Effect of arsenic acid on anaerobic methanogenic process: Kinetics, inhibition and biotransformation analysis

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A B S T R A C T

Arsenic acid (4-aminophenylarsonic acid) is widely used in the poultry and animal industries as a feed additive in the diets. Nearly all the added arsenic acid is excreted unchanged in manure resulting in the risk of arsenic contamination. In this study, the effects of arsenic acid on the kinetics, inhibition of methanogenic processes and its biotransformation were investigated. The methane yield was not affected by arsenic acid loading at concentration <0.46 mM, while the methane production was completely inhibited at concentration of 0.92 mM. The IC50 of arsenic acid in this study was 0.47 mM. After 115 days of incubation, 37–59% of the added arsenic acid was degraded. The species analysis indicated that at lower initial arsenic acid concentration, the soluble inorganic arsenic mainly existed in the species of arsenate (As(V)), while at higher initial arsenic acid concentration (>0.460 mM), the soluble inorganic arsenic mainly existed in the species of arsenite (As(III)), which explains why higher arsenic acid concentration has severe inhibition to methanogens.

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1. Introduction

Arsenic acid (4-aminophenylarsonic acid, C6H5NO3As), an aromatic organic-arsenic, has been extensively used as a feed additive in the poultry and pig farms for improving feed efficiency and growth rate, and controlling coccidial intestinal parasites [1–3]. The approved dosages as additives in animal feed are usually at 20–50 mg/kg, and most of the supplemented arsenic acid is excreted without chemical change in manure [4]. There were 800–1000 tons of arsenic acid being consumed every year in the United States, and the leaching and runoff resulted in the release of arsenic acid and its metabolites from manure into water body and soil, causing the risk of arsenic contamination [5]. Adsorption removal of organoarsenic from aqueous solution has been reported in the previous references [6,7].

Since arsenic acid is high water soluble, irrigation and runoff would mobilize it in the environment after land application of arsenic acid contaminated manure and litters and discharging of wastewater from farms [8], which results in the risk of arsenic contamination in the environment [9]. Guo et al. observed the inhibition of organoarsenic on the biological wastewater treatment processes at concentration exceeding 40 mg/L [10]. Yao et al. has reported that organic arsenic compounds accumulated in crops, and their degradation products arsenite (As(III)) and arsenate (As(V)) accumulated in water spinach and turnip [11–13]. Therefore, there are increased concerns on the arsenic pollution from organoarsenic additives.

Anaerobic digestion is a very common treatment way for the high concentration of organic wastewater generated during pig and poultry production. It has been reported that anaerobic methanogenesis was inhibited by arsenic acid at a concentration of 1 mM to acetate-utilizing methanogens [14]. However, to hydrogen-utilizing methanogens, there was little or no inhibition during the initial 4 days exposure to arsenic acid, and the inhibition significantly increased with the prolonged exposure time [14]. The reason why the inhibition markedly increases with exposure time is not clear. Several studies have reported that biological transformation of organoarsenic under anaerobic environments, which resulted in the formation of As(V) and As(III) [8,15]. As(III) and As(V) can be readily leached into surface water and groundwater [16,17], and are more toxic to organisms than organoarsenic. The actual concentration of arsenic acid in animal wastewater is lower...
than 1 mM. However, the impact of low concentration of arsenic acid on acetate-utilizing methanogens, and the fate and speciation of arsenic acid during anaerobic digestion process is very limited.

The aim of this study was to investigate the inhibition of arsenic acid on anaerobic acetate-utilizing methanogens, to analyze the inhibitory parameters through modified Gompertz equation, and to investigate the biodegradation and speciation of arsenic acid under anaerobic conditions. The investigation of fate and speciation of arsenic acid during anaerobic digestion might be able to explain the increased toxicity of arsenic acid to acetate-utilizing methanogens with prolonged exposure time, and to provide suggestion for the removal of arsenic from the effluent after anaerobic digestion.

2. Materials and methods

2.1. Chemicals

Arsenic acid was purchased from Alfa Aesar (98% purity) without further purification before use. Stock solution (1000 mg/L) was prepared by dissolving the determined amount of arsenic acid into deionized water. All other chemicals used in this study were of analytical grade.

2.2. Anaerobic methanogenic sludge

Anaerobic methanogenic sludge was collected from Zhu Zhanjing wastewater treatment plant, Hefei, China with volatile suspended solids (VSS) concentration of 27 g/L. After collection, the anaerobic sludge was acclimated in the laboratory at 37 °C for two months as the inoculums.

2.3. Anaerobic inhibitory assays

The inhibitory effect of arsenic acid on anaerobic methanogenesis was carried out in 250 mL serum bottles with work volume of 150 mL using batch cultures at 37 °C. The inhibition assays composed of two parts. In the first part, various sludge concentrations were investigated at an arsenic acid concentration of 0.092 mM, and the corresponding inoculated sludge concentration (as mixed liquor volatile suspended solids, MLVSS) was 482 mg/L, 1446 mg/L, 2450 mg/L, and 4820 mg/L, respectively. The acclimated sludge was diluted to the desired concentration with mineral medium under N₂ gas conditions. In the second part, various arsenic acid loadings (0 mM, 0.092 mM, 0.230 mM, 0.460 mM, 0.61 mM, 0.76 mM, and 0.920 mM) were applied at a MLVSS concentration of 1446 mg/L. Anaerobic assay without arsenic acid addition was used as the control. The nutrient medium used in the assays had the following composition (g/L): KH₂PO₄, 0.066; CH₃COONa, 3.81; NaHCO₃, 2.00; NH₄Cl, 0.287. Acetate was used as the sole carbon source and was added at the beginning of the experiment. Arsenic acid was supplemented into the bottles by injecting a calculated volume of stock solution. Serum bottles were incubated statically in an incubator and were mixed once per day. Each test was carried out in triplicate and the incubation was lasted over 115 days.

The mixed medium and headspace were flushed with N₂ for 2–3 min to maintain anaerobic conditions, and then the bottles were sealed with butyl rubber. Biogas volume was measured by a glass syringe once per day during the initial 20 days and the corresponding composition of the biogas was analyzed by a gas chromatography. The cumulative methane production was calculated. Liquid samples were collected at predetermined time intervals and centrifuged for 10 min at 10,000 rpm. The supernatant was filtered by 0.45 μm cellulose membrane under N₂ conditions for the analysis of arsenic acid and arsenic species.

2.4. Kinetics analysis

Modified Gompertz equation has been used to describe the accumulative hydrogen production process and anaerobic digestion process of cattail by rumen microorganisms [18,19]. In this study, the modified Gompertz equation was applied to describe the methane production for analyzing the inhibitory effect. The modified Gompertz equation was given as:

\[ P_{CH₄} = CH₄_{max} \times e^{- \frac{R_{max,CH₄} \times \exp \left( \frac{\exp \left( R_{max,CH₄} \times (\lambda - t) + 1 \right)}{CH₄_{max}} \right)}{R_{max,CH₄} \times (\lambda - t) + 1} + 1} \]  \quad (1)

By differentiating Eq. (1), the methane production rate was expressed as

\[ r_{CH₄} = \frac{R_{max,CH₄} \times \exp \left( \frac{\exp \left( R_{max,CH₄} \times (\lambda - t) + 1 \right)}{CH₄_{max}} \right)}{R_{max,CH₄} \times (\lambda - t) + 1} \]  \quad (2)

where \( P_{CH₄} \) is the cumulative methane production (mL CH₄/flask); \( CH₄_{max} \) is the potential maximum cumulative methane production (mL CH₄/flask); \( R_{max,CH₄} \) is maximum methane production rate (mL CH₄/flask/day); \( t \) is the incubation time (day); \( \lambda \) is the lag phase time (day); and \( R² \) is the correlation coefficient.

The experiment data were non-linearly simulated with software First Optimization (7D-Soft High Technology Ltd., China) and Origin 7.5 (OriginLab Ltd., USA). The corresponding parameters in both Eqs. (1) and (2) were estimated. Correlation coefficient (\( R² \)) values were used to evaluate the fitness between the experimental data and estimated values.

The inhibition of arsenic acid to methanogenesis was expressed by the specific methanogenic activities, which were calculated from the slope of the cumulative methane production versus time based on the results obtained from the second part of the inhibitory assays. Since the biomass concentrations added in the second part of the inhibitory assays were same, the specific methanogenic activities can be simplified as maximum methane production rate, which was calculated from Eq. (1). Thus, the inhibition of arsenic acid to methanogenesis was described by inhibition index as Eq. (3).

\[ \text{Inhibition index} (\%) = 100 - \left( \frac{100 \times \frac{R_{max,CH₄}}{R_{max,CH₄}^{\text{control}}}}{R_{max,CH₄}^{\text{control}}} \right) \]  \quad (3)

where \( R_{max,CH₄} \) is the \( R_{max,CH₄}^{\text{control}} \) of the control (mL CH₄/flask/day).

2.5. Analysis

Arsenical acid was analyzed by a high performance liquid chromatography (LC-20AD, Shimadzu Ltd., Japan) with an ultraviolet detector (UV) at 260 nm, a C18 Inertsil ODS-3 column (4.6 mm × 250 mm), and a HPLC guard column (4.6 mm × 10 mm). The mobile phase (pH 5.9) consisted of 46 mM potassium phosphate monobasic, 2 mM formic acid and 10% (v/v) methanol at a flow rate of 1.0 mL/min.

The methane content in the biogas was analyzed by a gas chromatography (Model SP-6890, Lunan Co., China) equipped with a thermal conductivity detector. The temperature of injector, detector and column was maintained at 100, 100 and 90 °C, respectively. Argon was used as carrier gas at a flow rate of 30 mL/min.

The concentration of inorganic arsenic in the solution was determined by an atomic fluorescence spectrometer (APS-8220, Beijing
Fig. 1. The daily methane production (a) and cumulative methane production (b) at various anaerobic MLVSS concentrations supplemented with 0.092 mM arsenic acid; daily methane production (c) and cumulative methane production (d) at different concentrations of arsenic acid and MLVSS concentration of 1446 mg/L.

3. Results and discussion

3.1. Methane production

Fig. 1 shows methane production with the addition of arsenic acid. As shown in Fig. 1a, at arsenic acid concentration of 0.092 mM, the maximum daily methane production increased from 28.0 mL/day to 54.5 mL/day when the anaerobic sludge concentration increased from 482 mg/L MLVSS to 4820 mg/L MLVSS, while the cumulative methane production increased from 176.0 mL to 236.5 mL, as shown in Fig. 1b. Fig. 1c shows the methane production at various arsenic acid concentrations. The maximal daily methane production was 26.5 mL/day, 29.5 mL/day, 29.75 mL/day, 19.7 mL/day, and 4.5 mL/day, corresponding the arsenic acid concentration 0 mM, 0.092 mM, 0.230 mM, 0.460 mM, and 0.920 mM, respectively. With the control (0 mM), there was no significant difference in the cumulative methane production when the added arsenic acid concentration was lower than 0.460 mM. However, when arsenic acid concentration increased to 0.460 mM, the lag time was significantly prolonged, as shown in Fig. 1c and d. When arsenic acid concentration increased to 0.920 mM, the methane production was completely inhibited.

3.2. Kinetics approach and inhibition analysis

3.2.1. Kinetics analysis of methane production

The accumulative methane production was analyzed using Eq. (1). As shown in Fig. 2a, the accumulative methane production increased with incubation time and was well described by Eq. (1) (solid line in Fig. 2a) with a high correlation coefficient value of 0.9984. R_{max,CH_4} and λ were 184.9 mL/day and 4.07 days, respectively. In a similar way, the corresponding methane production rate as a function of incubation time was calculated using Eq. (2), as illustrated in Fig. 2b. The maximum methane production rate was 44.6 mL/day at the 5.25th day of the incubation. The parameters calculated based on Gompertz equation describing methane production at various sludge concentrations and arsenic acid concentrations are listed in Table 1. The high correlation coefficient values (R^2) indicate that the modified Gompertz model was appropriate for describing the kinetics of methane production with and without arsenic acid addition.

3.2.2. Inhibition analysis

As shown in Table 1, the lag phase time of methane production was obviously affected by the supplement of arsenic acid. For quantitatively analyzing the relationship between the lag phase time and the arsenic acid loading, the lag phase time versus arsenic acid/MLVSS concentration was plotted, as shown in Fig. 3. At lower loading of arsenic acid/MLVSS, as shown in Fig. 3a, there was shorter lag phase time, indicating that methanogenesis was less affected at lower arsenic acid concentration and the inhibition to microorganisms can be recovered easily. With the increase of the
loading, the lag phase time was prolonged. For example, in the first part of the inhibition assays, the ratios of arsanilic acid/MLVSS were 0.019 mmol/g MLVSS, 0.038 mmol/g MLVSS, 0.064 mmol/g MLVSS, 0.191 mmol/g MLVSS, which corresponds to the lag phase time were 1.66 days, 2.73 days, 4.11 days, and 5.96 days, respectively. While at higher loading of arsanilic acid/MLVSS, as shown in Fig. 3b, the lag phase time was significantly prolonged with the increase of the loading. When the loading increased to 0.6345 mmol/g MLVSS (0.92 mM arsanilic acid), the inhibition was not recovered, indicating that methanogenesis was completely inhibited. Comparing Fig. 3a with Fig. 3b, it can be found that there is obviously trend for the inhibition that high arsanilic acid loading results in severe inhibition to anaerobic treatment system. Hence, increasing sludge concentration can effectively reduce the loading and the impact of toxic chemicals on methanogens.

Fig. 4a shows the methane production rate at various arsanilic acid concentrations. The $CH_{4}^{max}$ of the control was 184.9 mL, and the corresponding $\lambda$ value was 4.07 day (Table 1). As illustrated in Fig. 4a, the methane production rate of the control calculated from Eq. (2) was peaked at 43.4 mL/flask/day at the 5.5th day of the incubation, and then decreased gradually. It was very interesting that the maximal daily methane production and the cumulative methane production at arsanilic acid concentration of 0.092 mM and 0.23 mM were even slightly higher than that of the control (0 mM), confirming that lower arsanilic acid loading only affected the lag phase time, and the microbial activities could be completely recovered. As arsanilic acid concentration reached to 0.46 mM, the methane production rate decreased obviously, and the cumulative methane production decreased mildly, indicating that methanogen activities were partly inhibited at 0.460 mM. It has been reported that roxarsone could enhance microbial activity for the degradation of organic matters at concentration ~40 mg/L in a sequential batch reactor system [10], and 0.5 mM and 1.0 mM of roxarsone enhanced the microbial activity of Clostridium sp. strain OhILAs [8]. Therefore, the results obtained from this study and the references indicated that inhibition, toxicity or even stimulation to biological system depend on the arsanilic acid loading and the species of microorganisms [21,22].

To evaluate the inhibitory effect of arsanilic acid towards anaerobic methanogenesis, the Inhibition index was calculated using Eq. (3) which was shown in Fig. 4b. Negative numbers represent the

<table>
<thead>
<tr>
<th>Number</th>
<th>MLVSS (mg/L)</th>
<th>Arsanilic acid (mM)</th>
<th>$R^2$</th>
<th>$CH_{4}^{max}$ (mL/flask)</th>
<th>$R_{max,CH_4}$ (mL/flask/day)</th>
<th>$\lambda$ (day)</th>
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</tr>
<tr>
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<td>81.3</td>
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<td>0.8837</td>
<td>8.3</td>
<td>0.97</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 1**

Calculated parameters of Gompertz equation for the 8 runs.

**Fig. 2.** Simulation of cumulative methane production (a) and methane production rate (b) by Gompertz equation at 1446 mg MLVSS/L without arsanilic acid addition.

**Fig. 3.** Effect of low arsanilic acid loading (a) and high arsanilic acid loading (b) on the lag phase time ($\lambda$) of anaerobic methanogenesis.
degree of promotion to the maximum methane production rate. The inhibition index of 0.092 mM, 0.230 mM, 0.460 mM, 0.61 mM, 0.76 mM, and 0.920 mM in the second part of the inhibition assays was 5.33, 6.72, 45.73, 66.78, 92.54, and 97.75, respectively, indicating that at higher arsenic acid concentration, the inhibition increased very quickly. At 0.460 mM arsenic acid concentration (red line in Fig. 4a), the anaerobic sludge did not produce any methane during the initial 5 days. After 5 days incubation, the methane production began to recovered, indicating that anaerobic methanogenic bacteria had adapted to arsenic acid concentration. The elimination of inhibition after a certain exposure time could be attributed to factors such as acclimatization to contaminant of microorganisms, transition of dominant methanogenic species [23,24], adsorption of toxic compounds on flocs of sludge, and/or biological transformation of a toxic compound to a less toxic species [25] during exposure. As shown in Fig. 5a, the variation of arsenic acid concentration during incubation was very slow in the first 18 days of the incubation, suggesting that acclimatization of microorganisms to arsenic acid might be the main mechanisms for the recovery of the inhibition in this study.

The inhibition index of the anaerobic consortium supplied with 0.92 mM arsenic acid increased to 97.75 and the cumulative methane production decreased to 8.0 mL/flask. The methane was produced at the beginning of the incubation. It has been reported that arsenic acid didn't inhibit hydrogen-utilizing methanogens at the beginning of anaerobic digestion. The produced methane might be due to the activities of hydrogen-utilizing methanogens. Afterwards, organoarsenic compounds will be transformed to inorganic arsenic with the extending process of anaerobic digestion, which would exhibit severe toxicity to methanogens [8,26,27].

According to the report by Stasinakis et al. [28], 6.1–10.6 mg/L of As(III) or 81–198 mg/L As(V) give half of the maximum inhibition of respiration rate. As shown in Fig. 5b, trace amount of inorganic arsenic were detected, and the concentration was lower than 1.0 mg/L during the initial 10 days. Therefore, the methanogenic inhibition on methane production at the beginning of anaerobic digestion might be caused by arsenic acid itself, rather than the inorganic arsenics. Zhang et al. [29] has reported that arsenic acid at a low concentration of 50 μM caused DNA damage in Sertoli cells, and 0.5 mM resulted in oxidative stress and inhibition, which can also explain the inhibition of arsenic acid to anaerobic methanogenic sludge.

The inhibitory effects of arsenic acid can be expressed by half inhibitory concentration (IC50), which is the concentration of arsenic acid that causes a 50% reduction in the specific
methanogenesis activity relative to the control. The IC₅₀ value of arsenic acid in this study was calculated to be 0.47 mM (102.9 mg/L). Sierra-Alvarez et al. has reported the inhibition of HAPa to methanogenic activity, but the inhibition was only 14.2% at 1 mM HAPa under anaerobic conditions [14]. The difference might be caused by the inoculated methanogenic sludge and the inhibition of the generated inorganic arsenic in this study.

3.3. Biotransformation of arsenic acid

Fig. 5a shows the transformation of arsenic acid during anaerobic methanogenesis in the second part of the inhibitory assays in which arsenic acid was supplemented at various concentrations at MLVSS concentration of 1446 mg/L. It was found that only a small amount of arsenic acid was removed in the solution during the initial 18 days. After that, the removal rate gradually increased, as shown in Fig. 5a. The removal percentage of arsenic acid in the solution was 16.7%, 3.5%, 4.6%, and 10.8% on the 18th day and 46.0%, 36.9%, 37.6%, 58.7% on the 115th day for the 0.092 mM, 0.230 mM, 0.460 mM, 0.920 mM treatments, respectively. The low removal rate indicates that the biotransformation of arsenic acid under anaerobic conditions is very difficult. Because arsenic acid is high water soluble, the persistent existence increases the mobilization. Therefore, the removal of arsenic acid during anaerobic process is very important for the control of arsenic acid contamination.

Fig. 5b shows the generation of inorganic arsenic. The soluble inorganic arsenicals in the solution were 3.45 μM, 18.27 μM, 50.06 μM and 146.13 μM on day 115, which accounted for 7.5%, 19.2%, 24.3% and 23.8% of the arsenic contained in the removed arsenic acid, or 3.4%, 7.1%, 9.2% and 14.1% of total arsenic added at the beginning in the reactors for 0.092 mM, 0.230 mM, 0.460 mM and 0.920 mM treatments, respectively. It is very interesting that low initial arsenic acid concentration had lower molar yield of soluble inorganic arsenic production. Since the sludge concentration in the second part was same for all assays, the lower yield might be due to the fact that most of the produced inorganic arsenic was adsorbed and precipitated in the sludge. The activities of methanogens were completely inhibited at 0.920 mM arsenic acid, indicating that this concentration of arsenic acid was toxic to methanogens. However, the removal rate of arsenic acid in this assay was not lower than other assays with lower arsenic concentration addition, in which methanogenesis was not inhibited. Thus, it can be deduced that the removal of arsenic acid might be mainly contributed to the activities of bacteria, rather than archaea (methanogens).

The toxicity of arsenic depends on its species existing in the environment [30]. For example, As(III) is more toxic than As(V). The biodegradation of arsenic acid and roxarsone was associated with the release of inorganic arsenic [26], which results in a stronger toxicity than their parent compounds to the organisms. The species analysis can explain the toxicity or inhibition of arsenic acid and its degradation products to anaerobic methanogenic sludge. As shown in Fig. 5c, it was found that when the initial arsenic acid concentration was lower than 0.230 mM, the main species of inorganic arsenic in the solution was As(V). When the initial arsenic acid concentration was higher than 0.460 mM, the main species in the solution was As(III), which was 2-5 fold greater than the concentration of As(V). This further explains the complete inhibition of arsenic acid to methanogens at high arsenic acid concentration.

4. Conclusions

In this study, the effect of arsenic acid on the kinetics, inhibition of methanogenic process and its degradation and speculation was investigated. The methane yield was not affected by arsenic acid at concentration <0.46 mM, while the methane production was completely inhibited at concentration of 0.92 mM. The lag phase time increased with arsenic acid/MLVSS loading. The half inhibition concentration (IC₅₀) was 0.47 mM. The species analysis indicated that arsenate (As(V)) was the main species at lower initial arsenic acid concentration, and arsenite (As(III)) was main forms at higher initial arsenic acid concentration (>0.460 mM).

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