Ammonia Inhibition Level of Anaerobic Microbes Acclimated in the Anaerobic Digester Treating Pig Slurry under the High Ammonia Nitrogen

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Abstract

A biochemical methane potential (BMP) assay was conducted to determine ammonia inhibition level of acclimated methanogens inoculated from an anaerobic digester in high total ammonia nitrogen (TAN) concentration. Anaerobic methane production was assessed by batch anaerobic reactor in mesophilic condition (38℃). Ammonium chloride (NH_4C1) was added to adjust the TAN concentration to 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000 and 13,000 mg/L. The reactors of triplicate for each treatment and blank were incubated up to 50 days in a convection incubator. The biogas productions from the reactors below 7,000 mg TAN/L level were not different significantly, and the significant reduction of biogas production arose above from 8,000 mg TAN/L level. The theoretical methane potential of cellulose material, calculated by Buswell's equation, was 0.414 $Nm^3/kg\text{-}VS_{added}$. Ultimate methane potentials were in the range of 0.32-0.36 $Nm^3/kg\text{-}VS_{added}$ at the NH_4^+-N levels between 1,000 and 6,000 mg/kg. The ultimate methane potentials at the ammonia levels between 10,000 and 13,000 mg TAN/L showed the very low values of 0.10, 0.006 $Nm^3/kg\text{-}VS_{added}$. Results of this study have presented that the methanogens acclimated at

the high strength ammonium-nitrogen of about 4,000 – 5,000 mg/L could be tolerant to the ammonium nitrogen concentration of 7,000 mg/L. These results imply that the acclimation technology of methanogens is very useful for the improvement of digester microbial stability in the inhibitory anaerobic condition.

Keywords: *Biogas, Anaerobic digestion, Ammonia inhibition, Methanogen.*

Introduction

Anaerobic digestion process is an important technology for recovery of biogas from organic substrates as a source of renewable energy along with an efficient tool for waste management, but the process should be carefully controlled and monitored. Process inhibition is related to the particular characteristics of the substrate to be anaerobically digested, pH, process temperature (mesophilic or thermophilic), type of the seed sludge (inoculum), the reactor configuration and the concentrations of ammonia (NH_3) and ammonium ion (NH_4^+).

Inhibition by ammonia and ammonium ion makes anaerobic digestion process vulnerable. The excess of total ammonia nitrogen (TAN), i.e. free ammonia nitrogen and ammonium nitrogen in anaerobic process could cause an inhibitory effect in different ways. Firstly, free ammonia (which is more toxic for anaerobic microorganisms than ammonium-ion) is formed during the process. Secondly, amination of α-ketoglutaric acid by ammonia-nitrogen coupled with rapid disappearance of α-ketoglutaric acid from the metabolic pool of the tricarboxylic acid cycle could cause difficulties in the metabolism of organic compounds. Finally, the release of ammonia may result in accumulation of VFA because of the need to maintain the pH at

8.0 - 8.2 (Krylova, 1997).

Even though ammonia is an essential nutrient for bacterial growth (McCarty, 1964b), it may inhibit methanogenesis during anaerobic digestion process if it is available at high concentrations. Therefore, ammonia is regarded as a potential inhibitor during anaerobic digestion, particularly when dealing with complex type of substrates such as manure or the organic fraction of municipal solid waste (Yengün and Demirel, 2013). It is generally known that when the TAN concentration exceeds 3,000 mg/L, then the ammonium ion itself becomes quite toxic regardless of pH and the process can be expected to fail (McCarty, 1964a). Total ammonia nitrogen concentrations of around 1,700 - 1,800 mg/L in most of the full scale anaerobic digesters operated under mid-mesophilic conditions were completely inhibitory with unacclimated inoculum (Albertson, 1961; Melbinger and Donnellon, 1971). But the acclimation of archaea to higher TAN concentrations could increase inhibitory up to 5,000 mg/L (van Velsen, 1979) because acclimation can greatly increase the free ammonia tolerance of methanogenic archaea (Koster, 1986).

One of the first reports dealing with the possibility of adapting methanogenic microbes to ammonia by exposing them to slowly increasing concentrations was the sludge digestion study of Melbinger and Donnellon (1971). They succeeded in operating a stable high-rate sludge digester at ammonia concentrations up to 2,700 mg TAN/L, whereas at that time 1700 mg TAN/L was considered to be the upper limit. It was observed that within NH_4Cl concentration range of 2 and 10g/L in a laboratory-scale reactor using with poultry manure high in nitrogen compounds, production of methane was not affected, but at higher concentrations from 10 to 30 g/L, methane production significantly decreased (Krylova *et al*., 1997).

Pig slurry, especially the waste from pig pen, is used as the major feedstock of Korean biogas plants, which has the high water content above 95%, and the high total nitrogen content above 4,000 mg/L. Anaerobic digesters which use the pig slurry as a main feedstock has been operated under the high ammonia condition, and occasionally ammonia inhibition is to be the main operational factor resulting in the reduced biogas production and the malfunction of anaerobic digester. Thus, a biochemical methane potential (BMP) assay was conducted to investigate ammonia inhibition level of acclimated methanogens inoculated from an anaerobic digester in high ammonia concentration.

Material and methods

Inoculum

The inoculum used in this study was taken from a 190 m^3 farm-scale digester (Anseong, South Korea) operated at a temperature of 38℃ and a hydraulic retention time of 30 days. This digester has been running for more than 6 years by being fed with pig slurry. The solids concentration in the digester was maintained at 3 to 4% by weight. Characteristics of inoculum were shown in Table 1. The anaerobic inoculum was kept at the mesophilic condition (38℃) during one week for removing any remaining biodegradable fraction after 2 mm sieving.

BMP assay

Anaerobic methane production was assessed by batch anaerobic reactors in mesophilic condition (38℃). Methane is the primary catabolic product of methanogenesis. The methanogenic rate of a sample, which is typically incubated in a reactor serum bottle,

can be readily measured, and the effect of organic matter decomposition on the rate can be assessed.

<Table 1>

<Table 2>

The potential of the methanogens in question to degrade added methanogenic substrates or their precursors can be determined (Shelton and Tiedje, 1984). Cellulose powder (Sigmacell, Sigma-Aldrich Co., USA) was used for substrate in the experiment. For BMP assay 160 mL size serum bottles were used in working volume of 100 mL. For each serum bottle, 0.3g of cellulose powder (3g-$VS_{substrate}$/L) and 20 mL of inoculum were added. NH_4C1 was added to adjust the TAN concentration to 1,000 (N-1), 2,000 (N-2), 3,000 (N-3), 4,000 (N-4), 5,000 (N-5), 6,000 (N-6), 7,000 (N-7), 8,000 (N-8), 9,000 (N-9), 10,000 (N-10) and 13,000 (N-11) mg/L. The reactors were then filled to 80 mL with mineral salt medium (Table 2) modified from Shelton and Tiedje (1984). In order to prevent rapid pH drop 3.6 g/L of $NaHCO_3$ was finally added to each serum bottle and the head space of serum bottle was filled with N_2 gas, and sealed with butyl rubber stopper. The serum bottles of triplicate for each treatment and blank were incubated up to 50 days in a convection incubator, manually mixing every day during the incubation period. Methane production was corrected for standard temperature and pressure (STP), and BMP (Nm^3/kg-VS_{added}) was determined by unit of VS content of substrate added to bottle. Biogas production data were compared after single factor statistical significance analysis using Duncan's multiple range test (0.05 level). In order

to describe the progress of cumulative methane production, the modified Gompertz equation (Equation 1), employed to fit the cumulative methane production data, was as follows (Costa et al., 2012).

$$M = P \times exp\left\{-exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right\} \qquad (Equation\ 1)$$

where M was cumulative methane production (mL); e was exp (1); R_m is the maximum specific methane production rate (mL/d); P was methane production potential (mL); λ was lag phase time (days).

Analytical procedure

Total solid (TS), volatile solid (VS), pH, total chemical oxygen demand (TCOD), total nitrogen (T-N), TAN and alkalinity were determined according to standard methods (APHA, 1998). Total gas production by BMP assay was measured daily for the first 5 days and every 2 to 3 days afterward by displacement of an acidified brine solution in burette and recording the volume of displaced solution after correcting to atmospheric pressure (Beuvink *et al*., 1992; Williams *et al*., 1996). For analysis of VFA (Erwin *et al*., 1961), 1 mL of supernatant was mixed with 0.2 mL of metaphosphoric acid (250 g/L) in a 2 mL Eppendorf tube and kept at 4℃ for 30 min. After re-centrifugation of the mixture at 14,000×g for 10 min at room temperature, the supernatant was injected into the gas chromatograph (Shimadzu GC-2010, Kyoto, Japan) equipped with flame ionization detector (FID) and SGE BP21 column (SGE, Melbourne, Australia). The temperature of oven, injector and detector was 150℃, 250℃ and 200℃, respectively. To investigate the gas composition, CH_4 and CO_2

concentration in the gas samples were determined by gas chromatograph (Clarus 680, PerkinElmer, USA) equipped thermal conductivity detector with HayeSepQ packed column (CRS Inc., USA). Column was operated with helium as carrier gas at the constant flow rate of 5 mL/min. Injector was maintained at 150℃, oven was set at 90℃, and the detector was set at 150℃.

Results and Discussion

Characteristics of inoculum

The characteristics of inoculum from the anaerobic digester input with pig slurry were represented in Table 1. Inoculum used in the experiment was not in normal condition showing higher pH, ammonia and alkalinity. Swine manure is one of the main substances that are used in Korean farm scale biogas plant. Generally the high amount of pig manure is discharged as slurry phase that have a small fraction around 1-7% of TS from pig farmhouse, since most of Korean pig farmhouses have been adopting the slurry storage tank without a solid/liquid separation equipment (Yoon *et al*., 2009). Again, pig slurry is stored for a long time about 30 days in the slurry tank before it is used for biogas production in the anaerobic digester. Therefore, the organic contents in the pig slurry discharged from the farmhouse are highly fluctuant influencing the biogas production and stability of the digester operation. And the fluctuation of organic contents in the pig slurry may cause “wash-out” that methane producing bacteria are lost in anaerobic digester which disturbs microbial community of the anaerobic digester and consequently methane yield decreases steeply (Yu *et al*., 2005; Boe and Angelidaki, 2009). Adding with this, the higher ammonia contents in the pig slurry inhibits microbes

in the digester. The anaerobic digester from which the inoculum samples for this experiment was taken has been monitored for some parameters including the methane production and concentration in biogas and pH, TS, VS, VFA, alkalinity, TCOD, T-N and TAN in digestate during more than 900 days. During these days, the parameters were highly variable showing pH 7.82~8.30, TS 15,330~40,520 mg/L, VS 7,820~39,780 mg/L, VFA 900~4,688 mg/L, alkalinity 12,000~26,250 mg/L as $CaCO_3$, COD_{Cr} 19,000~46,124 mg/L, T-N 3,451~6,829 mg/L and TAN 2,686~5,787 mg/L. Fig. 1 shows methane production (m^3/ton-$substrate_{added}$) and methane concentration (%) of the anaerobic digester indicating methane production was high in fluctuation. It is assumed that the microorganisms in the digester are highly acclimated in the unstable environmental condition. Therefore, the acclimated microorganisms could resist in high ammonium nitrogen levels in BMP assay.

<Figure 1>

Ammonia inhibition assay

Averaged biogas productions and methane concentrations in ammonia inhibitory BMP assay produced during the incubation period were presented in Fig. 2. Ammonia inhibition started from 7,000 mg TAN/L, showing the significant reduction ($p<0.05$) of biogas production compared with the reactors in the lower ammonia concentrations except N-1 and N-4 reactors. Beyond 7,000 mg TAN/L level, biogas yields were dramatically decreased accompanying with lowered methane concentrations. From Fig. 2, it is assumed that microbial activity in the reactors was inhibited from 7,000 mg TAN/L but the inhibition of methanogenesis was started from the lower TAN concentration (6,000 mg).

Cumulative methane productions influenced with increased ammonia concentrations in the BMP assay were presented in Fig. 3. Ultimate methane potentials (B_u; $Nm^3/kg\text{-}VS_{added}$) for all treatments calculated according to Fig. 3 by Gompertz model were 0.33, 0.36, 0.34, 0.33, 0.34, 0.32, 0.26, 0.21, 0.23, 0.10 and 0.006 for N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10 and N-11, respectively. Methane production curves in a BMP assay implicates biodegradability characteristics of substrates and production of inhibitory intermediate products which will mainly control the kinetics of the different steps of anaerobic digestion and define the shape of the methane production curve. In this study the BMP assay was firstly controlled by increasing levels of ammonia concentration to determine inhibition levels of TAN. Cumulative methane production curves of the treatments beyond 6,000 mg TAN/L showed that methanogenesis was initially inhibited but resumed after inhibition. The delay period of methanogenesis seemed to be dependent on the ammonia concentration in the BMP assay. Labatut *et al*. (2011) suggested that in BMP assay as the concentration of a fermentation product such as volatile fatty acids or hydrogen reaches the homeostatic threshold of a certain microorganism or a group of microorganisms, the thermodynamic balance is changed, and one or several metabolic reactions may be inhibited, causing no further product accumulation and delay of substrate digestion. In most cases, product inhibition is reversible, and as soon as thermodynamic conditions become favorable, reactions resume. Again McCarty (1964a) suggested that microorganisms usually have the ability to adapt to some extent to inhibitory concentrations of most materials. The extent of adaptation is relative, and in some cases the activity after acclimation may approach that obtained in the absence of the inhibitory material, and in other cases the acclimation may be much less than this.

The theoretical methane potential (0.414 $Nm^3/kg\text{-}VS_{added}$) of cellulose substrate evaluated by the Buswell's formula was higher than observed methane productions of all treatments in Fig. 3. The difference between theoretical (B_{th}) and observed (B_u) methane productions were mostly from that the formula does not account for substrate biodegradability along with the 12% loss of the total carbon during the digestion of carbohydrates in the cell protoplasm which was not accounted for by the formula (Symons and Buswell, 1933). The observed methane productions up to 6,000 mg TAN/L were about 70% of the theoretical methane potential. There were further decreases of the observed methane production in the ammonia concentrations which are higher than 6,000 mg/L showing about 55% in 7,000, 8,000 and 9,000 mg/L, 24% in 10,000 mg/L and 1% in 13,000 mg/L of the theoretical methane production.

The results presented in Fig. 3 indicate that the microorganisms present in digestate, acclimated to a high ammonia concentration of 4,435 mg/L (Table 1), can produce methane at all ammonia concentrations investigated except the 13,000 mg TAN/L level. However, an increasing lag-phase occurred when the ammonia concentration was increased in BMP assay, extending to a 40 day period at an ammonia concentration of 10,000 mg TAN/L. As mentioned above in Fig. 2, the sharp increase of the lag-phase from N-7 to N-8 may point to a threshold level at some ammonia concentration in this range, beyond which a prolonged lag-phase is required for the acclimation of the inoculum. However, this threshold level is far beyond from the findings of van Velsen (1979) which methane production was not inhibited at ammonia concentrations in the range 605-3,075 mg TAN/L in digested piggery manure, acclimated to an ammonia concentration of 2,420 mg TAN/L. It is suggested that the higher threshold level in this study be caused by using an inoculum highly acclimated at the higher ammonia

concentration. Table 3 shows characteristics of digestates in all batch reactors with increasing levels of ammonia concentration after 50 days incubation. The pH of all treatments ranged between 7.46 and 8.02. Alkalinity ranged between 6,425.0 and 9,983.3 mg/L as $CaCO_3$ in all treatments. Total N and TAN concentrations directly reflected the increased NH_4Cl addition levels. TS, VS and TCOD concentrations in digestates were increased by the increased addition levels of NH_4Cl, which implicates the inhibited degradation of cellulose by microorganisms in the reactors.

<Figure 2>

<Figure 3>

<Table 3>

CONCLUSIONS

Anaerobic digestion processes are widely used for the biological degradation of concentrated organic wastes and for biogas production. And high ammonium-nitrogen content and long chain fatty acid, and volatile fatty acids (VFAs) accumulation by high organic loading could result in variable inhibitory effects during anaerobic digestion. Pig slurry which has been used as the feedstock of farm scale biogas production facilities has the high ammonium nitrogen content above 4,000 mg/L. Therefore, anaerobic digesters which use the pig slurry as a main feedstock could have vulnerability at the operation of anaerobic digester. Results of this study have presented

that the methanogens acclimated at the high strength ammonium-nitrogen of about 4,000 – 5,000 mg/L could be tolerant to the ammonium nitrogen concentration of 7,000 mg/L. This result imply that the acclimation technology of methanogens is very useful for the improvement of digester microbial stability in the inhibitory anaerobic condition

Acknowledgements

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Table 1. Characteristics of inoculum (Values in parentheses are standard deviations) from an anaerobic digester operated for 6 years with pig slurry.

Parameters	Inoculum
pH	8.51 (0.006)
TS (%)	4.14 (0.014)
VS (%)	2.53 (0.022)
TCOD (mg/L)	47,433 (1,002)
T-N (mg/L)	6,322 (596)
TAN (mg/L)	4,435 (153)
VFAs (g/L)	3,031 (88).
Alkalinity (mg/L as $CaCO_3$)	25,213 (160)

TS: total solid; VS: volatile solid; TCOD: total chemical oxygen demand; T-N: Total nitrogen; TAN: total ammonia nitrogen; and VFAs: volatile fatty acids.

Table 2. Composition of mineral salt medium for the biochemical methane potential assay in the experiment.

Ingredient	Chemical	Concentration(g/L)
Phosphate buffer	KH_2PO_4	0.27
	K_2HPO_4	0.35
Mineral salts	$CaCl_2 \cdot 2H_2O$	0.075
	$MgCl_2 \cdot 6H_2O$	0.1
	$FeCl_2 \cdot 4H_2O$	0.02
Trace metals	$MnCl_2 \cdot 4H_2O$	0.0005
	H_3BO_3	0.00005
	$ZnCl_2$	0.00005
	$CuCl_2$	0.00003
	$NaMoM_4 \cdot 2H_2O$	0.00001
	$CoCl_2 \cdot 6H_2O$	0.0005
	$NiCl_2 \cdot 6H_2O$	0.00005
	Na_2SeO_3	0.00005

Table 3. Characteristics of digestates in anaerobic batch reactors with increasing levels of ammonia concentration after 50 days incubation (Values in parentheses are standard deviations).

Parameters	Ammonia treatment										
	N-1	N-2	N-3	N-4	N-5	N-6	N-7	N-8	N-9	N-10	N-11
pH	7.97 (0.07)	7.46 (1.06)	8.02 (0.13)	7.94 (0.04)	7.97 (0.02)	7.96 (0.02)	7.94 (0.05)	7.90 (0.02)	7.89 (0.06)	7.77 (0.02)	7.52 (0.04)
TS (%)	0.95 (0.01)	1.06 (0.02)	1.26 (0.04)	1.40 (0.11)	1.73 (0.13)	2.28 (0.03)	2.54 (0.08)	2.87 (0.09)	3.27 (0.04)	3.76 (0.18)	4.70 (0.03)
VS (%)	0.38 (0.07)	0.45 (0.03)	0.64 (0.03)	0.80 (0.13)	1.11 (0.14)	1.67 (0.03)	1.93 (0.08)	2.31 (0.05)	2.71 (0.10)	3.09 (0.06)	4.11 (0.03)
$TCOD_{Cr}$ (mg/L)	6,197 (25)	6,210 (149)	6,617 (188)	6,647 (182)	6,570 (160)	7,313 (420)	8,103 (214)	8,147 (35)	8,865 (247)	10,283 (491)	13,877 (168)
T-N (mg/L)	1,519 (206)	2,688 (159)	3,644 (244)	4,250 (348)	5,598 (128)	6,099 (362)	7,512 (903)	8,386 (479)	9,539 (280)	10,659 (111)	13,211 (36)
NH_4^+-N (mg/L)	1,149 (21)	2,167 (55)	3,304 (123)	4,022 (391)	4,998 (338)	5,982 (143)	7,174 (250)	8,183 (89)	9,153 (321)	10,024 (176)	12,654 (262)
VFA (mg/L as acetate)	ND	ND	ND	4.9 (0.3)	4.9 (0.4)	6.0 (0.3)	11.9 (1.7)	30.8 (3.1)	142.0 (20.1)	554.8 (13.4)	1,587.7 (261.3)
Alkalinity (mg/L as $CaCO_3$)	7,550 (189)	8,221 (1551)	9,983 (62)	7,308 (213)	7,188 (315)	7,621 (328)	7,496 (88)	6,975 (331)	6,267 (1,543)	7,063 (75)	6,425 (130)

N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, and N-11 are 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, and 13,000 mg NH_4^+-N/L in the reactors, respectively.

TS: total solid; VS: volatile solid; TCOD: total chemical oxygen demand; T-N: Total nitrogen; TAN: total ammonia nitrogen; VFAs: volatile fatty acids; and ND : not detected.

Figure 1

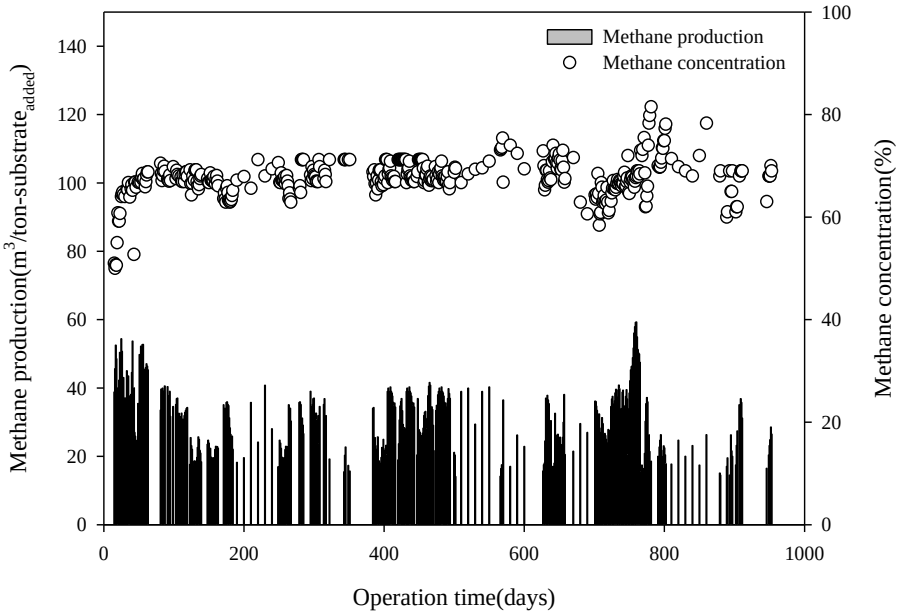

Fig. 1. Methane productions (m^3/ton-$substrate_{added}$) and methane concentrations (%) changes monitored from an inoculum-donor anaerobic digester for a period of 1,000 days. The anaerobic digester is a 190 m^3 farm-scale digester (Anseong, South Korea) operated at a temperature of 38℃ and a hydraulic retention time of 30 days using pig slurry.

Figure 2

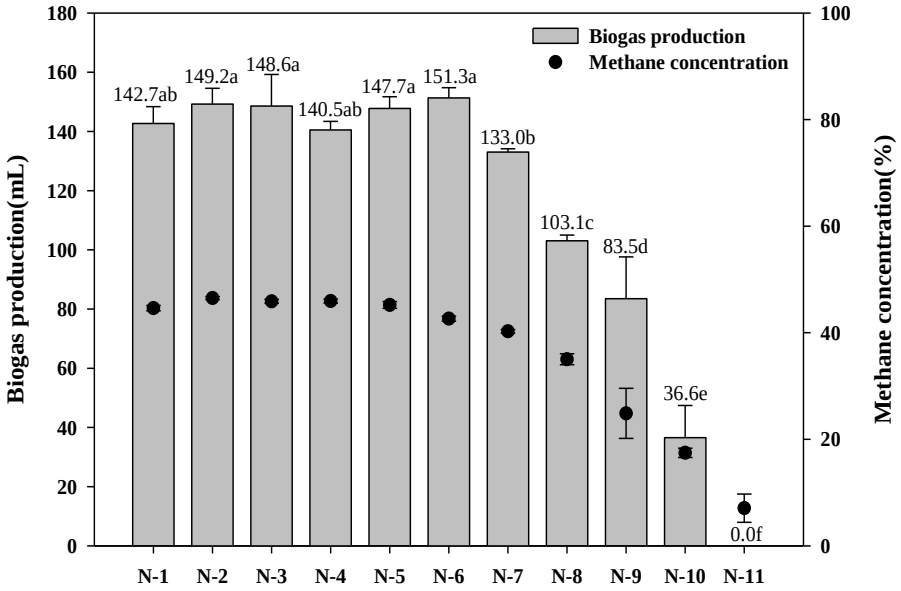

Fig. 2. Biogas productions and methane concentrations influenced with increased ammonia concentrations in anaerobic batch reactors (Means with different character differ significantly ($p<0.05$) (N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, and N-11 are 1,000, 2,000, ,3000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, and 13,000 mg-total ammonia nitrogen/L in the reactors, respectively).

Figure 3

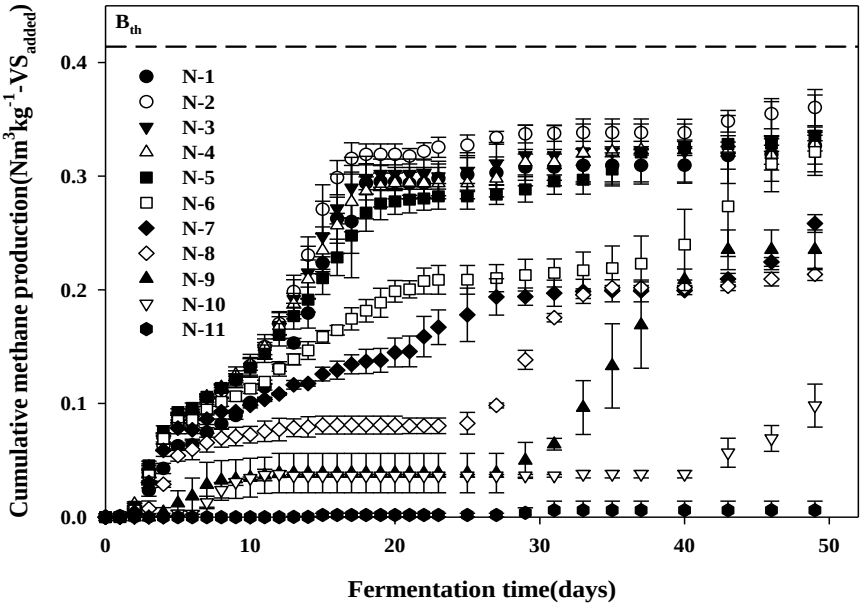

Fig. 3. Cumulative methane production curves influenced with increased ammonia concentrations in anaerobic batch reactors (Vertical bars mean standard deviations, B_{th} is theoretical methane potential of cellulose, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, and N-11 are 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, and 13,000 mg-total ammonia nitrogen/L, respectively).