Effects of different potassium and nitrogen pretreatment strategies on anaerobic digestion performance of rice straw

Juan Luo, a Juan Li, a Liang Zhang, a Nankun Li, ac Akiber Chufo Wachemo, ad Chunmei Liu, a Hairong Yuan, a and Xiujin Li a

Received 6th March 2020
Accepted 22nd April 2020
DOI: 10.1039/d0ra02136a
rsc.li/rsc-advances

1. Introduction

China is one of the largest agricultural countries in the world. The annual production of rice straw (RS) averages in the range of 180–270 million tons. However, a considerable amount of RS has been burned in the open air, resulting in serious environmental pollution and the waste of a reusable resource. Anaerobic digestion (AD) is a highly recommended waste-to-energy conversion method and the most promising method for renewable energy production from agricultural residues.

However, two shortcomings were observed in direct utilization of RS. One is the recalcitrant lignocellulosic structure, resisting hydrolysis of the substrate and further conversion by anaerobic microorganisms; the other is the excessive carbon/nitrogen ratio (C/N, 53 : 1–78 : 1), while the suggested optimum C/N for AD is in the range from 20 : 1 to 30 : 1. Excessive C/N can inhibit the methane production due to the changes occurred in the microbial composition and metabolic pathway transfer.

Therefore, overcoming the recalcitrance of lignocellulosic biomass and optimize the C/N in digesters are critical to further improve the AD performance of RS.

Pretreatment of straw prior to AD can effectively destroy the inherent recalcitrant lignocellulosic structure and increase biodegradability of lignocellulosic biomass. Several different single alkali pretreatments were conducted. Shetty et al. conducted AD at room temperature with 1% NaOH pretreated RS, the biogas production increased by more than 34%. Mancini et al. using 1.6% (w/w) NaOH for the pretreatment of RS achieved an enhancement of 21.4% for the final biomethane production yield (318 mL g VS⁻¹) compared to the untreated substrate. However, the high load of Na⁺ in the biogas slurry is harmful to environment because it is difficult to recover and might cause salinization. Potassium hydroxide (KOH) pretreatment is accepted due to potassium ability to be recycled and used as fertilizer after pretreatment and digestion. Muhammad et al. received a 41% higher biomethane yield of 258 mL g VS⁻¹ with 6% KOH pretreatment than untreated wheat straw, and yielded a digestate with higher fertilizer values potassium (138%). Liu et al. studied ambient temperature KOH pretreatment at a loading of 20% and achieved a 52.5% higher methane yield compared to untreated wheat straw. Although these single alkali pretreatments can promote the AD performance of straw, the C/N in the digestion system cannot be regulated and extra nitrogen is needed during AD. Oji et al. indicated that ammonia monohydrate (NH₃·H₂O) pretreatment could show enhanced biodegradability of straw for better AD
performance and increase the nitrogen content in the digestion system. Zhang et al.,11 pretreated rice straw with 2% NH₃·H₂O, which showed a 17.5% biogas yield increase over the untreated one. Yuan et al.16 reported that 4% NH₃·H₂O pretreated wheat straw achieved 427.1 mL g VS⁻¹ of biogas yield, which was 26.7% higher than that of untreated sample. However, there has been few studies paying attention to the combined alkali pretreatment and single NH₃·H₂O pretreatment. Because KOH is a strong monobasic alkali, which can quickly weaken the ester bond connecting the hemicellulose and lignin in the substrate. This may increase the damaging effect of NH₃·H₂O on lignocelluloses. Moreover, KOH + NH₃·H₂O combined pretreatment can provide nitrogen for AD system and has potential potassium and nitrogen nutrient for digestate. This method can recover the cost of pretreatment agents and reduce the negative environmental impacts such as soil salinization and water pollution.

Different pretreatment methods could result in differences in the substrate bioconversion characteristics and further the microbial community structure, diversity and activity of specific populations.17 In the study of Zhou et al., the overall performance of waste activated sludge digestion was significantly dependent on the initial chemical pretreatments, which in turn influenced and was related to the microbial community structures.18 A few studies have been studied on the biogas performance for single KOH and single NH₃·H₂O pretreatment.19 However, little information is available on the effects of KOH + NH₃·H₂O combined pretreatment on AD performances and micro-organisms.

The objectives of this research were to: (1) investigate the biomethane production performance and conversion rate of main compositions for different nitrogen and organic pretreatment strategies (KNPSs). (2) Compare the microbial community structures for different KNPSs. (3) Analyze the nutritional value of digestate for different KNPSs.

2. Methods and materials

2.1. Materials

The RS used in this study was collected from Tianjin District, China and then air-dried in open field. The collected RS was chopped by a knife mill (YSW-180, Zhengde, China) for size reduction and then grounded into a size of 20 meshes by a universal pulverizer (YSW-180, Yanshan Zhengde Co., Beijing, China) to increase the surface area for the next pretreatment and microbial attack. The inoculum for AD process was taken from a biogas continuously operated stable station in Shunyi District, Beijing, China. The characteristics of RS and inoculum are listed in Table 1.

2.2. Pretreatment

In order to compare the biogas production performance of RS with different pretreatments, NH₃·H₂O and KOH were used as pretreatment reagents in this study. The RS was pretreated by 1% KOH +1% NH₃·H₂O (1K + 1N), 2% KOH (2K) and 2% NH₃·H₂O (2N), which was based on dry weight of RS at 30 °C for 2 d, and the water amount addition of all pretreatment groups was 6 times that of the dry weight of RS. Then, the pretreated RS was directly used for AD tests.

2.3. Start-up and operation of reactors

Four lab-scale continuously stirred tank reactors (CSTRs) were applied for AD experiment. The total volume of each CSTR was 10 L with the working volume of 8 L. The four reactors (R1, R2, R3 and R4) were respectively started up with 1K + 1N, 2K, 2N and untreated RS at 60 g VS L⁻¹ and inoculum at mixed liquid suspended solids (MLSS) of 30 g L⁻¹. Stable reactor temperature was maintained at 35 ± 1 °C by circulating water through the surrounding water jacket. Each reactor was agitated with a stainless-steel stirrer at a speed of 80 rpm for 10 min every 2 h.

After 30 days of adaption period, the reactors were considered ready for semi-continuous feed. The feeding organic loading rate (OLR) of each CSTR was 1.7 g VS L⁻¹ d⁻¹ and hydraulic retention time (HRT) was 45 d.

The pretreatment method of feed in each reactor respectively corresponded to that at start-up period. Moreover, to supplement the N-source, 2% NH₃·H₂O was daily added to R2 (2K_2N) and R4 (untreated_2N) during AD process after being fed for 75 d. Different KNPSs in four reactors were shown in Fig. 1.

The biogas residue and biogas slurry samples were periodically collected from reactors at different time which was used for chemical composition analysis and system stability measurements. The steady state was defined to be the point when biogas production rates varied within 5% of their average values after an operating time to more than one HRT period.20

2.4. Analytic methods

2.4.1. Biogas analysis. Daily biogas production (DBP) was recorded using the water displacement method. The volume of the biogas produced was corrected to standard temperature (273.15 K) and pressure (101.325 kPa). Daily methane

---

**Table 1** Characteristics of RS and inoculum

| Items         | Value (%) |
|---------------|-----------|
| **Rice straw**|           |
| TS (%)        | 93.70 ± 0.09 |
| VS (%)        | 80.82 ± 0.48 |
| MLSS (g L⁻¹)  | —         |
| TC (%)        | 38.14 ± 0.13 |
| TN (%)        | 3.51 ± 0.03 |
| C/N (%)       | 74.78 ± 0.06 |
| K (g kg⁻¹)    | 10.42 ± 0.23 |
| P (g kg⁻¹)    | 2.86 ± 0.08  |
| Cellulose (%) | 36.44 ± 0.75 |
| Hemicellulose (%) | 26.87 ± 2.35 |
| Lignin (%)    | 4.84 ± 0.18  |
| **Inoculum**  |           |
| TS (%)        | 11.95 ± 0.06 |
| VS (%)        | 8.31 ± 0.13  |
| MLSS (g L⁻¹)  | 112.00 ± 3.50 |
| TC (%)        | 35.15 ± 0.11 |
| TN (%)        | 2.56 ± 0.28  |
| C/N (%)       | 13.15 ± 0.45 |
| K (g kg⁻¹)    | 20.09 ± 0.42 |
| P (g kg⁻¹)    | 36.07 ± 0.67 |

---

"a Values are means ± SD (n = 3). b Content of fresh matter. c Content of dry matter."
production (DMP) was determined from CH$_4$ content. Daily methane production per volume (DMP-V) was determined from DMP and system’s working volume. In the same way, the daily methane production per total solid (DMP-TS) was calculated from DMP and TS fed, and daily methane production per volatile solid (DMP-VS) was calculated from DMP and VS fed. The biogas components (CH$_4$, CO$_2$, N$_2$ and H$_2$) were measured using a gas chromatograph (Zhongkehuijie corporation, SP-2100, Beijing, China) equipped with a thermal conductivity detector and a TDX-01 column. Argon was used as the carrier gas. The biogas components (CH$_4$, CO$_2$, N$_2$ and H$_2$) were measured using a gas chromatograph (Zhongkehuijie corporation, SP-2100, Beijing, China) equipped with a thermal conductivity detector and a TDX-01 column. Argon was used as the carrier gas.

### 2.4.2. Chemical composition analysis

Samples were taken from the discharging port of each reactor every 5 days to determine total solid (TS), volatile solid (VS), pH value, volatile fatty acids (VFAs), ammonia-nitrogen (NH$_3$–N) and total alkalinity concentration (TAC). TS, VS and MLSS were determined according to the standard methods. The total carbon (TC) and total nitrogen (TN) were analyzed with the elemental analyzer (Vario EL/micro cube elemental analyzer, Germany). The concentrations of potassium (K) and phosphorus (P) were measured by inductive coupled plasma emission spectrometer (iCAP 6000, Thermo, USA). The cellulose, hemicellulose and lignin contents were measured using fiber analyzer (A2000i, ANMOM, USA).

The samples for VFAs, TAC and NH$_3$–N concentration analysis were centrifuged at 10 000 rpm for 15 min and then the supernatants divided into two parts. A portion of the supernatants were filtered with a 0.22 µm membrane for VFAs determination, and the other supernatant was directly used for NH$_3$–N concentration and TAC analysis. The VFAs containing ethanol, acetic, propionic, n-butyric, iso-butyric, n-valeric and iso-valeric acids concentrations were analyzed using a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with a flame ionization detector. TAC was determined by pH titration method using 0.1 M HCl and expressed in g equivalent CaCO$_3$ L$^{-1}$ using the APHA standard methods. The concentration of NH$_3$–N was measured by a Kjeldahl analyzer (KT-260, Foss, Suzhou, China). The pH value of each digester was detected by a pH meter (Thermo Electron, USA).

#### 2.4.3. Microbial community analysis

The microbial communities in each anaerobic digester with different KNPSs were investigated. The functional roles of specific microbial populations were evaluated in relation with methane production in each digester. The sample for DNA extraction was prepared and analyzed based on the method adapted from the manufacturer protocol of Fast DNA Spin Kit (MP Biomedicals, U.S.A). The sample (slurry) for DNA analysis was collected from the effluent of AD reactors. The V3–V4 region of the bacterial 16S ribosomal RNA gene were amplified by PCR (95 °C for 3 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min) using primers 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’). In the same way the archaeal 16S ribosomal RNA gene were amplified by PCR (95 °C for 3 min, followed by 33 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min) using primers 524F_10_ext (5’-TGCACGGGCGGCGGTAA-3’) and Arch 958R (5’-YCCGGGTGTGATCCTAAT-3’). The PCR reactions were performed in triplicate using 20 µL mixture which contained 4 µL of 5× FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of forward primer (5 µM), 0.8 µL of reverse primer (5 µM), 0.4 µL of FastPfu Polymerase, 0.2 µL of BSA and 10 ng of template DNA, then add ddH$_2$O to 20 µL.

Amplions were extracted from 2% agarose gels and purified using the AsyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer’s instructions and quantified using QuantiFluor™-ST (Promega, U.S.). The purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

### 3. Results and discussion

#### 3.1. Biogas production performance

Different KNPSs of anaerobic digestion were carried out. The DBP trend in each CSTR was shown in Fig. 2(a). After initial steady state start-up (1–30 d), reactors were operated at OLR of 90 g TS L$^{-1}$(31–180 d). The R1 and R3, in which respectively fed with 1K + 1N and 2N pretreated RS, showed the DBP kept at 8.78 L d$^{-1}$ (105–131) and 6.80 L d$^{-1}$ (105–131), respectively. However, the average DBP of 1K + 1N pretreatment was higher than that of other KNPSs. However, the average daily methane content of different KNPSs had no significant difference (52.5–53.0%), but were a little higher than untreated RS (50.2%) (Fig. 2(b)).
The average DMP-V and DMP-VS of CSTRs with different KNPSs were shown in Table 2. The 1K + 1N pretreatment resulting the highest average DMP-V and DMP-VS of 473 mL L⁻¹ d⁻¹ and 274 mL g VS⁻¹ respectively, followed by 2N pretreatment (446 mL L⁻¹ d⁻¹, 258 mL g VS⁻¹); the DMP-V and DMP-VS of 2K_2N and untreated_2N experiment groups were higher than that of 2K pretreated and untreated RS. Compared to the sample without NH₃·H₂O supplementation during AD, the methane production from the ammonia added groups increased by 6.6–25.6%. The DMP-VS of 1K + 1N pretreatment was 6.2%, 13.8% and 39.8% higher than that of 2N, 2K_2N and untreated_2N groups, respectively. This result was 32.2% higher than that of RS pretreated with 3% KOH, as reported by Muhammad et al.¹² The DMP-VS of the 1K + 1N pretreated RS was also 63.7% higher than that for corn stalk pretreated with 2K reported by Liu et al.¹³ The results illustrated that KOH + NH₃·H₂O pretreatment showed good system stability and achieved a significant effect on the biodegradability of RS during AD in long-term CSTR operation. This was mainly because the addition of KOH and NH₃·H₂O enhanced the destruction of rigid lignocelluloses structures, which was benefit to the effects of the RS pretreatment.

### 3.2. Conversion rate of main compositions

The main characteristics of RS digestate collected from reactors with different KNPSs are shown in Table 3. RS, like any other lignocellulosic biomass, is composed of cellulose, hemicellulose and lignin (LCH), which can be converted to biomethane through AD process. Additionally, the reduction of LCH components during the process of AD can be used to evaluate the performance of digestion process.²³,²⁴ The conversion rates of cellulose and hemicellulose in reactors with different KNPSs were analyzed (Table 3). Lignin is a complex molecule composed of phenylpropane units linked in a three dimensional structure which is particularly difficult to biodegrade by anaerobic bacteria.²⁵ In this study, the lignin content from the feedstock was low (below 5%) and its conversion was very insignificant, as a result, the data of lignin was not shown in tables.

1K + 1N pretreatment achieved 55.4% and 46.3% removal efficiencies for cellulose and hemicellulose, respectively, which was 3.4–18.1% and 5.7–21.2% higher than that of single alkali pretreatment and untreated groups (46.9–53.6% and 38.2–43.8%) (Table 3). The TS and VS removal rate of all groups was 33.9–43.9% and 46.0–57.2%, respectively, and 1K + 1N

### Table 2: Average daily methane production per volume (DMP-V) and daily methane production per g VS (DMP-VS) of different KNPSs

| Reactors | Experiment groups | DMP-V (mL L⁻¹ d⁻¹) | DMP-VS (mL g VS⁻¹) |
|----------|-------------------|--------------------|--------------------|
| R1       | 1K + 1N           | 473 ± 11           | 274 ± 7            |
| R2       | 2K                | 390 ± 34           | 226 ± 18           |
| R3       | 2N                | 446 ± 11           | 258 ± 6            |
| R4       | Untreated         | 269 ± 40           | 156 ± 23           |
| R2       | 2K_2N             | 416 ± 15           | 241 ± 9            |
| R4       | Untreated_2N      | 337 ± 10           | 196 ± 6            |
group received the highest TS and VS removal rate of 43.9% and 57.2%, which were 4.3–29.5% and 2.7–24.3% higher than other groups. The 2K and untreated groups showed low TS conversion rate (33.9–38.6%). This indicated that ammonia directly added to the digesters during AD can regulate nutritional balance and also had better effect on DBP as well as the substrate conversion rate. The substrate conversion rate is arranged in descending order as follows: 1K + 1N > 2N > 2K > untreated. 2N > untreated, indicating that high main compositions conversion rates correspond to high biogas production. Ammonia added during feeding period promoted the conversion of RS showing that lack of nitrogen leading to lower TS conversion and biogas production. Whereas, KOH + NH₃·H₂O combined pretreatment promoted further conversion of RS into biogas production.

### 3.3. System stability

Stability is a critical factor during the operation of anaerobic digesters whereas instability is a problem that has plagued digesters for many years which can be resulted from any material or related effects that interfere with methane formation.⁵⁶ The stability of AD system mainly reflected in the indicators of NH₃-N, pH, VFAs, and alkalinity.

#### 3.3.1. NH₃-N concentration

Suitable NH₃-N concentration controls buffer capacity of methanogenic medium in mesophilic anaerobic reactor and strengthens the stability of AD process. The negative effect of low NH₃-N concentration on biomass can cause not only low buffer capacity but also resulted in low nitrogen as nutrient.⁵⁷ Fig. 3(a) shows the changes in NH₃-N concentration of four reactors with different KNPSs.

As shown in Fig. 3(a), R1 and R3 had a relatively high NH₃-N concentration of 770 mg L⁻¹ and 1270 mg L⁻¹ at the beginning due to the 1% and 2% NH₃·H₂O addition during pretreatment, respectively. Then, the NH₃-N concentration in R1 and R3 gradually decreased when the feeding continued, and then kept at a relatively stable level of 265–580 mg L⁻¹. The NH₃-N concentration in R2 and R4 was 410 mg L⁻¹ and 490 mg L⁻¹ on the first feeding day, and continuously decreased with the feeding proceeding. On the 105th day, The NH₃-N concentration in R2 and R4 dropped to 39 mg L⁻¹ and 24.5 mg L⁻¹ respectively. When ammonia supplemented with feeding, the NH₃-N concentration of R2 and R4 gradually recovered and reached up to 280–565 mg L⁻¹. This is consistent with the reports of Rajagopal et al.⁶⁸ who regulated the NH₃-N concentration by adjusting the carbon and nitrogen ratio of the system.

As can be seen in Fig. 2(a) and 3(a), the biogas production of each reactor maintained stable when the NH₃-N concentration is in between 55-1270 mg L⁻¹, and then gradually declined while the NH₃-N concentration is less than 55 mg L⁻¹. This result is not consistent with other researchers. For instance, Procházká et al.⁶⁷ indicated that low NH₃-N concentration (500 mg L⁻¹) caused low methane yield, loss of biomass and loss of the aceticlastic methanogenic activity. Additionally, Benjamin et al.⁷⁰ reported that the optimum growth conditions for Methanoseta concilii, which is the most ammonia-sensitive methanogen, were in the range of 250–1100 mg L⁻¹ NH₃-N concentration. Wang and Yuan⁵⁶,⁶⁷ achieved the highest biogas yield with 0.7% and 4% ammonia concentration, respectively. When 1% and 2% NH₃·H₂O were used in our study, the ammonia concentration in the digesters maintained 265–580 mg L⁻¹, which were lower than the levels mentioned above. Therefore, no ammonium toxicity existed in our study.

#### 3.3.2. pH value

Fig. 3(b) shows the difference in pH values of four reactors throughout the experimental periods. Experiment groups 1K + 1N, 2K and 2N had a relatively stable pH values (6.8–7.4), and untreated experiment group showed a relatively fast fall in pH, which was decreased below 6.7 on the 105th day. With the external addition of ammonia during AD, the pH value of untreated_2N groups gradually recovered to 6.9. The pH value showed the same change trends as the NH₃-N concentration, which could be explained as the hydrolysis of ammonia nitrogen in the compound accompanied by release of hydroxide.

#### 3.3.3. TVFAs and TVFAs/TAC

High concentration of VFAs can inhibit the activity of the methanogenic bacteria. The value of TVFAs/TAC can be used as an important indicator for evaluating the stability of AD system.⁷⁷ TVFAs and TVFAs/TAC of different KNPSs are shown in Fig. 3(c). To maintain the buffer capacity of AD, the alkalinity should be above 2000 mg L⁻¹. In this study, TAC of four reactors ranged at 2200–6800 mg L⁻¹ during the whole operation period.

As shown in Fig. 3(c), the change in trend of TVFAs/TAC was consistent with TVFAs. After feeding for five days, TVFAs values of R1, R2 and R3 were less than 300 mg L⁻¹ and the TVFAs/TAC ratio fluctuated in a small range (0.01–0.08) during the whole operation period. The TVFAs of R4 rose to more than 600 mg L⁻¹ and the TVFAs/TAC had a sudden fluctuation that

---

**Table 3** Main characteristics of digestate and main components conversion rate of anaerobic digestion with different KNPSs

| Reactors | Experiment groups | Cellulose (%) | Hemicellulose (%) | TS (%) | VS (%) | Cellulose (%) | Hemi-cellulose (%) | TS (%) | VS (%) |
|----------|------------------|---------------|------------------|--------|--------|---------------|-------------------|--------|--------|
| R1       | 1K + 1N          | 16.2 ± 1.2    | 14.4 ± 0.9       | 5.1 ± 0.2 | 4.5 ± 0.1 | 55.4 ± 3.5    | 46.3 ± 2.6        | 43.9 ± 1.2 | 57.2 ± 1.0 |
| R2       | 2K               | 18.4 ± 2.2    | 16.3 ± 2.2       | 5.5 ± 0.5 | 5.1 ± 0.5 | 49.6 ± 6.4    | 39.4 ± 4.7        | 38.6 ± 2.1 | 51.2 ± 2.2 |
| R3       | 2N               | 16.9 ± 1.3    | 15.1 ± 0.8       | 5.2 ± 0.2 | 4.6 ± 0.2 | 53.6 ± 3.1    | 43.8 ± 2.4        | 42.1 ± 1.3 | 55.7 ± 1.2 |
| R4       | Untreated        | 21.2 ± 3.6    | 17.9 ± 3.7       | 6.0 ± 0.6 | 5.6 ± 0.5 | 41.8 ± 7.8    | 33.4 ± 5.3        | 33.9 ± 4.5 | 46.0 ± 4.7 |
| R2_2K    | Untreated_2N     | 17.9 ± 0.6    | 15.9 ± 0.8       | 5.5 ± 0.2 | 4.8 ± 0.1 | 50.8 ± 3.1    | 40.8 ± 2.0        | 39.4 ± 1.4 | 53.8 ± 1.5 |
| R4_2N    | Untreated_2N     | 19.3 ± 0.8    | 16.5 ± 0.8       | 5.6 ± 0.1 | 5.2 ± 0.1 | 46.9 ± 2.7    | 38.2 ± 2.1        | 37.7 ± 1.4 | 49.8 ± 1.7 |
increased from 0.05 to 0.33 (80–105 d), which restored stability after ammonia addition with feeding (Fig. 3(c)). TVFAs concentration was kept at a low level, indicating that no inhibition occurred during the fermentation of RS in CSTRs. Under the same conditions, the lower VFAs value was, the better biogas production performance showed. The sudden increase in TVFAs concentration in R4 was attributed to the nitrogen deficiency. This resulted in low methanogenic activity and less VFAs that converted to methane in R4. Moreover, Hun et al.\textsuperscript{33} pointed out that the TVFAs/TAC ratio must be observed in a dynamic way during AD period and a sudden increment of the ratio reflects a potential instability of the process. The reason for the instability of R4 was mainly due to low buffering capacity and VFAs accumulation for insufficient nitrogen and low methanogenic activity. However, R2 was in low nitrogen content but no fluctuation of TVFAs/TAC and instability was observed due to the enhanced buffer capacity for KOH pretreatment.\textsuperscript{13} Compared to pretreatment without ammonia, the 1K + 1N and 2N pretreatments were more suitable for long-term stable AD operation. Similarly, N-source added during AD also could promote the stability of anaerobic digester.

3.4. Microbial community structure of different KNPSs

3.4.1. Microbial diversity and richness. Different operational conditions had significant impacts on the community structure of microorganisms in anaerobic reactor.\textsuperscript{24} Table 4 shows the comparison of microbial richness and diversity of 1K + 1N, 2K, 2N, untreated, 2K_2N, and untreated_2N groups. As can be seen from Table 4, the coverage index was more than 99.70% for all samples, thereby confirming representativeness. Both in R2 and R4, the richness and diversity of the bacteria communities varied greatly after \(\text{NH}_3\cdot\text{H}_2\text{O}\) addition, while the archaea communities in R4 changed much more than that in R2. This indicated that the lack of nitrogen nutrition has a great influence on the richness and diversity of bacteria, and the archaea communities are more related to the system stability. Moreover, there was a much higher shannon and lower

![Fig. 3](NH3-N concentration (a), pH (b), TVFAs and TVFAs/TAC (c) of four reactors with different KNPSs.)
simpson indices in R1 than that in other KNPSs groups under stable operating condition, which suggested that KOH + NH₃·H₂O pretreatment could increase diversity of both bacteria and archaea communities. This might be that the KOH + NH₃·H₂O pretreatment increased VFAs, alkalinity and NH₃–N concentrations and changed the microbial environment. Ramirez et al. reported that the biodiversity was thought to be positively related to system stability in terms of resistance and resilience, and promote the degradation of refractory substances during AD and lead to high processing performance.

3.4.2. Bacterial communities. The sequence distribution of each sample at the level of the phyla level is shown in Fig. 4(a). Bacteroidetes was typical hydrolyzed bacteria and Firmicutes was the main cellulose-degrader and played an important role in the AD of methanogenesis. As shown in Fig. 4(a), Bacteroidetes and Firmicutes were the dominant phyla in all samples of representing 16.6–68.4%, and R1 showed the highest abundance of 68.4%. Moreover, the relative abundance of Bacteroidetes and Firmicutes in R1, R2 and R3 (49.8–68.4%) was higher than that in R4 (16.6%). This may be due to the addition of KOH and/or NH₃·H₂O in pretreatments. The total proportion of Bacteroidetes and Firmicutes in R2_2N (64.1%) and R4_2N (56.0%) was higher than that in R2 (49.8%) and R4 due to the NH₃·H₂O addition during AD.

The genus level in the six samples was observed. The result showed that the dynamic profiles of bacterial genus differed significantly in different KNPSs (Fig. 4(b)). Twenty-nine major genera (represented by greater than 2.5% of total bacterial sequences in at least one sample) were found in all samples, and the 10 top species genus were DMER64, norank_c_WS6_Dojkabacter, norank_f_Prolixibacteria, norank_c_Bacteroidetes_vadinHA17, Rumininofilibacter, Christensenellaceae_R-7_group, Syner-01, norank_f_Anaerolineaceae, Olsenella, Clostridium_sensu_stricto_1. DMER64 and Christensenellaceae_R-7_group can establish

![Figure 4](image-url)

**Fig. 4**  Bacterial and archaeal sequence distributions at phylum (a and c) and genus (b and d) level of different KNPSs.

| Items          | Experiment groups | Samples | OTUs | Ace | Chao | Coverage | Shannon | Simpson |
|---------------|-------------------|---------|------|-----|-----|----------|---------|---------|
| Bacteria      | 1K + 1N           | R1      | 552  | 603 | 618 | 0.9986   | 4.34    | 0.0359  |
|               | 2K                | R2      | 415  | 510 | 516 | 0.9972   | 3.52    | 0.0914  |
|               | 2N                | R3      | 531  | 575 | 578 | 0.9984   | 4.10    | 0.0392  |
|               | Untreated         | R4      | 551  | 584 | 586 | 0.9983   | 3.90    | 0.0758  |
|               | 2K_2N             | R2_2N   | 489  | 564 | 568 | 0.9976   | 3.98    | 0.0431  |
|               | untreated_2N      | R4_2N   | 516  | 592 | 591 | 0.9976   | 3.65    | 0.0759  |
| Archaea       | 1K + 1N           | R1      | 47   | 47  | 47  | 1.0000   | 1.44    | 0.3406  |
|               | 2K                | R2      | 19   | 19  | 19  | 1.0000   | 1.57    | 0.2795  |
|               | 2N                | R3      | 27   | 30  | 29  | 0.9999   | 1.38    | 0.3430  |
|               | Untreated         | R4      | 32   | 32  | 32  | 1.0000   | 1.63    | 0.2990  |
|               | 2K_2N             | R2_2N   | 21   | 23  | 21  | 0.9999   | 1.38    | 0.3439  |
|               | untreated_2N      | R4_2N   | 19   | 19  | 19  | 1.0000   | 0.95    | 0.5150  |
magnetite-mediated direct electron transfer with methanogens during methanogenic degradation of VFAs. In addition, *Christensenellaceae_R-7_group* also promotes hydrolysis acidification, especially for cellulose that is difficult to degrade. The total abundance of *DMER64* and *Christensenellaceae_R-7_group* in R1, R3, R2_2N and R4_2N was 24.2%, 23.0%, 15.6% and 8.2%, respectively. In R2 and R4, there were no *DMER64* and *Christensenellaceae_R-7_group* observed. This indicated that the NH$_3$·H$_2$O addition increases the diversity of acid hydrolytic microorganisms. In addition, the bacterial diversity in R1 was higher than the other KNPSs groups, which demonstrating that the KOH + NH$_3$·H$_2$O combined pretreatment improved the ability of bacteria to hydrolyse lignocellulosic matter, thereby resulting in the remarkable increment of the hemicellulose and cellulose reduction efficiency. As a result, the diversity of bacteria increased in the final stage of AD.

### 3.4.3. Archaeal communities

Archaea are believed to be responsible for many biochemical reactions in the methanogenic phase. The distributions of archaeal sequences at the phylum level from each sample are shown in Fig. 4(c). *Euryarchaeota* was the most dominant phylum among the archaeal phyla with the relative abundances over 93.5% in all samples.

The dynamic profiles of the archaeal community at the genus level were significantly different in the six samples (Fig. 4(d)). *Methanosarcina* and *Methanosaeta* are the methanogens have been found to decomposing acetic acid for methane production, and metabolic transformation could occur under certain conditions from *Methanosaeta* to *Methanosarcina*. The genus *Methanobacterium* is a typical hydrogen-nutrient methanogen existing in anaerobic system and has strong viability in producing methane from H$_2$ and CO$_2$. As can be seen in Fig. 4(d), archaeal communities demonstrated a clear dominance of the genus *Methanosarcina* and *Methanosaeta* (20.3–55.2%) and *Methanobacterium* (40.1–69.7%). The relative abundance of *Methanosarcina* and *Methanosaeta* in R1 (55.2%) and R3 (52.5%) was higher than those in R2 (28.4%) and R4 (20.3%). After NH$_3$·H$_2$O addition during AD, the *Methanosarcina* and *Methanosaeta* accounted for 39.2% and 38.6% in samples of R2_2N and R4_2N, respectively. This demonstrated that the KOH and/or NH$_3$·H$_2$O added in the digesters make more genera to use acetate as a substrate for methanogenesis. During the AD process, more than 70% of methane in biogas comes from the cleavage of acetate. Therefore, the addition of KOH and/or NH$_3$·H$_2$O can enrich the abundance of acetate-nutrient methanogen and the higher biomethane production.

It was observed that the proportion of *Methanosarcina* and *Methanosaeta* species in the 1K + 1N group was higher than that of 2K, 2N and untreated groups, which was contributed to the increment concentration of VFAs after KOH + NH$_3$·H$_2$O combined pretreatment. The data presented in this study indicated that both KOH and NH$_3$·H$_2$O pretreatment had significant impacts on microbial community structures and the metabolic pathway for biomethane production in an AD digester.

### 3.5. Fertilizer value analyses of the digestate

Digestate, as a multi-component quick-acting compound organic fertilizer, plays an important role in improving soil fertility and improving the quality of agricultural products, and is getting more and more attention. In this study, a simple fertilizer value analysis of digestate was provided (Table 5). The content of nitrogen ranged from 24.0 g kg$^{-1}$ TS$^{-1}$ to 31.9 g kg$^{-1}$ TS$^{-1}$. The content of the ammonia addition samples were 2.0–7.9 g kg$^{-1}$ TS$^{-1}$ more than that of none ammonia addition samples. This indicated that most of the nitrogen, whether added in pretreatment or during AD process, remains in the digestate. A small portion of nitrogen may be volatilized in the form of NH$_3$ during pretreatment or AD. As shown in Table 5, it was found that there was no much difference in the content of phosphorus (4.1–4.2 g kg$^{-1}$ TS$^{-1}$) in each sample for there was no extra phosphorus addition in each digester. The potassium contents in digestate were 15.2–29.9 g kg$^{-1}$ TS$^{-1}$ in all samples. Compared to groups without potassium addition, the content of the potassium addition groups increased 6.8–14.7 g kg$^{-1}$ TS$^{-1}$, which was consistent with the theoretical addition of potassium (7.0–14.0 g kg$^{-1}$ TS$^{-1}$). This indicated that the potassium added in pretreatment was not consumed as the AD proceeding, and retained in the digestate. The more potassium in digestate, the better fertilizer effect because sufficient potassium in the soil plays an active role in activation of enzymes for healthy plant growth.

Therefore, the potassium and nitrogen added in the digesters can significantly increase fertilizer value of the digestate. Compared with untreated RS, although different KNPSs require a certain amount of reagents cost, these costs can be recovered by the increasing fertilizer value in the digestate. Moreover, three different KNPSs used in this study could all increase methane production efficiency. It was found that 1K + 1N pretreatment achieved the highest biomethane production and provided an environmental solution that had no potential environment pollution. Therefore, 1K + 1N pretreatment provides a meaningful insight for exploring efficient potassium and nitrogen pretreatment strategy to enhance AD performance for practical application in an affordable and environmentally friendly way.

### 4. Conclusions

KOH + NH$_3$·H$_2$O combined pretreatment was proved to be efficient for bioconversion of rice straw to biomethane. The rice

| Groups   | N (g kg$^{-1}$ TS$^{-1}$) | P (g kg$^{-1}$ TS$^{-1}$) | K (g kg$^{-1}$ TS$^{-1}$) |
|----------|-------------------------|-------------------------|-------------------------|
| 1K + 1N  | 28.2 ± 0.9              | 4.2 ± 0.1               | 23.1 ± 1.0              |
| 2K       | 26.2 ± 1.2              | 4.1 ± 0.2               | 29.9 ± 1.4              |
| 2N       | 30.3 ± 0.6              | 4.1 ± 0.1               | 15.2 ± 0.7              |
| Untreated| 24.0 ± 1.1              | 4.1 ± 0.2               | 16.3 ± 0.8              |
| 2K_2N    | 31.9 ± 0.9              | 4.2 ± 0.0               | 28.5 ± 0.9              |
| untreated_2N | 31.3 ± 1.2         | 4.1 ± 0.1               | 15.2 ± 0.5              |
straw pretreated by KOH + NH3·H2O achieved the highest methane production of 274 mL g VS−1, which was 6.2–75.8% higher than that of single alkali pretreatments and untreated RS. KOH + NH3·H2O pretreatment also showed higher abundance of the dominant bacterial phyla (Bacteroidetes and Firmicutes) and the acetate methanogen (Methanosarcina and Methanosaeta). Moreover, digestate nutritional value was improved due to the K and N remaining in digestate after AD. KOH + NH3·H2O combined pretreatment could be one of alternative methods for high-efficient biogas production and environmentally friendly from RS.

Conflicts of interest
There are no conflicts to declare.

Acknowledgements
The authors are grateful to the fund supports from National “Thirteenth Five-Year” Plan for Science & Technology Support (2018YFC1900901).

References
1 M. Elsayed, A. E. Abomohra, P. Ai, D. Wang, H. M. El-Mashad and Y. Zhang, Bioprocess. Technol., 2018, 268, 183–189.
2 X. Qian, G. Shen, Z. Wang, X. Zhang, X. Chen, Z. Tang, Z. Lei and Z. Zhang, Bioresource Technology Reports, 2019, 7, 100208.
3 J. Li, A. C. Wachemo, H. Yuan, X. Zuo and X. Li, Bioprocess. Technol., 2019, 288, 121518.
4 R. Guan, X. Li, A. C. Wachemo, H. Yuan, Y. Liu, D. Zou, X. Zuo and J. Gu, Sci. Total Environ., 2018, 637–638, 9–17.
5 J. Xu, X. Wang, S. Sun, Y. Zhao and C. Hu, Sci. Rep., 2017, 7, 10897.
6 G. Tian, W. Zhang, M. Dong, B. Yang, R. Zhu, F. Yin, X. Zhao, Y. Wang, W. Xiao, Q. Wang and X. Cui, Energy, 2017, 139, 571–579.
7 M. J. Fernández-Rodríguez, D. de la Lama-Calvente, A. Jiménez-Rodríguez, R. Borja and B. Rincón-Llorente, Process Saf. Environ. Prot., 2019, 128, 167–175.
8 J. Du, Y. Qian, Y. Xi and X. Lü, Renew. Energy, 2019, 139, 261–267.
9 D. J. Shetty, P. Kshirsagar, S. Tapadiamaheshwari, V. Lanjekar, S. K. Singh and P. K. Dhakephalkar, Bioprocess. Technol., 2016, 226, 80–88.
10 G. Mancini, S. Papirio, G. Riccardelli, P. N. L. Lens and G. Esposito, Bioprocess. Technol., 2018, 247, 897–903.
11 L. Li, C. Chen, R. Zhang, Y. He, W. Wang and G. Liu, Energy Fuels, 2015, 29, 5841–5846.
12 M. Jaffar, Y. Pang, H. Yuan, D. Zou, Y. Liu, B. Zhu and R. M. Korai, Chin. J. Chem. Eng., 2016, 24(3), 404–409.
13 X. Liu, S. M. Zicari, G. Liu, Y. Li and R. Zhang, Bioprocess. Technol., 2015, 185, 150–157.
14 U. I. Oji, H. E. Etim and F. C. Okoye, Small Rumin. Res., 2007, 69(1), 232–236.
15 R. Zhang and Z. Zhang, Bioprocess. Technol., 1999, 68(3), 235–243.
16 H. Yuan, R. Li, Y. Zhang, X. Li, C. Liu, M. Ying, M. Lin and Z. Yang, Biosyst. Eng., 2015, 129, 142–148.
17 J. Li, A. C. Wachemo, H. Yuan, X. Zuo and X. Li, Waste Manag., 2019, 97, 52–62.
18 A. Zhou, J. Zhang, C. Varrone, K. Wen, G. Wang, W. Liu, A. Wang and X. Yue, Energy, 2017, 137, 457–467.
19 Q. Yu, R. Liu, K. Li and R. Ma, Renewable Sustainable Energy Rev., 2019, 107, 51–58.
20 D. G. Mulat, H. F. Jacobi, A. Feilberg, A. P. S. Adamsen, H. H. Richnow and M. Nikolauzs, Appl. Environ. Microbiol., 2015, 82(2), 438–449.
21 APHA, A. W., Standard Methods for the Examination of Water and Wastewater, American Water Works Association, 2005.
22 J.-H. Park, J.-J. Yoon, G. Kumar, Y.-S. Jin and S.-H. Kim, Waste Manag., 2018, 80, 218–223.
23 S. D. Poe, A. Engineer, S. S. Dagar and P. K. Dhakephalkar, Bioprocess. Technol., 2019, 279, 25–33.
24 Z. Weizhang, Z. Zhongzhi, L. Yijing, S. Shanshan, Q. Wei and X. Meng, Bioprocess. Technol., 2011, 102(24), 11177–11182.
25 M. T. Jin, S. G. Sommer, H. B. M Ller, M. R. Weisbjerg and Y. J. Xion, Bioprocess. Technol., 2011, 102(20), 9395–9402.
26 J. Keenan, Environ. Lett., 1976, 11(8–9), 525–548.
27 J. Procházká, J. Máca and M. Dohányos, Appl. Microbiol. Biotechnol., 2012, 93(1), 439–447.
28 R. Rajagopal, D. I. Masse and G. Singh, Bioprocess. Technol., 2013, 143(17), 632–641.
29 S. Benjamin, M. L. Garcia, A. Q. Shen and L. T. Angenent, Appl. Environ. Microbiol., 2007, 73(5), 1653.
30 D. Wang, Y. Xin, H. Shi, P. Ai, L. Yu, X. Li and S. Chen, Closing ammonia loop in efficient biogas production: recycling ammonia pretreatment of wheat straw, Biosyst. Eng., 2019, 180, 182–190.
31 H. Yuan, R. Guan, A. Wachemo, Y. Zhang, X. Zuo and X. Li, Chin. J. Chem. Eng., 2020, 28(2), 541–547.
32 Y. Q. Li, C. M. Liu, A. C. Wachemo, H. R. Yuan, D. X. Zou, Y. P. Liu and X. J. Li, Bioprocess. Technol., 2017, 235, 380–388.
33 K. S. Hun and K. G. Krishna, Biosystems Engineering, 2010, 35(1), 53–62.
34 B. Si, Z. Liu, Y. Zhang, J. Li, R. Shen, Z. Zhu and X. Xing, Int. J. Hydrog. Energy, 2016, 41(7), 4429–4438.
35 Y. Liu, A. C. Wachemo, H. Yuan and X. Li, Bioprocess. Technol., 2019, 287, 121339.
36 I. Ramírez, E. I. P. Volcke, R. Rajnikanth and J. Steyer, Water Res., 2009, 43(11), 2787–2800.
37 M. Kim, M. Abdulazeez, B. M. Haroun, G. Nakhla and M. Kelemen, Microbial communities in co-digestion of food wastes and wastewater biosolids, Bioprocess. Technol., 2019, 289, 121580.
38 L. Sun, T. Liu, B. Müller and A. Schnürer, Biotechnol. Biofuels, 2016, 9(1), 128.
39 J. Lee, T. Koo, A. Yulisa and S. Hwang, J. Environ. Manage., 2019, 241, 418–426.
40 L. Dong, G. Cao, J. Wu, S. Yang and N. Ren, Bioprocess. Technol., 2019, 284, 248–255.
41. J. H. Ko, N. Wang, T. Yuan, F. Lù, P. He and Q. Xu, *Bioreour. Technol.*, 2018, **266**, 516–523.

42. M. B. Kurade, S. Saha, E. Salama, S. M. Patil, S. P. Govindwar and B. Jeon, *Bioreour. Technol.*, 2019, **272**, 351–359.

43. S. Feng, X. Hong, T. Wang, X. Huang, Y. Tong and H. Yang, *Bioreour. Technol.*, 2019, **288**, 121509.

44. D. G. Mulat, H. F. Jacobi, A. Feilberg, A. P. Adamsen, H. H. Richnow and M. Nikolausz, *Appl. Environ. Microbiol.*, 2016, **82**, 438–449.

45. D. Holthusen, S. Peth and R. Horn, *Soil Tillage Res.*, 2010, **111**(1), 75–85.