eddy Inc. <u>Wastewater Engineering Treatment and Reuse.</u> pp. 1000. New York: McGraw Hill Inc.