The effect of propionic acid accumulation on methane production in dry mesophilic anaerobic fermentation

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Abstract. The dry anaerobic digestion performances were investigated at the temperature of 35±1°C with addition of propionic acid at different concentrations in five lab-scale reactors (R1-R5) to investigate the effect of propionic acid accumulation on methane production in dry mesophilic anaerobic digestion. And the volatile fatty acids, biogas and microbial community were detected. The active of microbial community and the methane generation would be inhibited when the propionic acid exceeded 30 g·L⁻¹, which affected not only syntrophic propionate-oxidizing bacteria but also acetate-utilizing methanogens. Eubacteria decreased sharply accompany with the higher concentration of propionic in R4 and R5. The relative abundance of acetoclastic methanogens was directly correlated with volatile solids removals and methane generation in dry anaerobic reactors. Otherwise, the quantity of syntrophic propionate-oxidizing bacteria was positively linear correlated with propionic acid when the concentration was below 30 g·L⁻¹. The methanogenic bacteria activity in R3 was the best with the methane yield of 27.01 mLCH₄ g⁻¹ VS d⁻¹. The results suggested that appropriate propionate could enhance methane production in the dry anaerobic fermentation.

1. Introduction
Solid waste is increasingly produced annually from industrial, municipal, and agricultural sources. Anaerobic digestion had been extensively used as a suitable treatment for organic waste, including livestock manure for society benefit by providing a clean fuel[1,2]. Dry anaerobic digestion (≥20% total solids) was demonstrated to be feasible[3] and had become attractive because it required relatively simple pre-treatment process and added less water before digestion than the liquid form (<5% total solids) [4]. The rate of mass transfer was lower than liquid form for less water in dry anaerobic digestion, which might hamper the activity of microorganisms. However, dry anaerobic fermentation of solid waste provides opportunities for energy recovery and nutrient reclamation because it requires less space and input than landfilling, composting and incineration[5]. So high efficient dry anaerobic digestion had attracted attention for increasing production of solid wastes.

Dry anaerobic digestion was carried out by the synergistic action of various groups of microorganisms in common with wet systems and went through hydrolysis, acidogenesis, acetogenesis and methanogenesis[6]. Overall process enhancement must be based on the understanding of the acidogens behavior which dominate the process and play a primary role in the major intermediates producing for the methanogens. These major substances in methanogenesis were also that could aggravate the process stability at high concentrations[7]. The dominant major intermediates were volatile fatty acids (VFAs). When the anaerobic food chain was unbalanced during the phases of
overloading or start-up of anaerobic process, the VFAs would accumulate and even inhibit methanogens, such as propionic and butyric acids[8], resulting in a reduction in methanogenic activity.

Once propionate or butyrate had been generated by acidogenic bacteria, acetogenic bacteria were essentially necessary for further anaerobic process. But slow conversion of propionic acid will lead to acidification and even failure if no counteractions are taken[9]. Highe-rate dry anaerobic digestion depends on syntrophic interaction of acetogens to avoid VFA accumulation[10]. If fatty acids have been accumulated, propionic acid is the most critical intermediate products, since its degradation may depend on the established pathway, the hydrogen partial pressure and acetate level[11]. And high degradation rates require close interspaces distances between propionate degraders, hydrogenolytic and aceticlastic methanogens[12].

Until now, the mechanisms of propionic acid accumulation had not been well understood. Some researchers found that propionic acid accumulation seemed to be independent of hydrogen partial pressure[13], and that often occurred in the condition of high yield of NADH. These conflicted results implied that the reasons for propionic acid accumulation and the relationship between hydrogen generation and accumulation in the anaerobic process were not still totally clarified[14]. And little is known about the community structure in dry anaerobic digestion reactors. The methanogens a dominance of Methanosarcinales, either of Methanosaeta spec. or Methanosarcina spec. was reported, whereas propionate degraders were only investigated in wet anaerobic digestion systems[11,15-18].

The main objective of this work was to estimate the evolution of dominant microorganisms of mesophilic-dry anaerobic reactors in different propionic acid accumulation. The interaction between microorganisms and substances (not only propionic acid accumulation, but also methane generation) were researched in the digestion process. This was accomplished by Fluorescent in situ Hybridization (FISH), employing different oligonucleotide probes, which was a technique that uses fluorescently labeled probes to detect and quantify specific cells/organisms in biological samples[6], since FISH was a valuable tool for the study of microbial dynamics in natural environment[19,20].

2. Materials and methods

2.1. Materials
The cattle manure was from a cattle farm beyond Zhengzhou city. The inoculum was anaerobic sludge from anaerobic digestion of the cattle farm. The characteristic of feedstock was shown in Table 1.

| Parameter         | Organic matter (%) | TS(%)   | VS(%)   | pH     | Total VFA (gAcH/L) | N-NH4(g/L) | TN(g/L) | C/N               |
|-------------------|--------------------|---------|---------|--------|--------------------|------------|---------|-------------------|
| Cattle manure     | 62.14±6.59         | 84.46±3.23 | 57.72±6.02 | 8.5±0.2 | 1.16±0.79          | 0.72±0.23  | 1.46±0.87 | 24.72±2.68       |
| Inoculum          | 38.05±6.12         | 13.61±3.02 | 32.66±6.32 | 7.2±0.2 | 2.08±0.59          | 2.24±1.12  | 10.63±2.01 |                  |

2.2. Experimental equipment
The experiments were carried out in lab-scale reactors with capacity of 5.0 L, and operated with 20% of total solid (Figure 1). The operational temperature was 35.0±0.5 °C, monitored by thermostatic bath.
Figure 1. Diagram of experimental device.
1. intelligent digital constant temperature controller, 2. heater, 3. temperature sensor, 4. water tank, 5. Thermometer, 6. sample connection, 7. fermentation tank, 8. wet gas flowmeter, 9. air pocket.

The reactor was loaded with 4 kg of cattle manure with 10% inoculum of total solid. The concentration of propionic acid was added in five reactors (R1, R2, R3, R4, R5) of 0, 4, 8, 12, 16 g·L⁻¹ based on the previous pilot test, respectively, and it was added every other day for eight times till to the stability stage.

2.3. Analysis methods
The parameters of initial feedstock were detected as follows: total solids (TS), volatile solids (VS), pH, total volatile fatty acid (VFA), ammonium nitrogen (N-NH₄) and total nitrogen (TN). The analytical techniques of all parameters except total VFA were based on Standard Methods[21]. And in the digestion process, we detected the variety and quantity of all the microbial community in the reactor by FISH, but also the volume and composition of the biogas (CH₄ and CO₂) by wet gas flowmeter (LML-1) and gas chromatography. And biogas composition was analyzed by a gas chromatography (GC1120, Shanghai shunyu gas chromatography) equipped with a thermal conductivity detector and GDX-01 column, with the injection port and detector temperature of 100°C, the column temperature of 80°C. Hydrogen of 8.78 mL·min⁻¹ was the carrier gas.

Liquid sample for VFAs detection was taken from the reactors every other day. The samples were pre-treated by adding same volume of 3% formic acid in order to adjust the pH less than 3.0, then the samples were centrifuged at 13000 rpm for 10 min, and the supernatant was to analyze for VFAs. Total VFA was calculated by addition of individual VFA levels.

The VFA levels were determined by the gas chromatography (GC1120, Shanghai shunyu gas chromatography) equipped with a flame-ionisation detector and FFAP capillary column. The injection port and detector temperature were 200°C, respectively, and the column temperature was 150°C. Nitrogen gas of 22.27 mL·min⁻¹ was the carrier gas. Hydrogen and air flow rates were 43.90 mL·min⁻¹ and 159.38 mL·min⁻¹, respectively.

All the analysis method was in accordance with Hong-li Li[22].

2.4. Fluorescence in situ hybridization (FISH) analyzing
The samples were digestive liquor in the mesophilic dry anaerobic reactors and collected every other day. The FISH technique used for cell fixation, consequent permeabilization and hybridization and the analytical techniques were performed in according with the procedures described by Montero[23]. In this study, the probes and the operational conditions were in according with the procedures described by Hong-li Li [24]. All the probes were labeled with FITC at the 5’ terminal, synthesized and fluorescently labeled by Dalian TaKaRa company.
3. Results and discussion

3.1. The evolution of pH and VFAs

All the reactors except R5 progressed successfully. And the ammonium nitrogen values in all reactors were below 1000 mg·L⁻¹, in the safety range for anaerobic digestion[25].

The evolution of pH and VFAs was shown in Figure 2. The pH was stable and remained in the neutral range (between 6.5 and 7.8) in R1, R2 and R3. Although the pH in R3 was below 6.5 in the day from 10 to 14 for the concentration of propionic acid or the total VFA exceeding 30 g·L⁻¹ or 100 g·L⁻¹, respectively, the pH adjusted slowly to the neutral range accompany with the slowly drop of the acid concentration to the safe range after the 14th day. However, the pH in R5 was below and remained 6.2 since the 8th day because the concentration of propionic acid or total VFA was far more than 30 g·L⁻¹ or 100 g·L⁻¹, respectively. In R4 the concentration of propionic acid or the total VFA exceeded 30 g·L⁻¹ or 100 g·L⁻¹, respectively, after 8 days, while it dropped from the 18th day and then the pH adjusted slowly to the neutral range from the 20th day.

Figure 2. The evolution of pH (a), various forms and Total VFA for five reactors (R1-R5).
3.2. The evolution of microbial community in reactors

Figure 3 showed the evolution of microbial community and accumulative methane in five reactors. *Eubacteria* predominated absolutely in the start-up stage of dry anaerobic digestion process in all reactors, and then dropped sharply with the enhancing of *Archaea* bacteria and propionic acid concentration.

![Figure 3. The evolution of microflora and accumulative methane in different reactors.](image)

H$_2$-utilising methanogens predominated the *Archaea* and decreased sharply in the first 6 days in all the reactors. The *acetate-utilizing methanogens* played the main role of methane transformation from the 6$^{th}$ day. The hydrolysis and acidogenesis of substrate was carried out in the initial stage of anaerobic digestion. Then the evolution of *Archaea* was in common with *acetate-utilizing methanogens*. And the quantity of *acetate-utilizing methanogens* decreased with the increasing of propionic acid in reactor R2 to R5. The *acetate-utilizing methanogens* had two peaks in R2 and R3 after the concentration of propionic acid dropped to about 30 g·L$^{-1}$. While in R4 and R5, the quantity of *acetate-utilizing methanogens* dropped below 0.25×10$^{10}$ cell·mL$^{-1}$ from 14$^{th}$ and 10$^{th}$ day, respectively, furthermore, the active was declined sharply and the methan generated hardly.

The quantity of syntrophic propionate-oxidizing bacteria was lower than other microbial community, and it changed gently with concentration of propionic acid. But the quantity of acetate-
utilizing methanogens dropped from the 10th and 8th in R2 and R3, respectively, accompany with the concentration of propionic acid exceeding 30 g·L⁻¹ (Figure 2 and Figure 3). The quantity of acetate-utilizing methanogens rose again when the concentration of propionic acid was below 30 g·L⁻¹. So not only syntrophic propionate-oxidizing bacteria but also acetate-utilizing methanogens were affected by propionic acid. And the accumulation of propionic acid had nothing to do with hydrogen partial pressure and H₂-utilising methanogens, which was in agreement with the result of Inanc[13].

Table 2 showed the cellular quantity of Eubacteria, Syntrophic propionate-oxidizing bacteria, Archaea, H₂-utilizing methanogens and acetate-utilizing methanogens in the reactors. The higher quantity was in R2 and R3 in accord with the methane accumulation in Figure 3.

| Reactors | Eubacteria | Syntrophic propionate-oxidizing bacteria | Archaea | H₂-utilizing methanogens | Acetate-utilizing methanogens |
|----------|------------|------------------------------------------|---------|--------------------------|-----------------------------|
| R1       | 5.48±0.69  | 0.43±0.05                                | 2.74±0.37 | 0.80±0.19                | 1.90±0.22                   |
| R2       | 5.89±0.82  | 0.50±0.05                                | 2.85±0.33 | 0.71±0.21                | 2.09±0.24                   |
| R3       | 4.85±0.86  | 0.43±0.06                                | 2.63±0.28 | 0.70±0.21                | 1.89±0.28                   |
| R4       | 3.47±0.33  | 0.09±0.03                                | 0.16±0.12 | 0.13±0.09                | 0.28±0.10                   |
| R5       | 2.18±0.58  | 0.08±0.05                                | 0.19±0.23 | 0.11±0.14                | 0.29±0.19                   |

3.3. Biogas generation and methane content
In the five reactors, the biogas product reached the maximum in the first week (Figure 4). In R2 and R3, there had two biogas generation peaks similar with the evolution of acetate-utilizing methanogens. The methane content exceeded 50% after the 10th days in R1, R2 and R3, while the methane content dropped slowly and biogas generation dropped sharply in R5 for the high concentration of propionic and total VFA. In R4, the methane content decreased in the first 12 days, and then it increased over 50% from the 20th days when the concentration of propionic acid dropped under 60 g·L⁻¹, with the slowly increasing of biogas production.

Table 3 showed a summary of performance data at the end of the process in five reactors. Over 32 day’s digestion process, the greatest methane yield was obtained in R3 followed R2, with values of 27.01 and 25.22 mLCH₄·g⁻¹VS·d⁻¹, respectively.

**Figure 4.** The biogas generation and methane content.
Table 3. Summary performance in five reactors after 32 days digestion.

| Reactors | Elim. VS (%) | Yield (mL•g-1•VS•d-1) Biogas Methane | Average content of biogas (%) CH4 CO2 |
|----------|--------------|--------------------------------------|-------------------------------------|
| R1       | 13.92        | 45.87                                | 22.67                                | 49.25 50.75 |
| R2       | 16.28        | 46.21                                | 25.22                                | 53.62 46.38 |
| R3       | 15.42        | 46.11                                | 27.01                                | 56.06 43.94 |
| R4       | 11.46        | 44.85                                | 21.36                                | 48.18 51.82 |
| R5       | 6.21         | 45.51                                | 18.29                                | 33.03 66.97 |

Elim. VS = organic removal efficiency as a percentage of VS elimination.  
Biogas: volume of gas generated as m3 of biogas per m3 of reactor per day.  
Methane: volume of methane generated as m3 of methane per m3 of reactor per day.

Figure 5. Linear correlations between physicochemical parameters and microbial concentration.  
Archaea with volume of methane generated and VS removal (a, c), respectively, acetate-utilizing methanogens with volume of methane generated and VS removal (b, d), respectively and syntrophic propionate-oxidizing bacteria with propionic acid concentration (e, f).
3.4. The correlation between Methanogenic microorganisms and physicochemical parameters

Some correlations were obtained between physicochemical parameters and microbial concentrations (see Figure 5).

These correlations had shown that changes in microbial population, mainly methanogens, could be linked to the traditional performance parameters.

The results of total methanogenic population (Archaea) and specifically acetate-utilizing methanogens were positive linear correlation with the volume of methane generated in the digester ($R^2=0.8077$ and $R^2=0.7838$, respectively). These results were in accordance with the mixing mesophilic anaerobic digestion treating other substance[26]. Archaea and acetoclastic methanogens were positive linear correlation with VS removal ($R^2_{Archaea}=0.8024$ and $R^2_{acetoclastic methanogens}=0.8431$).

The correlation between syntrophic propionate-oxidizing bacteria and propionic acid was positive linear with the coefficient of 0.8133 when the propionic acid concentration was below 30 g·L$^{-1}$, while the correlation was negative ($R^2=-0.87$) linear once the concentration of propionic acid exceeding 30 g·L$^{-1}$. It was accordance with the result that syntrophic propionic-oxidizing bacteria decreased in the 6th or 8th day when the concentration of propionic acid increased higher than 30 g·L$^{-1}$ (see Figure 2 and Figure 3).

4. Conclusions

It had been shown that the microbial community and the methane generation would be inhibited when the propionic acid exceeded 30 g·L$^{-1}$, and the inhibition would be eliminated once the concentration dropping.

Eubacteria decreased sharply accompany with the higher concentration of propionic acid. The active of acetate-utilizing methanogens, which played an important role in the anaerobic fermentation process, was affected by the concentration of propionic acid. And the syntrophic propionate-oxidizing bacteria was positive linear correlated with propionic acid below 30 g·L$^{-1}$, while it was negative linear once the propionic acid exceeding that. Therefore, propionate could enhance methane production with the increase of propionate concentration below 30 g·L$^{-1}$ in dry anaerobic digestion.

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