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*Original Research*

# Unionized Acetate Degradation at 45ºC Anaerobic Digestion: Kinetics and Inhibition

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## Abstract

This study demonstrated the degradation of unionized acetate in anaerobic digestion at 45ºC through the identification of kinetics and inhibition parameters. The kinetic parameters, $K_s$ and $r_{max}$, were determined using Monod-based model for three different conditions, namely uninhibited, inhibited, and systems with high substrate condition. The $K_s$ values for uninhibited condition were in the range of 0.124 to 0.191 mg/L as unionized HAc. $K_s$ value of inhibited condition were at 0.027 mg/L as unionized HAc. $K_s$ values for systems with high substrate condition were found to be in the range of 0.237 to 0.279 mg/L as unionized HAc. As for $r_{max}$, a 35ºC anaerobic digestion system showed the highest value at 0.166 mg/L/day – greater than the values of all 45ºC systems under all experimental conditions. Additionally, the inhibition parameter $K_I$ was also determined using the Michaelis-Menten model. The parameter was determined for inhibitory conditions resulting from high free $NH_3$ content. The inhibition type was uncompetitive with $K_I$ value of 0.072 mg/L as unionized HAc. The outcomes suggested that the methanogens responsible for the digestion process at 45ºC were thermo-tolerant acetate-utilizing methanogens of *Methanosarcinaceae* species, and the system will be totally inhibited with the presence of high free $NH_3$ content.

**Keywords**: 45ºC anaerobic digestion, acetate metabolism, monod kinetic, kinetic constant, inhibition constant

## Introduction

Numerous studies have assessed the performances of mesophilic and thermophilic anaerobic digestion systems [1-5]. Nevertheless, only a handful have investigated the feasibility of running anaerobic digesters in the intermediate zone of 45ºC, between the mesophilic and thermophilic ranges. This is due to the reason that within this intermediate zone of 40ºC to 50ºC, neither mesophilic nor thermophilic microorganisms would flourish, thus suppressing them from demonstrating high activity. This is because at these temperatures microorganisms are trying to adapt to the changing environment [6]. However, some researchers have found that at 45ºC, their anaerobic digestion systems were still functioning well by generating a comparable amount of gas and at times were higher than the conventional mesophilic systems [7-9]. This finding was similar to observations by Gao et al., Mohd et al.,

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and Peces et al. [10-12], who discovered that there were no major discrepancies between anaerobic digestion systems operating at 37ºC and 45ºC in terms of methane production, volatile solids (VS) content, and pH, except for volatile fatty acid (VFA) concentrations that increase significantly as temperatures rise [10-12].

Generally, an increase in the concentration of VFA, primarily acetate, usually indicates a failure of the anaerobic digestion process. This was mainly because acetate degradation was believed to be the rate-limiting step that brings the reaction to completion [13-14]. The degradation of acetate is a process of methane formation that undergoes either one of two mechanisms involving two different types of methanogens. The first mechanism is aceticlastic, a single-step methane fermentation carried out by acetate-utilizing methanogens such as *Methanosarcinaceae* or *Methanosaetaceae* [15-16]. The second mechanism consists of a two-step reaction in which the acetate is initially oxidized to $H_2$ and $CO_2$ prior to its final conversion to methane. This reaction is executed by acetate-oxidizing bacteria such as *Clostridium* spp., together with hydrogen-utilizing methanogens, such as *Methanomicrobiales* or *Methanobacteriales* [17-18]. As methanogens convert acetate and $H_2$ to methane and $CO_2$, it will therefore help in maintaining a low partial pressure of $H_2$, thus encouraging acetogenesis to proceed, and shifting the equilibrium of the reactions toward the formation of more $H_2$ and acetate [19]. However, if the methanogens are not present in suitable numbers, or are being slowed down by unfavorable environmental conditions, they will not utilize $H_2$ and acetate as rapidly as they are produced. It will then disturb balanced acetogenic processes and ultimately result in an accumulation of VFAs [19-20].

Despite the importance of acetate degradation for methane fermentation, none of the studies have elucidated the kinetics of the reaction at the intermediate temperature of 45ºC. Many of the reported kinetic parameters were either from mesophilic digestion or thermophilic digestion. Aynur and Mani et al. reported $K_s$ values of 233 and 180 mg/L as HAc in experiments with mesophilic-acclimatized sludge metabolizing acetate at 35ºC, respectively [21, 22]. While Gavala et al. and Mladenovska and Ahring examined the metabolization of acetate in thermophilic sludge at 55ºC and 52ºC, and reported $K_s$ of 511 and 390 mg/L as HAc, respectively [23-24].

Anaerobic digestion operating at high temperature is also susceptible to environmental influences such as pH and temperature that significantly contribute to the abundant generation of other toxic compounds such as ammonia – an essential nutrient for anaerobic microorganisms, but when available at high concentrations it can inhibit microbial activities. In aqueous phase, the fractions of ammonia, which are ammonium ion ($NH_{4\,(aq)}^{+}$), free ammonia ($NH_{3(aq)}$), and ammonia ($NH_{3(g)}$) exist in equilibrium with hydrogen ($H^+$) and hydroxyl ($OH^-$) ions. Among all, free or unionized ammonia is reported to be the most toxic due to its capability to penetrate through the cell membrane [25-26].

Several studies have been conducted to investigate free ammonia inhibition mechanisms experienced by anaerobic digestion systems. Bai et al. in their mesophilic study reported that ammonia inhibition in anaerobic digestion showed non-competitive inhibition characteristics with a $K_I$ reported value of 0.02 [27], while Angelidaki et al. in their study described ammonia inhibition as following a non-competitive inhibition behavior [28]. With regard to that, they reported a $K_I$ value of 260 mg/L as HAc in their thermophilic anaerobic digestion system.

The present work was done to study the kinetics of acetate metabolism at the intermediate temperature of 45ºC. Additionally, the kinetic coefficients of half saturation constant, $K_s$, were evaluated. In corresponding to the inhibitory condition posed by free ammonia, the inhibitory kinetics coefficient $K_I$ was also determined. The rational that motivates this study was that the kinetic study at this intermediate temperature was scarce and that the very limited studies that have been done had indicated its more cost-effective performance. Thus, the understanding of the kinetics of methane fermentation at this temperature is crucial, as this option is more economical, while at the same time it is able to function at the same par if not better than the conventional thermophilic digestion system.

## Experimental

### Anaerobic Digestion Systems

Three batch reactors were run at 45ºC, 35ºC, and 55ºC for more than 3 years and fed with municipal sludge collected from the Blue Plains Advanced Wastewater Treatment Plant. The 45ºC digester was operated at an operational volume of 15 L, organic loading rate (OLR) of 6.96 kg VS/$m^3$/day, and solid retention time (SRT) of 7.5 days. The mesophilic digester was operated at 35ºC with an operational volume of 13 L, OLR of 4.07 kg VS/$m^3$/day, and SRT of 10 days, while the thermophilic digester was run at 55°C with an operational volume of 15 L, OLR of 5.22 kg VS/$m^3$/day and SRT of 10 days. The characteristics of the sludge fed to the digester are presented in Table 1.

### Batch Kinetic Tests

Effluent from the digesters was taken out and used as inoculations for batch kinetic tests. The characteristics of the effluent of 35ºC, 45ºC, and 55ºC anaerobic digestion systems were presented in Table 1. However, no kinetic test was performed for the effluent of the 55ºC system because of the difficulties in reducing the background acetate to a baseline level of less than 100 mg/L.

Initially, the effluent was diluted in order to remove the potential inhibition effects of existing soluble

Table 1. The characteristics of the feed, 45ºC AD effluent, 35ºC AD effluent, 55ºC AD effluent, and baseline condition used for batch kinetic tests.

| | Feed | Effluent 45ºC AD | Effluent 35ºC AD | Effluent 55ºC AD | Baseline |
|---|---|---|---|---|---|
| pH | 6.09±0.13 | 7.40±0.05 | 7.31±0.06 | 7.42±0.08 | 7.45±0.08 |
| TS | 6.64±0.65 | 4.14±0.40 | 3.31±0.35 | 2.16±0.24 | 3.54±0.29 |
| (%)<br>VS | 5.21 ±0.50 | 2.64±0.21 | 5.07±0.58 | 3.53±0.38 | 2.20±0.20 |
| (%) TAN (mg/L) | 412±67 | 1559±95 | 1382±85 | 2392±221 | 426±33 |
| Free $NH_3$ (mg/L) | 1.2±0.2 | 76.5±5.9 | 29.9±2.7 | 239.0±37.9 | 23.4±2.0 |
| Acetate (mg/L) | 1322±271 | 541±99 | 94±27 | 2510±209 | 67±10 |
| Total VFA (mg/L) | 2491±350 | 974±191 | 126±24 | 3944±15 | 67±10 |

AD = Anaerobic digestion; TS = Total Solids; VS = Volatile Solids; TAN = Total Ammoniacal Nitrogen; $NH_3$ = Ammonia; VFA= Volatile Fatty Acids

compounds such as ammonia and acetate. 400 mL of the samples were placed in 500 mL glass bottles and the substrates in a form of acetic acid were added. Following that, the headspace was flushed with nitrogen gas in order to create anaerobic condition within the bottles. The samples in the bottles were then mixed continuously using a magnetic stirrer and incubated at desired temperature. All tests were run in duplicate.

The kinetic tests were first conducted at a baseline condition at which no inhibition was observed. At baseline condition, the temperature was 45ºC, pH was 7.45, total solids (TS) was 3.54%, volatile solids (VS) was 2.20%, TAN concentration was 426 mg/L, free $NH_3$ concentration was 23.4 mg/L, background acetate concentration was 67 mg/L, and total VFA concentration was 67 mg/L (Table 1). The condition was subsequently varied at different temperature, different SRT, different free $NH_3$ concentration, and different background acetate concentration. For each test, acetic acid was added as a substrate until the total concentration of acetate was approximately 1000 mg/L. Acetic acid was added in a diluted form of synthetic acetic acid 2.0 N (Fisher Chemical, Waltham, Massachusetts) while ammonia was added in a solution form of crystalline Ammonium Chloride ($NH_4Cl$) (Fisher Chemical, Waltham, Massachusetts). For each condition, the tests were run for at least two times, with each of them having a duplicate. During the tests, the concentrations of the remainder of the acetate were measured at every one-hour or 30-minute interval and the tests were run until all acetate was exhausted or when there was no further degradation of the acetate left in the system.

## Sampling and Analysis

The concentration of acetate and total VFA were measured by a Shimadzu Gas Chromatograph Model GC-2010 (Shimadzu, Kyoto, Japan). The gas chromatography system was equipped with flame ionization detector (FID) at 250°, and Restek Stabilwaxfi-DA (Restek, Bellefonte, Pennsylvania) capillary column with column temperature of 145ºC. The calibration curves were generated using five types of organic acids, namely acetic, propionic, butyric, valeric, and caproic, with concentrations ranging from 25 to 1000 µL/L.

Prior to the analysis, the samples were centrifuged for 20 minutes at 13,000 rpm and filtered using Millipore Millex-HN filters (Fisher Scientific, Waltham, Massachusetts) with 0.45 µm pore size. The solution phase or supernatant was then acidified using phosphoric acid prior to use for analysis. The measured acetate was presented as the unionized acetate calculated according to Equation (1) [28].

$$C_{HAc} = \frac{C_{H^+} \cdot \gamma_1^2}{K_{D,Ac} + C_{H^+} \cdot \gamma_1^2} \cdot C_{Ac} \quad (1)$$

…where $C_{HAc}$ = Concentration of unionized acetic acid (mol/L); $C_{H+}$ = Concentration of hydrogen ion, mol/L; $C_{Ac}$ = Concentration of total acetic acid (mol/L); $\gamma_1$ = Monovalent ion activity coefficient = 0.73; $K_{D,Ac,35}$ = Dissociation constant at 35ºC = 1.700 x $10^{-5}$ mol/L; and $K_{D,Ac,45}$ = Dissociation constant at 45ºC = 1.644 x $10^{-5}$ mol/L.

pH was measured with Denver Instrument Model 250 pH meter (Denver Instrument, Bohemia, New York) while the analysis of TS, VS, and TAN were done in accordance to Standard Methods 2540 B, 2450 E, and 4500-$NH_3$ B and C, respectively [29].

The analysis on microbial population of the effluent was done using the PCR method. For DNA extraction, freeze-dried samples were homogenized and physically disrupted using a bead beater (BioSpec Products). DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen) following the manufacturer's instructions. DNA concentration and quality were measured by spectrophotometry corrected according to initial sample weight and dilutions. Absolute concentrations

of DNA from methanogens were determined using qPCR and serial dilutions (from 101 to 105) of specific DNA standards [30].

## Kinetics Model: Monod Model

During kinetic tests, the concentrations of remaining unionized acetate, $S$ at sampling time $t$ was measured and the data were used to plot the unionized acetate degradation curves. The Monod-based model proposed by Smith et al. was then used to fit these curves (Equation (2)) [31].

$$t = \left[ -\frac{Y}{r_{\max}} \right] \cdot \left[ K_s \cdot \ln \frac{S}{S_0} + S - S_0 \right] \quad (2)$$

Where $t$ = Sampling time (days); $Y$ = Yield coefficient (0.035 g VSS/ g BOD [32]; $r_{\max}$ = Maximum bacterial growth rate (mg/L.day); $K_s$ = Half saturation constant (mg/L); S = Concentration of growth-limiting substrate (mg/L); $S_o$ = Initial concentration of growth-limiting substrate (mg/L).

The kinetic coefficients $K_s$ and $r_{max}$ were determined by minimizing the discrepancies between $t$ values predicted by the model and $t$ values obtained through the experiments using the assumed value of $Y$ and the initial estimates of $K_s$. This method is known as non-linear least square analysis.

## Inhibition Factor I

Inhibition factor I was also determined to express the degree of inhibition caused by any particular condition. I is a ratio of the initial slope of acetate degradation curve of inhibited condition to the slope of the curve of uninhibited condition. I was expressed as $I_{Temp}$, $I_{SRT}$, $I_{NH3}$, and $I_{HAc}$ for inhibited condition caused by temperature, SRT, free $NH_3$ content, and background acetate concentration, respectively. I value of 1.0 represents zero inhibition.

## Inhibition Kinetics Model: Michaelis-Menten Model

Based on the outcomes of the kinetics model, free $NH_3$ has been shown to inhibit methanogenic activity in a 45ºC anaerobic digestion system, and the inhibition was classified as uncompetitive inhibitory condition [33]. The type of inhibition was determined through the trend of increasing or decreasing $K_s$ and reaction rate at different free $NH_3$ concentrations. The inhibition kinetic coefficient $K_I$ was subsequently determined using the Michaelis-Menten-based model proposed by Shuler and Kargi (Equation (3)) [34].

$$\nu = \frac{dP}{dt} = -\frac{dS}{dt} = \frac{V_{m,inh} \cdot S}{K_{m,inh} + S} = \frac{\frac{V_m}{\left(1 + \frac{I}{K_I}\right)} \cdot S}{\frac{K_m}{\left(1 + \frac{I}{K_I}\right)} + S} = \frac{k \cdot S \cdot X}{\left(\frac{K_s}{\left(1 + \frac{I}{K_I}\right)} + S\right) \cdot \left(1 + \frac{I}{K_I}\right)} \quad (3)$$

…where $V_m$ = Maximum specific rate ($day^{-1}$); $V_{m,inh}$ = Maximum specific rate under inhibited condition ($day^{-1}$); $K_m$ = Substrate utilization constant (mg/L); $K_{m,inh}$ = Substrate utilization constant under inhibited condition (mg/L); $K_I$ = Inhibitor's dissociation constant (mg/L); $I$ = Inhibitor concentration (mg/L).

## Results and Discussion

### Monod Kinetic Constants Determination: Baseline Condition

At 45ºC, $r_{max}$ was observed to be at 0.102 mg/L and $K_s$ was reported at 0.170 mg/L as unionized HAc or 171 mg/L as HAc (Table 2). This value was lower in comparison to the value reported by Aynur with $K_s$ of 233 mg/L as HAc, but approximately similar to the value reported by Mani et al. at 180 mg/L as HAc [21-22]. These studies were done at 35ºC and 37ºC, respectively. When the comparison was made with thermophilic digestion values, much higher differences were observed. Thermophilic studies done by Gavala et al. and Mladenovska and Ahring reported $K_s$ of 511 and

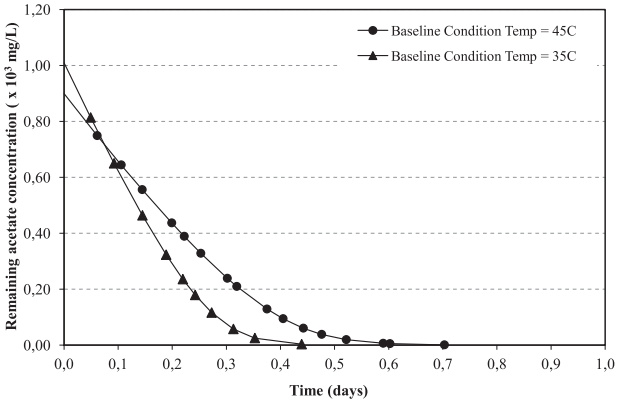


Fig. 1. Progress curve of acetate degradation at 35ºC and 45ºC. Different temperatures: $r_{max}$ (t-test, $\alpha = 0.05$, $p = 0.012$ for $\mu_{45} = \mu_{35}$), $K_s$ (t-test, $\alpha = 0.05$, $p = 0.397$ for $\mu_{45} = \mu_{35}$).

Table 2. Model-estimated kinetic coefficients, $K_s$, $r_{max}$, and I at different temperatures, SRTs, free $NH_3$ contents, and background acetate concentrations.

| | $r_{max}$ (mg/L/day) | $K_s$ (mg/L as unionized HAc) | $K_s$ (mg/L as HAc) | I (dimensionless) |
|---|---|---|---|---|
| Baseline | | | | |
| 45ºC Baseline | 0.102 | 0.170 | 171 | 1.00 |
| Different Temperature | | | | |
| 45ºC | 0.102 | 0.170 | 171 | 0.68 |
| 35ºC | 0.166 | 0.191 | 190 | 1.00 |
| Different SRT | | | | |
| 45ºC SRT 7.5 days | 0.102 | 0.170 | 171 | 1.00 |
| 45ºC SRT 10 days | 0.095 | 0.128 | 130 | 0.94 |
| 45ºC SRT 15 days | 0.083 | 0.124 | 125 | 0.83 |
| Different Free $NH_3$ Content | | | | |
| 45ºC Free $NH_3$ 20 mg/L | 0.102 | 0.170 | 171 | 1.00 |
| 45ºC Free $NH_3$ 70 mg/L | 0.085 | 0.138 | 139 | 0.94 |
| 45ºC Free $NH_3$ 90 mg/L | 0.095 | 0.147 | 149 | 0.96 |
| 45ºC Free $NH_3$ 110 mg/L | 0.044 | 0.027 | 26 | 0.05 |
| Different background HAc | | | | |
| 45ºC HAc b/g 0 + add 1000 mg/L | 0.102 | 0.170 | 171 | 1.00 |
| 45ºC HAc b/g 500 + add 1000 mg/L | 0.143 | 0.237 | 237 | 1.36 |
| 45ºC HAc b/g 1000 + add 1000 mg/L | 0.128 | 0.279 | 279 | 1.26 |
| 45ºC HAc b/g 1500 + add 1000 mg/L | 0.097 | 0.257 | 257 | 0.98 |

$r_{max}$ = Maximum bacterial growth rate; $K_s$ = Half saturation constant; $I$ = Inhibitor factor; HAc = Acetic Acid;
SRT = Solid retention time; $NH_3$ = Ammonia; b/g = background; add = additional.
Different temperature: $r_{max}$ (t-test, α = 0.05, $p$ = 0.012 for $\mu_{45} = \mu_{35}$), $K_s$ (t-test, α = 0.05, $p$ = 0.397 for $\mu_{45} = \mu_{35}$).
Different SRT: $r_{max}$ (t-test, α = 0.05, $p$ = 0.187 for $\mu_{7.5} = \mu_{10}$; $p$ = 0.109 for $\mu_{7.5} = \mu_{15}$; $p$ = 0.281 for $\mu_{10} = \mu_{15}$),
$K_s$ (t-test, α = 0.05, $p$ = 1.515E-4 for $\mu_{7.5} = \mu_{10}$; $p$ = 2.588E-4 for $\mu_{7.5} = \mu_{15}$; $p$ = 0.256 for $\mu_{10} = \mu_{15}$).
Different Free $NH_3$ Content: $r_{max}$ (t-test, α = 0.05, $p$ = 0.003 for $\mu_{20} = \mu_{110}$; $p$ = 0.007 for $\mu_{70} = \mu_{110}$; $p$ = 0.007 for $\mu_{90} = \mu_{110}$),
$K_s$ (t-test, α = 0.05, $K_s$: $p$ = 1.658E-5 for $\mu_{20} = \mu_{110}$; $p$ = 7.682E-3 for $\mu_{70} = \mu_{110}$; $p$ = 4.257E-3 for $\mu_{90} = \mu_{110}$).
Different background HAc: $r_{max}$ (t-test, α = 0.05, $p$ = 0.054 for $\mu_0 = \mu_{500}$; $p$ = 0.176 for $\mu_0 = \mu_{1000}$; $p$ = 0.569 for $\mu_0 = \mu_{1500}$),
$K_s$ (t-test, α = 0.05, $p$ = 2.354E-3 for $\mu_0 = \mu_{500}$, $p$ = 8.060E-4 for $\mu_0 = \mu_{1000}$, $p$ = 1.050E-3 for $\mu_0 = \mu_{1500}$).

390 mg/L as HAc [23-24]. These studies were done at 55ºC and 52ºC, respectively.

## Monod Kinetic Constants Determination: Different Temperatures

In order to observe the implication of temperature on the digestion process, another set of tests were also conducted to the effluent of the digesters operating at 35ºC and 55ºC with the optimum SRT of 10 days. The comparisons were made on a basis that the optimum SRT of 45ºC was 7.5 days and the optimum SRTs of 35ºC and 55ºC were 10 days. The other parameters that remained identical were free $NH_3$ concentration of approximately 20 mg/L and background acetate concentration of approximately 100 mg/L. However, the kinetic test with the effluent of 55ºC digester was unsuccessful because of the difficulties of reducing the background acetate to a baseline level of less than 100 mg/L. Fig. 1 showed the degradation rate of acetate in a 45ºC system in comparison to the 35°C system. In general, methanogens in 45ºC systems were able to degrade acetate to a minimum level even though the rate was slightly slower compared to those in the mesophilic system. This finding was supported by significantly less $r_{max}$ value of 0.102 mg/L/day in 45ºC systems as opposed to 0.166 mg/L/day in the mesophilic system (Table 2). Similarly, as temperature increased, $K_s$ value also decreased. $K_s$ value was 0.191 mg/L in the 35ºC system and 0.170 mg/L in the 45ºC system (Table 2). However, the variation of $K_s$ value was insignificant. Corresponding to the declining trend of $r_{max}$, $I_{Temp}$ values were also decreasing as the temperature increased, with a 32% reduction in acetate degradation

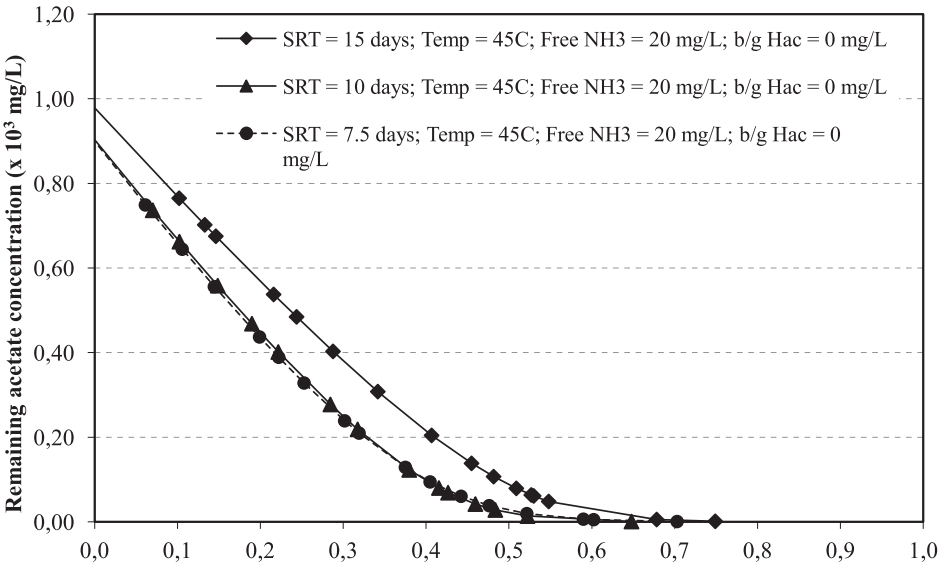


Fig. 2. Progress curve of acetate degradation at different SRTs of 7.5, 10, and 15 days (Temp = temperature; b/g = background). Different SRT: $r_{max}$ (t-test, $\alpha = 0.05$, $p = 0.187$ for $\mu_{7.5} = \mu_{10}$; $p = 0.109$ for $\mu_{7.5} = \mu_{15}$; $p = 0.281$ for $\mu_{10} = \mu_{15}$), $K_s$ (t-test, $\alpha = 0.05$, $p$ = 1.515E-4 for $\mu_{7.5} = \mu_{10}$; $p$ = 2.588E-4 for $\mu_{7.5} = \mu_{15}$; $p = 0.256$ for $\mu_{10} = \mu_{15}$).

rate relative to the rate at mesophilic condition (Table 2).

## Monod Kinetic Constants Determination: Different SRTs

Other than temperature, the kinetics of anaerobic digestion at 45ºC was also compared at different SRTs of 15 days, 10 days, and 7.5 days. Correspondingly, as the SRTs were varied, the other parameters remained unchanged. Fig. 2 illustrated the degradation pattern of acetate at different SRTs. A slight reduction in degradation rate was observed as the SRT was increased and when the results were compared, a notable declining trend can be seen in $K_s$ and $r_{max}$. However, the decreasing trend was only significant in $K_s$ but not in $r_{max}$. $K_s$ values were 0.170, 0.128, and 0.124 mg/L for a 45ºC system with 7.5 days, 10 days, and 15 days SRT, respectively (Table 2). $r_{max}$ values were 0.102, 0.095, and 0.083 mg/L/day for a 45ºC system with 7.5 days, 10 days, and 15 days each (Table 2). Apparently, the insignificant declining trend of $r_{max}$ suggested that increasing the SRT will only reduce 6 to 17% methane production of the digestion process at 45ºC (Table 2). However, these $I_{SRT}$ values were not low enough to be considered as inhibitory; rather, the methanogens might have been harvested beyond their capability at SRT longer than 7.5 days, thus explaining the decreasing $K_s$ in the system with longer SRT.

## Monod Kinetic Constants Determination: Different Free Ammonia Concentrations

Kinetic tests were also performed at different levels of free $NH_3$ of 20, 70, 90, and 110 mg/L. From Fig. 3 we observed that lower free $NH_3$ concentrations of 20, 70, and 90 mg/L did not have any harmful effect on acetate degradation, while higher free $NH_3$ concentration at 110 mg/L caused an obvious inhibition. It was also shown by the sharp decline of $K_s$, $r_{max}$, and $I_{NH3}$ values as the concentration approached 110 mg/L (Table 2). A major decrease in parameter values at free $NH_3$ concentration of 110 mg/L was statistically significant. The $K_s$ values were 0.170, 0.138, 0.147 and 0.027 mg/L for 45ºC system with 20, 70, 90 and 110 mg/L free $NH_3$, each, while $r_{max}$ values were 0.102, 0.085, 0.095 and 0.044 mg/L/day, respectively (Table 2). The distinctively low value of $K_s$ and $r_{max}$, coupled with almost 95% reduction in methanogenesis at free $NH_3$ concentration of 110 mg/L explained the non-existence of acetate degradation at high free $NH_3$ content (Table 2).

## Monod Kinetic Constants Determination: Different Background Acetate Concentrations

As anaerobic digestion may as well suffer from existing substrate inhibition, the kinetic tests were also performed at different concentrations of background acetate, which were less than 100 mg/L, 500, 1000, and 1500 mg/L. For simplicity, tests with less than 100 mg/L acetate were labelled as 0 mg/L acetate content. For each test, 1000 mg/L acetate as acetic acid was added as additional substrate. Fig. 4 and Table 2 show that $r_{max}$ value was increasing as the background acetate concentration increased to 500 mg/L, and started to decrease when the concentration approached 1000 mg/L, though the rate remained either higher than or similar to the baseline test. However, the increment was insignificant. The $r_{max}$ values were 0.102, 0.143, 0.128, and 0.097 mg/L/day for the system with 0,

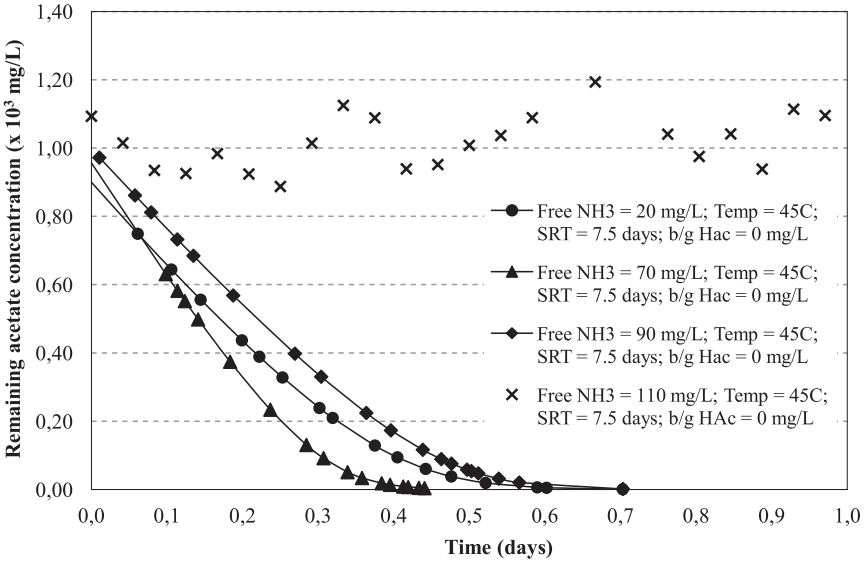


Fig. 3. Progress curve of acetate degradation at different free $NH_3$ concentrations of 20, 70, 90, and 110 mg/L (Temp = temperature; b/g = background).
Different Free $NH_3$ Content: $r_{max}$ (t-test, $\alpha = 0.05$, $p = 0.003$ for $\mu_{20} = \mu_{110}$; $p = 0.007$ for $\mu_{70} = \mu_{110}$; $p = 0.007$ for $\mu_{90} = \mu_{110}$), $K_s$ (t-test, $\alpha = 0.05$, $K_s$: $p = 1.658E\text{-}5$ for $\mu_{20} = \mu_{110}$; $p = 7.682E\text{-}3$ for $\mu_{70} = \mu_{110}$; $p = 4.257E\text{-}3$ for $\mu_{90} = \mu_{110}$).

500, 1000, and 1500 mg/L background acetate each (Table 2). The similar inclining trend before slightly declining at high concentration was also observed in $K_s$ value. The $K_s$ values were 0.170, 0.237, 0.279 and 0.257 mg/L for the system with 0, 500, 1000, and 1500 mg/L background acetate, respectively, and high $K_s$ values at higher concentration were significantly higher than the baseline condition (Table 2). The increase in $K_s$ values was mainly because at higher background acetate concentration, part of the available acetate helped supply more substrate to the system, thus increasing its methanogenic activity. Additionally, $I_{HAc}$ of more than 1.0 also suggested that an increase in background acetate concentration does not exert an inhibitory condition to the system.

## Overall Analysis of $K_s$ and $r_{max}$

$K_s$ and $r_{max}$ values for each condition were then plotted as shown in Fig. 5. Generally, it can be concluded that $K_s$ values could be categorized under 3 distinct ranges. $K_s$ values of uninhibited condition ranged

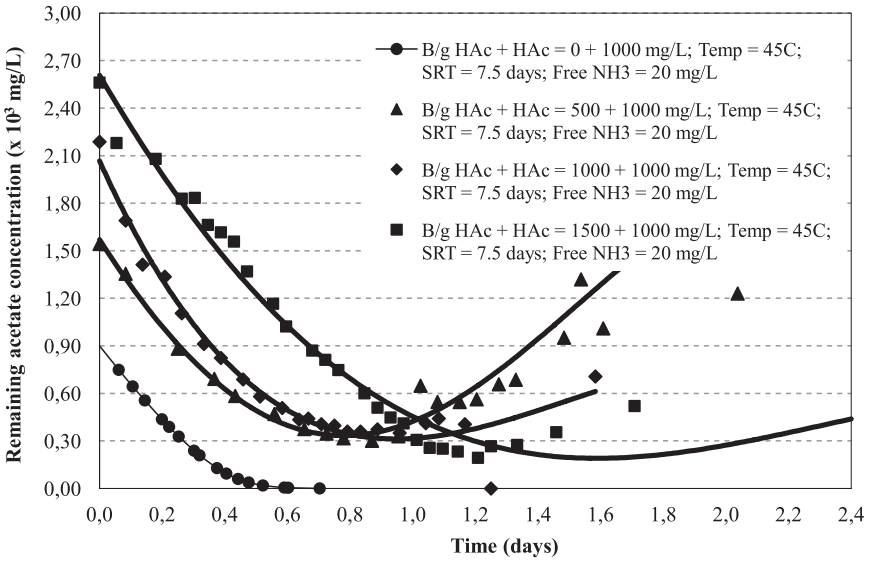


Fig. 4. Progress curve and trendline of acetate degradation at different background acetate concentrations of 0, 500, 1000, and 1500 mg/L (with the addition of synthetic acetate).
Different background HAc: $r_{max}$ (t-test, $\alpha = 0.05$, $p = 0.054$ for $\mu_0 = \mu_{500}$; $p = 0.176$ for $\mu_0 = \mu_{1000}$; $p = 0.569$ for $\mu_0 = \mu_{1500}$), $K_s$ (t-test, $\alpha = 0.05$, $p = 2.354E\text{-}3$ for $\mu_0 = \mu_{500}$, $p = 8.060E\text{-}4$ for $\mu_0 = \mu_{1000}$, $p = 1.050E\text{-}3$ for $\mu_0 = \mu_{1500}$).

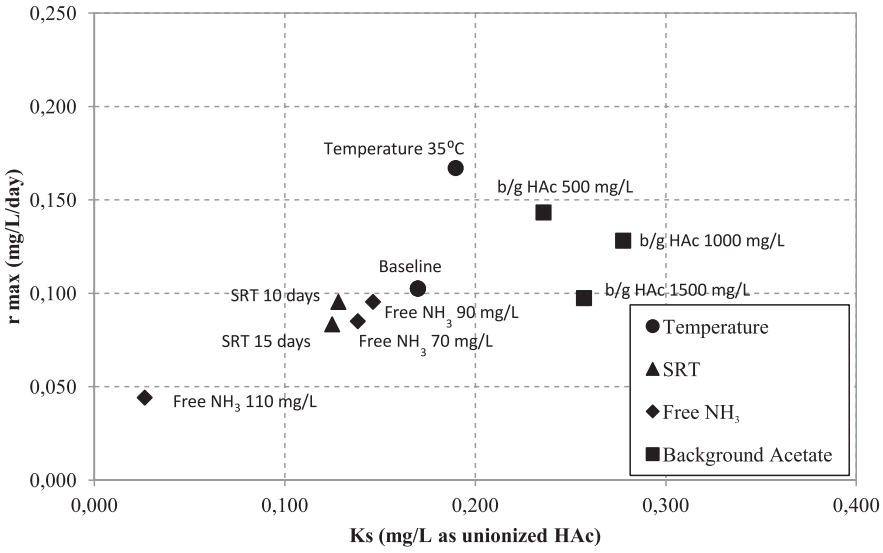


Fig. 5. $K_s$ and $r_{max}$ at different temperatures, SRTs, free $NH_3$, and background acetates.

from 0.124 to 0.191 mg/L, with the lowest $K_s$ in the 45ºC system with 15 days SRT and the highest in the 35ºC system, while $K_s$ values of inhibited condition were at 0.027 mg/L, which was observed in the 45ºC system with 110 mg/L free $NH_3$. Higher $K_s$ values were observed in systems with more substrate, ranged from 0.237 to 0.279 mg/L. For $r_{max}$, the highest rate was seen in the 35ºC system, even exceeding the degradation rate of the 45ºC system with higher substrate concentration. Thus, this implied that at 45ºC the methanogens did not work at their maximum capability as the temperature was not the optimum temperature for the involved methanogens. Additionally, these observations also suggested that the methanogens in the 45ºC system were similar to the ones in 35ºC system. This was because of their ability to degrade acetate to a very low level, and was further supported by the significantly similar $K_s$ values of uninhibited conditions with the value of $K_s$ in the 35ºC system (Table 2). The said methanogens were probably thermo-tolerant acetate-utilizing mesophilic methanogens. In comparison to $K_s$ values of two prevalent mesophilic methanogens, *Methanosarcinaceae* with $K_s$ of approximately 200 mg/L as HAc and *Methanosaetaceae* with $K_s$ of approximately 30 mg/L as HAc, it was concluded that the methanogens of our 45°C systems belonged to *Methanosarcinaceae* type [35]. Further DNA investigation, which demonstrated that *Methanosarcinaceae* existed in significant numbers of 82.45% in the 45°C system and 85.40% in 35ºC system, ultimately confirmed the findings Table 3).

## Inhibition Kinetic Constants Determination

Free $NH_3$ has been shown to inhibit methanogenic activity in our 45ºC anaerobic digestion system. The finding was based on the absence of acetate utilization when free $NH_3$ concentration reached 110 mg/L (Fig. 3). Though under normal operating conditions an ammonia-stressed environment was rarely observed, the understanding of the kinetics under inhibited condition remained crucial. As observed previously, as free $NH_3$ concentration increased, $r_{max}$ and $K_s$ values decreased substantially, showing that the system was experiencing uncompetitive inhibitory condition (Table 2). Hence, it was calculated using Equation 3, $K_I$ value was 0.073 mg/L as unionized HAc or 72 mg/L as HAc (Table 4). In comparison to a study done by Angelidaki et al., they reported a much lower value of 260 mg/L as HAc and the ammonia inhibition experienced by their system was characterized as non-competitive inhibition [28]. As in non-competitive inhibition $K_I$ was expected to be similar with $K_s$ values, this explained the reason why their $K_I$ value was similar with our $K_s$ value rather than our $K_I$ value. In addition, $K_I$ is also a parameter used to represent a substrate

Table 3. Proportion of the methanogen abundance in 35ºC, 45ºC, and 55ºC anaerobic digestion systems.

| | 35ºC AD (%) | 45ºC AD (%) | 55ºC AD (%) |
|---|---|---|---|
| Mesophiles *Methanosarcinales* | 85.44±2.05 | 82.47±2.02 | 80.67±3.68 |
| Thermophiles *Methanobacteriales & Methanomicrobiales* | 14.56±2.05 | 17.53±2.02 | 19.33±3.68 |

Table 4. $K_I$ values of 45ºC anaerobic digestion system.

| Technique: Non-linear least square analysis<br>Type of inhibition: Uncompetitive inhibition | | | | |
|---|---|---|---|---|
| $K_{s,inhibitory}$ | 0.026 | mg/L as unionized HAc | 26 | mg/L as HAc |
| $K_{s,uninhibitory}$ | 0.170 | mg/L as unionized HAc | 171 | mg/L as HAc |
| $K_I$ | 0.073 | mg/L as unionized HAc | 72 | mg/L as HAc |

$K_{s,inhibitory}$ = Half saturation constant under inhibitory condition; $K_{s,uninhibitory}$ = Half saturation constant under uninhibitory condition; $K_I$ = Inhibitor's dissociation constant

affinity of the methanogens. As $K_s$ is used to represent methanogen's substrate affinity under uninhibited conditions, $K_I$ is used to represent methanogen's substrate affinity under inhibited conditions. Furthermore, in comparison to $K_{s,inhibitory}$ of 0.026 mg/L (Table 2), $K_I$ value of 0.072 mg/L represented methanogen's substrate affinity in any condition regardless of the free $NH_3$ concentration (Table 4), while $K_{s,inhibitory}$ was only applicable when free $NH_3$ concentration in the system is 110 mg/L.

## Conclusions

Statistically similar $K_s$ values and their ability to degrade acetate to a very low level suggests that the methanogens in the 45ºC anaerobic digestion system were similar to those in the 35ºC system. The methanogens were most probably thermo-tolerant acetate-utilizing methanogens from *Methanosarcinaceae* species. However, its lower $r_{max}$ values implied that at 45ºC, the methanogens did not work at their maximum capability as the temperature was not the optimum temperature for the involved methanogens. The performance was 32% reduced as the temperature rises from 35ºC to 45ºC. Additionally, total inhibition was observed in a 45ºC anaerobic digestion system when the concentration o f free $NH_3$ was high, with 95% reduction in acetate degradation. Other conditions such as SRT and background acetate concentration did not have a significant detrimental effect on the system.

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