Effect of urea addition on giant reed ensilage and subsequent methane production by anaerobic digestion

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Abstract

The effect of urea addition on giant reed ensilage and sequential anaerobic digestion (AD) of the ensiled giant reed was evaluated. The dry matter loss during ensilage (up to 90 days) with or without urea addition was about 1%. Addition of 2% urea enhanced production of lactic acid by about 4 times, and reduced production of propionic acid by 2-8 times. Besides, urea addition reduced degradation of cellulose and hemicellulose, and increased degradation of lignin in giant reed during ensilage. Ensilage with or without urea addition had no significant effects on the enzymatic digestibility of giant reed, but ensilage with urea addition achieved a cumulative methane yield of 173 L/kg VS, which was 18% higher than that of fresh giant reed. The improved methane yield of giant reed could be attributed to the production of organic acids and ethanol during ensilage.

Keywords: Giant reed; Ensilage; Urea addition; Lactic acid; Anaerobic digestion

1. Introduction

Giant reed (*Arundo donax* L.) is a C3 perennial rhizomatous grass that is currently widespread in India, China, the USA, Australia, Southern Africa, and the Mediterranean regions (Scordia et al., 2013). Different from typical C3 plants, which are less productive than C4 plants, giant reed has an unusually high photosynthetic capacity (Rossa et al., 1998), and thus achieves a biomass yield even higher than those of typical C4 plants, such as *Miscanthus × giganteus* (Corno et al., 2014). As a result, giant reed has been recognized as one of the most promising energy crops for providing biomass feedstocks for the production of fuels and value-added products (Corno et al., 2014). One approach for bioenergy production from giant reed is anaerobic digestion (AD) which is a well-established and widely-used technology for treating biomass and producing methane as energy (Yu and Schanbacher, 2010). AD of giant reed has been reported in a few publications, and is considered to be an effective and reliable method for harnessing energy from giant reed with low greenhouse gas emissions (Di Girolamo et al., 2013; Dragoni et al., 2011; Ragaglini et al., 2014; Yang and Li, 2014).

In order to maintain a viable biofuel supply chain, storage of raw biomass feedstocks is generally required. Ensilage is a well-known technology for wet biomass storage (Darr and Shah, 2012). During the ensilage, lactic acid bacteria (LAB) consume non-structural carbohydrates under anaerobic conditions, and produce organic compounds, such as lactic acid, acetic acid and ethanol. The accumulation of organic acids, mainly lactic acid, reduces the pH to about 4.0, thus preventing further degradation of biomass by inhibiting the activity of microorganisms (Zheng et al., 2011). Under favorable conditions, the silage can be stable for a long period, e.g. one year, with organic dry matter (ODM) loss of less than 10% (Herrmann et al., 2011). Besides being used for preserving wet feedstocks in the livestock industry, ensilage is also considered a

promising biomass storage method for bioenergy production (Darr and Shah, 2012; Herrmann et al., 2011; Neureiter et al., 2005). The ensiled biomass is particularly suitable for production of methane by AD, because the organic acids and ethanol are also intermediates for biogas production in the AD process. Studies on ensilage of various crops, such as maize and grasses for methane production have been reported (Neureiter et al., 2005; Pakarinen et al., 2011; Pakarinen et al., 2008; Vervaeren et al., 2010; Yahaya et al., 2001). However, published data on ensilage of giant reed for methane production by AD is scarce.

Urea treatment has been frequently used in ensilage for improved silage quality, e.g. nutritive value and digestibility (Dias-da-Silva and Sundstøl, 1986; Guedes et al., 2006; Mascarenhas-Ferreira et al., 1989; Sarwar and Khan, 2004). Breakdown of urea to ammonia via hydrolysis during 2-3 weeks of ensiling without any urease addition was reported by Ibrahim et al., (1985). In addition, urea treatment also increased lignin degradation during ensilage of hemp and, subsequently, increased its enzymatic digestibility by 46% (Pakarinen et al., 2011). To date, there have been no reports on urea treatment for ensilage of giant reed.

The objective of this study was to examine the effect of urea addition on giant reed ensilage, and subsequent methane production from ensiled giant reed via AD. Giant reed ensilage experiments with and without urea addition were conducted for different periods of time. Dry matter loss, lignocellulose degradation, and organic acid accumulation during the ensilage processes were examined. Enzymatic hydrolysis and AD of giant reed with or without urea addition were then carried out, and the effects of ensilage and urea addition on enzymatic digestibility and methane production were studied. Finally, the correlation between the content of organic acids and ethanol in giant reed (fresh and ensiled) and the corresponding methane yields was further evaluated in order to explain the improved methane yield by ensilage.

2. Methods

2.1. Feedstock and inoculum

Giant reed biomass was harvested from the Ohio State University (OSU) research farm in Columbus, OH, USA on October 3, 2014, ground to pass through a 12 mm sieve using a shredder-chipper (Mighty Mac, Mackissic Inc., Parker Ford, PA, USA), and ensiled on the same day. Effluent from a mesophilic liquid anaerobic digester (KB BioEnergy, Akron, OH, USA) fed with sewage sludge was used as an inoculum for AD. Characteristics of giant reed biomass and the inoculum for AD are presented in Table 1.

2.2. Ensilage of giant reed

The processed giant reed biomass was ensiled with and without urea addition, respectively. For ensilage with urea addition, the giant reed biomass was mixed with 16% (w/v) urea to reach a urea content of 2% (based on dry weight of biomass) and a moisture content of 40.6% (w/w). For ensilage without urea addition, the giant reed biomass was mixed with water to reach the same moisture content (40.6%). Ensilage was conducted by packing 1 kg of the mixture (giant reed biomass with additional water and/or urea) into 1-gallon-size zipper bags (Ziploc vacuum Freezer System, SC Johnson Inc., Racine, WI, USA). For each mixture and the control, 12 bags were prepared. The bags were then vacuumed to minimize the presence of oxygen and placed at room temperature ($25\pm 3^{\circ}\text{C}$). Ensilage was run for different periods of time (30, 60, and 90 days). At each time interval including day 0, three bags were selected randomly from trials with and without urea treatment and the mixture in each of the three bags was taken out and mixed thoroughly. After sampling for composition analysis, the remaining silage samples were stored at -20°C for AD and determination of enzymatic digestibility.

2.3. Determination of enzymatic digestibility

Enzymatic digestibility of treated and untreated giant reed was determined according to a Laboratory Analytical Procedure (LAP) reported by National Renewable Energy Laboratory (NREL) (Selig et al., 2008). The activity of the cellulase (Cellic CTec 2, Novozymes, Denmark) was measured according to an NREL LAP (Adney and Baker, 2008). Samples (in duplicate) supplemented with cellulase (60 FPU per gram of cellulose) were incubated at 50°C with shaking at 180 rpm for 72 hours, and each hydrolysate was filtered through a 0.2 µm nylon membrane filter prior to sugar analysis. The enzymatic digestibility was defined as the glucose yield from cellulose by enzymatic hydrolysis, and calculated as follows:

$$\text{Enzymatic digestibility (\%)} = \frac{M_{\text{glucose}}}{f \times M_{\text{cellulose}}} \times 100$$

Where M_{glucose} is the amount of glucose released from cellulose by enzymatic hydrolysis, $M_{\text{cellulose}}$ is the amount of cellulose in the sample (determined by a method described in section 2.5), and $f = 180/162$ is the conversion factor for cellulose to glucose (Zeng et al., 2007).

2.4. Anaerobic digestion of ensiled giant reed

AD was set up by mixing fresh or ensiled giant reed, inoculum, and deionized (DI) water to obtain a feedstock to effluent (F/E) ratio of 0.5 (based on volatile solids, VS) and a total solids (TS) content of 5%. AD with only inoculum was also run as a control. All AD reactors were conducted in triplicate, and incubated at a mesophilic ($37 \pm 1^\circ\text{C}$) condition for 30 days. A 5-L Tedlar gas bag (CEL Scientific, Santa Fe Springs, CA, USA) was connected to the outlet of each reactor for biogas collection. Biogas volume and composition were determined every 2 to 4 days.

2.5. Analytical methods

TS, VS, total Kjeldahl nitrogen (TKN), ammonia nitrogen ($\text{NH}_3\text{-N}$), and pH of samples were measured based on the Standard Methods for the Examination of Water and Wastewater

(APHA, 2005). A 5 g sample was suspended in 50 mL of DI water prior to pH measurement. The carbon to nitrogen (C/N) ratio was calculated based on total carbon (TC) and total nitrogen (TN) contents which were determined using an elemental analyzer (Elementar Vario Max CNS, Elementar Americas, Mt. Laurel, NJ, USA). Crude protein content was calculated by determining total organic nitrogen (TKN minus $\text{NH}_3\text{-N}$) and multiplying by a factor of 6.25 (Hattingh et al., 1967).

Cellulose, hemicellulose, and lignin content was analyzed based on an NREL LAP (Sluiter et al., 2008). Briefly, biomass was extracted with DI water and ethanol in series using an automated extraction unit (Dionex ASE 300 extraction system, Thermo Scientific, Sunnyvale, CA, USA) and then the extractive content was determined. Extractive-free biomass was hydrolyzed to release mono-sugars via a two-step acid hydrolysis, and the concentrations of mono-sugars were measured by high performance liquid chromatography (HPLC). The amount of cellulose was calculated from glucose using a conversion factor of 0.90. The amount of hemicellulose was calculated from the sum of xylose, arabinose, galactose, and mannose, using a conversion factor of 0.90 for C-6 sugars and 0.88 for C-5 sugars, respectively. Acid-soluble lignin was measured by ultraviolet-visible (UV-vis) spectroscopy (BioMate 3S spectrophotometer, Waltham, MA, USA), and acid-insoluble lignin was determined by gravimetric analysis. The HPLC (Shimadzu LC-20AB, MD, USA) used for sugar analysis was equipped with a Biorad Aminex HPX-87P column and a refractive index detector (RID). Temperatures of the column and detector were maintained at 60°C and 55°C, respectively, and HPLC grade water was used as the mobile phase with a flow rate of 0.3 mL/min.

In order to determine the content of lactic acid, acetic acid, propionic acid, butyric acid, and ethanol in biomass, a 10 g sample was suspended in 100 mL of DI water and incubated at 4 °C

for 10 h. The suspension was filtered through a 0.2 μm nylon membrane filter, and the filtrate was analyzed by a HPLC that was equipped with a Phenomenex Rezex RFQ-Fast Fruit H^+ column (Phenomenex Inc., Torrance, CA, USA), a micro-guard cartridge (Catalog No. 125-0129, 30 $\times$ 4.6 mm), a RID, and an ultra violet (UV) detector. Temperature of the column and the detector were set at 60°C and 55°C, respectively, and 2.5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.4 ml/min. RID was used to determine concentrations of lactic acid, acetic acid, and propionic acid. Since butyric acid and ethanol were difficult to separate from each other, their concentrations were determined based on the fact that butyric acid has a high UV absorbance at 190 nm, while ethanol does not have a significant absorbance at this wavelength. Briefly, butyric acid concentration was first determined via the UV detector. The RID peak area of butyric acid at this concentration was calculated based on a pre-established calibration curve for butyric acid, and subtracted from the RID peak area for butyric acid/ethanol. The peak area after subtraction was then used to determine the ethanol concentration.

Biogas composition (methane and carbon dioxide) was analyzed by a gas chromatograph (Agilent, HP 6890, Wilmington, DE, USA) equipped with a 30 m $\times$ 0.53 mm $\times$ 10 μm Rt®-Alumina Bond/KCl deactivation column and a thermal conductivity detector (TCD). Helium gas was used as a carrier gas at a flow rate of 5.2 mL/min. The column oven was initially set at 40°C for 4 minutes and then increased to 60°C with an increment of 20°C/minute and held at 60°C for 5 minutes. The temperatures of the injector and detector were set at 150°C and 200°C, respectively. Volume of biogas in the Tedlar gas bag was determined by using a diaphragm pump to draw the biogas out from the bag and inject into a drum-type gas meter (Ritter, Bochum, Germany).

2.6. Statistical analysis

Statistical significance was assessed by analysis of variance (ANOVA, $\alpha = 0.05$) using Minitab (Version 16, Minitab, Inc., State College, PA, USA).

3. Results and discussion

3.1. Effect of urea addition on compositions of ensiled giant reed

Generally, stable storage was achieved after 30 days of ensilage with or without urea addition (Table 2). During ensilage of giant reed without urea addition, the DM loss was about 1% in the first 30 days with a significant ($p < 0.05$) decrease in extractive content and pH, while these values remained relatively constant in the following 60 days. The decrease in extractive content was mainly attributed to the degradation of water soluble carbohydrates (WSC), most of which had been degraded in the first 30 days. For mixtures without urea addition, the $\text{NH}_3\text{-N}$, TN, and TC contents and the C/N ratio were stable throughout the 90 days of ensilage without significant variations ($p > 0.05$) (Table 2). During ensilage of giant reed with urea addition, the DM loss and the extractive, WSC, and carbon contents were comparable to those without urea addition (Table 2). Addition of urea led to an increased TN content and a decreased C/N ratio. Due to the degradation of urea to ammonia, the $\text{NH}_3\text{-N}$ content increased sharply from 0.86 to 1.80 g/kg DM in the first 30 days, which was associated with the increase of pH from 5.47 to 7.35. The $\text{NH}_3\text{-N}$ content increased slowly (up to 2.04 g/kg DM) in the following 60 days, while the pH value decreased to 5.11 indicating further accumulation of acids (Table 2).

As shown in Figure 1, organic acids and ethanol were produced during the ensilage process, while urea addition significantly ($p < 0.05$) changed the profile of lactic acid and propionic acid accumulation. During ensilage of giant reed without urea addition, the contents of lactic acid,

acetic acid, propionic acid, and ethanol were in the range of 6.5-10.7, 7.4-19.2, 21.5-35.2, and 8.1-14.0 g/kg DM, respectively (Figure 1). When urea was added, contents of lactic acid and acetic acid were increased to 23.0-36.0 (about 4-fold) and 16.7-21.9 (up to 2-fold) g/kg DM, respectively, while propionic acid content decreased to 3.7-10.1 g/kg DM (2- to 8-fold) (Figure1). Urea addition increased ethanol content to 13.8 g/kg DM on day 30, but resulted in ethanol content on days 60 and 90 comparable ($p>0.05$) to those without urea addition. Butyric acid was undetectable during the ensilage, except that 3g/kg DM of butyric acid was observed at 90 days of ensilage without urea addition.

The toxicity of organic acids to microorganisms generally depends on the pH level. At low pH, organic acids are undissociated, and can easily pass through the cell membrane and inhibit growth of microorganisms. At high pH, organic acids are in the form of ionic compounds, which cannot enter the cells by free diffusion alone (Warnecke and Gill, 2005). Therefore, during the ensilage process, higher organic acid contents can be reached at a higher pH due to the reduced toxicity of organic acids. In addition, pH levels can also affect the profile of organic acids during ensilage. According to Hofvendahl and Hahn-Hägerdal's study (2000), the optimum pH for lactic acid production by microorganisms ranges between 5.0 and 7.0. As a result, the pH values (5.1-7.4) during ensilage with urea addition could favor lactic acid production compared to those without urea addition (pH 4.6). It should be noted that overloading of urea may cause inhibitory effects on microorganisms due to toxicity of ammonia and increase of pH. An optimal urea loading rate could be determined prior to industrial applications.

Propionic acid accumulation in silage may occur through two mechanisms. One is propionic production by propionic acid bacteria, which is inhibited at a pH lower than 4.8 (Driehuis et al., 1999); 2) Another one is the degradation of lactic acid to propionic acid by hetero-fermentative

bacteria, such as *Lactobacillus buchneri*, with 1,2-propanediol as an intermediate (Driehuis et al., 1999; Elferink et al., 2001). According to literature, the degradation of lactic acid by *Lactobacillus buchneri* occurred when pH was about 4.0, but was inhibited at a pH above 5.8 (Elferink et al., 2001). As shown in Figure 1c and Table 2, propionic acid levels in silage at pH 4.6 were significantly higher than those at pH 5.1-7.4. Therefore, the propionic acid accumulation in this study could mainly be attributed to the second mechanism, i.e. lactic acid degradation by hetero-fermentative bacteria, and thus urea addition could reduce the propionic acid generation through increasing pH.

3.2. Effect of urea addition on lignocellulose degradation in giant reed during ensilage

Without urea addition, 1.8% of cellulose, 11.0% of hemicellulose, and 3.2% of lignin were degraded in 30 days of ensilage (Figure 2). Degradation of cellulose, hemicellulose, and lignin increased to 3.7%, 12.5%, and 6.3%, respectively, during 60 days of ensilage. More cellulose was degraded from day 60 to day 90, while no significant ($p>0.05$) increase in hemicellulose and lignin degradation was observed during this period. Urea addition significantly reduced cellulose degradation by at least 2-fold (Figure 2a), but had a minimal effect on hemicellulose degradation (Figure 2b). Besides, urea addition resulted in higher lignin degradation, especially on day 30 and day 90 (Figure 2c).

Since hemicellulose is easier to degrade than cellulose during ensilage (Mcdonal et al., 1991), higher hemicellulose degradation than cellulose degradation has been observed during ensilage of various feedstocks, such as corn stover and orchard grass (Ren et al., 2006; Yahaya et al., 2001). However, the lignin degradation during ensilage varied dramatically depending on the feedstock. For example, lignin degradation of 3-4% was observed during 4-month ensilage of hemp, but not for ensilage of maize or fava beans (Pakarinen et al., 2011). Improved lignin

degradation by urea addition was also observed during the ensilage of hemp (Pakarinen et al., 2011).

3.3. Effect of urea addition on enzymatic digestibility of ensiled giant reed

Neither ensilage nor the urea addition significantly enhanced enzymatic digestibility of giant reed ($p>0.05$) (Figure 3). Improved enzymatic digestibility (35-42% increase) during the ensilage process has been reported in studies of sugar beet, rye, and clover (Oleskowicz-Popiel et al., 2011; Zheng et al., 2011). However, Oleskowicz-Popiel et al. (2011) reported that no improvement of enzymatic convertibility was observed during ensilage of maize. Pakarinen et al. (2011) suggested that ensilage can lead to decreased enzymatic hydrolysis yield of corn, as highly digestible carbohydrates have been converted into acids during the ensilage process. In addition, Walton et al. (2010) mentioned that organic acids generated during ensilage may interfere with enzymatic hydrolysis. Therefore, ensilage may not necessarily improve the enzymatic digestibility of feedstocks.

Pakarinen et al. (2011) reported that hemp preserved with 3% urea achieved a carbohydrate increase of 46% in enzymatic hydrolysis compared to the fresh material. The urea dosage (2%) used in this study might be not enough to obtain a significant enhancement in enzymatic digestibility of giant reed. Besides, the effect of urea addition on enzymatic digestibility of ensiled biomass could also vary for different feedstocks.

3.4. Effect of urea addition on methane production via anaerobic digestion

Daily methane yield and methane content during 30-day AD of fresh giant reed and 90-day ensiled giant reed are presented in Figure 4. The daily methane yields all peaked in about 2-4 days, while ensiled giant reed (90-day ensilage) showed higher peak daily methane yields than fresh giant reed (0-day ensilage). The peak daily methane yields of giant reed ensiled with and

without urea addition (18.6 and 17.7 L/kg VS) were 16% and 10% higher than those of fresh giant reed, respectively (Figure 4a). This is likely attributed to the presence of organic acids in the ensiled biomass, which expedites methane production. After the initiation of AD, methane contents increased quickly reaching 64.1-69.5% in 4 days, and then maintained at these levels with minimal fluctuations until the end of the AD (Figure 4b). Besides, removal efficiencies of organic matter during all the AD tests were 18-21%.

AD of fresh giant reed with and without urea addition obtained similar cumulative methane yields of about 147.3 L/kg VS (Figure 5), which was close to the methane yield (150.8 L/kg VS) obtained by L-AD of giant reed in a previous study (Yang and Li, 2014). As shown in Figure 5, the cumulative methane yield was generally improved by ensilage, and longer ensilage time resulted in higher methane yields. Besides, 60-day and 90-day ensilage with urea addition achieved significant ($p<0.05$) improvements in the cumulative methane yield compared to those of fresh giant reed. Specifically, with urea addition, 60-day and 90-day ensilage increased the cumulative methane yields to 171 and 173 L/kg VS, respectively, which were 16% and 18% higher than those of fresh giant reed. Similarly, Pakarines et al. (2008) reported that ensilage increased the methane yield of different grasses (ryegrass and mixture of timothy, red clover and meadow fescue) by up to 19%.

The improved methane yields by AD of ensiled giant reed could be attributed to the production of organic compounds, especially lactic acid, acetic acid and ethanol, during ensilage, because these organic compounds can be readily utilized by microorganisms in AD systems for biogas production. Weak correlations ($R^2 < 0.04$) were obtained between cumulative methane yield and propionic acid or butyric acid, which indicated that these acids may not have contributed to the improved methane yield. In contrast, strong linear relationships were found

between cumulative methane yield and lactic acid ($R^2 = 0.85$) (Figure 6a) or acetic acid ($R^2 = 0.91$) (Figure 6b) or ethanol ($R^2 = 0.78$) (Figure 6c). A stronger linear relationship was observed between cumulative methane yield and a combination of lactic acid, acetic acid and ethanol ($R^2 = 0.96$) (Figure 6d). This result supports the above mentioned hypothesis that methane yield of giant reed can be improved by ensilage, especially with urea addition, due to production of organic acids and ethanol during ensilage.

4. Conclusions

Ensilage with or without urea addition was found to be a stable method for storage of giant reed with minimal dry matter loss. Urea addition enhanced production of lactic acid and acetic acid production, while it reduced propionic acid production during ensilage of giant reed. Urea addition resulted in reduced cellulose and hemicellulose degradation, and improved lignin degradation in giant reed during ensilage. Although ensilage didn't significantly increase the enzymatic digestibility of giant reed, methane yield during AD of giant reed was still improved by the ensilage process probably due to the production of lactic acid, acetic acid and ethanol.

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Tables

Table 1. Characteristics of giant reed biomass and inoculum for AD

Parameters	Giant reed	Inoculum for AD
DM, %	43.53 ± 0.55	6.71 ± 0.01
ODM, %DM	92.19 ± 0.10	63.85 ± 0.05
TN, % DM	0.77 ± 0.03	3.83 ± 0.07
TC, % DM	49.98 ± 0.03	39.06 ± 0.52
C/N	64.55 ± 2.47	10.20 ± 0.06
pH	5.57 ± 0.03	7.99 ± 0.01
Extractives, %DM	21.80 ± 0.76	14.13 ± 0.91
WSC, %DM	4.94 ± 0.25	0.46 ± 0.08
Cellulose, %DM	1.70 ± 0.08	ND
Glucose, %DM	1.47 ± 0.01	ND
Hemicellulose, %DM	15.65 ± 0.29	ND
Lignin, %DM	16.66 ± 0.38	NA
Crude protein, %DM	4.06 ± 0.66	ND
NH ₃ -N, % DM	0.06 ± 0.02	5.25 ± 0.52
Ash, %DM	7.81 ± 0.09	36.15 ± 0.05

DM-Dry matter; ODM - Organic dry matter; WSC - Water soluble carbohydrates (a fraction of extractives); ND- not detectable; NA- not applicable

Table 2. Dry matter (DM) loss, water soluble carbohydrates (WSC), extractives, ammonia nitrogen (NH₃-N), total nitrogen (TN) and total carbon (TC) content; carbon to nitrogen (C/N) ratio; and pH during 90-day ensilage of giant reed with and without urea addition.

Time (day)	DM loss (%)	WSC (% DM)	Extractives (% DM)	NH ₃ -N (g/kg DM)	TN (% DM)	TC (% DM)	C/N	pH
0% urea								
0	0.00 ± 0.00c	4.94 ± 0.25a	21.81 ± 0.69a	0.42 ± 0.06c	0.67 ± 0.01d	45.12 ± 0.04c	67.53 ± 1.02b	5.40 ± 0.03bcd
30	0.98 ± 0.02b	0.36 ± 0.02b	14.98 ± 0.51b	0.39 ± 0.02c	0.62 ± 0.03d	46.32 ± 0.53ab	74.82 ± 3.25a	4.60 ± 0.08de
60	1.14 ± 0.14ab	0.22 ± 0.09b	15.16 ± 0.11b	0.36 ± 0.08c	0.63 ± 0.02d	45.79 ± 0.53ab	72.66 ± 2.97a	4.56 ± 0.07e
90	1.20 ± 0.22a	0.40 ± 0.04b	15.26 ± 0.86b	0.39 ± 0.03c	0.64 ± 0.05d	46.17 ± 0.21a	72.43 ± 5.84ab	4.56 ± 0.06e
2% urea								
0	0.00 ± 0.00c	4.94 ± 0.25a	21.81 ± 0.69a	0.86 ± 0.01b	1.42 ± 0.01a	45.26 ± 0.08bc	31.95 ± 0.31e	5.47 ± 0.12bc
30	0.87 ± 0.10b	0.23 ± 0.01b	15.61 ± 0.09b	1.80 ± 0.09a	0.82 ± 0.02c	46.09 ± 0.27ab	56.26 ± 0.95c	7.35 ± 0.15a
60	0.91 ± 0.11b	0.20 ± 0.03b	15.89 ± 0.46b	1.95 ± 0.13a	1.14 ± 0.04b	45.99 ± 0.42ab	40.37 ± 1.84d	6.20 ± 0.63b
90	0.97 ± 0.15b	0.26 ± 0.04b	15.67 ± 0.27b	2.04 ± 0.19a	1.12 ± 0.01b	46.05 ± 0.29abc	41.25 ± 0.56d	5.11 ± 0.47cde

Within columns, means that are not sharing a letter are significantly different at $p < 0.05$.

Figures

Figure 1. Effect of urea addition on organic acid and ethanol production during giant reed ensilage for different periods of time

In each figure, means that are not sharing a letter are significantly different at $p < 0.05$.

(a)

(b)

(c)

Figure 2. Effect of urea addition on degradation of cellulose (a), hemicellulose (b), and lignin (c) in giant reed during ensilage for different periods of time

In each figure, means that are not sharing a letter are significantly different at $p < 0.05$.

Figure 3. Effect of urea addition and ensilage time on enzymatic digestibility of giant reed

(a)

(b)

Figure 4. Effect of urea addition and ensilage on the daily methane yield (a) and methane content (b) of giant reed during 30-day AD

Figure 5. Effect of urea addition and ensilage time on cumulative methane yield of giant reed during 30-day AD

Figure 6. Correlation between cumulative methane yield and contents of organic acids and ethanol in the feedstock of AD
(Lactic acid and ethanol content have been converted into acetic acid equivalent values based on their theoretical methane yields, i.e. 373, 373 and 730 L/kg for lactic acid, acetic acid and ethanol, respectively)