Effect of liquid digestate recirculation on biogas production and enzyme activities for anaerobic digestion of corn straw

Shuaixing Xue, Ling Qiu, Xiaohui Guo and Yiqing Yao

ABSTRACT

To accelerate the degradation of substrate, 50% liquid digestate recirculation (LDR) was used in the anaerobic digestion (AD) of corn straw. The effects of recirculation on the enzyme activities and biogas production were investigated by comparing with control reactor ($Reactor_{CK}$). During the AD process, the fermentation system with 50% LDR was more stable. The average biogas and methane production in $Reactor_{LDR}$ were 7,891 mL·d^{-1} and 347 mL CH_4·g^{-1} VS_{added}·d^{-1} respectively. The total volatile fatty acids (TVFAs) concentration in the two reactors both increased at first and then decreased with time. The LDR made the VFAs accumulation significant, especially propionic acid accumulation in 4 ~ 16 days. The maximum peak value of cellulase, xylanase, dehydrogenase and coenzyme F_{420} activities in $Reactor_{LDR}$ were 0.51 mg·g^{-1}·h^{-1}, 0.29 mg·g^{-1}·h^{-1}, 4.88 mL·g^{-1}·h^{-1} and 6.69 μmol·L^{-1}, respectively, which were higher than that in $Reactor_{CK}$. With or without recirculation, the concentration of TVFAs was positively correlated with cellulase, xylanase and dehydrogenase activities, while was negatively correlated with coenzyme F_{420} activity. Besides, a very significant correlation existed between hydrolase and dehydrogenase activities and daily biogas production in $Reactor_{CK}$. And the peaks of cellulase, xylanase and dehydrogenase activities appeared ahead of the peak of daily biogas production with the LDR.

Key words | biogas production characteristics, corn straw, enzyme activities, liquid digestate recirculation

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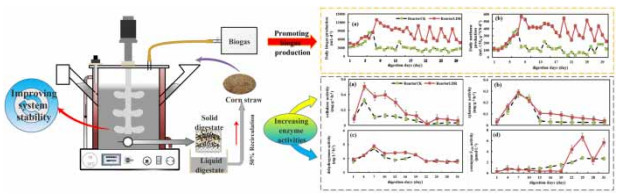
HIGHLIGHTS

- LDR improves the quality and efficiency of crop straw AD.
- LDR promotes the biogas production and enzyme activities.
- The peaks of enzyme activities appeared ahead of biogas production.
- The trail of biogas production resembled some enzyme activities delay.
- Coenzyme F_{420} activity was inhibited when the concentration of TVFAs exceeded 950 mg·L^{-1}.

GRAPHICAL ABSTRACT



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INTRODUCTION

Lignocellulosic waste is the most abundant resource in the world to produce bioenergy by anaerobic digestion (AD) (Talebnia *et al.* 2010). In China, about 10.4 billion tons of crop straw are produced every year, but the actual utilization rate is less than 40%. More than 60% of the straw is randomly stacked, discarded or returned to the fields as fertilizer, fuel for daily use, which potentially pollutes the environment. Straw is an abundant, cheap and accessible agricultural residue, and it is often regarded as an excellent raw material for AD due to its high content of organic matter. However, one drawback with corn straw as raw material for biogas production is its poor nutritional components, especially nitrogen content. Anaerobic microorganisms need these nutrients for physiological and enzymatic reactions (Peng *et al.* 2016; Ni *et al.* 2017; Xia *et al.* 2019). Lei *et al.* (2010) reported that nutrient deficiency was the main reason for the poor process performance of straw AD. In addition, the abundant cellulose, hemicellulose and lignin construct complicated the structure in straw (Fernando *et al.* 2006), which is highly resistant to AD and is difficult to be broken down by anaerobic bacteria (Xiao & Clarkson 1997; Buffiere *et al.* 2006; Fernandes *et al.* 2009).

As a by-product of AD, liquid digestate has the characteristics of large quantity and complex composition. Liquid digestate directly discharged into water streams could contaminate the soil and water, and the odors released by liquid digestate will also pollute the air (Li *et al.* 2018). Besides, liquid digestate produced by AD often exceeds the consumption ability of surrounding farmland. It is also economically unfeasible to transport liquid digestate from the point of surplus to distant farmlands (Ni *et al.* 2017). Therefore, liquid digestate needs to be treated by a laborious process before discharge or reuse. Due to the high post-treatment cost of digestate, AD has been limited in large application (Hu *et al.* 2014). On the contrary, liquid digestate is abundant in biodegradable lignocelluloses, microbes and soluble nutrients. Therefore, liquid digestate recirculation (LDR) to the anaerobic reactor can reduce the discharge of liquid digestate, promote the hydrolysis of organic matter and improve the buffer capacity and stability of the system (Hao *et al.* 2008; Peng *et al.* 2016; Li *et al.* 2018). However, too high LDR often leads to high ammonia nitrogen (NH_4^+-N) content and/or VFAs content, which may inhibit the activities of some bacteria or archaea and reduce the biogas production (Zamanzadeh *et al.* 2016). Previous researches have reported that the concentration of TVFAs in treatment with LDR of 21 $L{\cdot}d^{-1}$ was 61.5% higher than that with LDR of 9 $L{\cdot}d^{-1}$, the concentration of $NH4^+$-N in treatment with 100% LDR was about 3 times that of the control group, and the biogas production from both reports was inhibited during a batch AD experiment with food waste (Sponza & Ağdağ 2004; Shahriari *et al.* 2012). And Wu *et al.* (2016) discovered that with 100% LDR, the concentration of NH_4^+-N increased from 2,600 $mg{\cdot}L^{-1}$ to 5,000 $mg{\cdot}L^{-1}$, the concentration of VFAs increased from 1,600 $mg{\cdot}L^{-1}$ to 8,000 $mg{\cdot}L^{-1}$, and maximum daily methane production decreased by about 43% during the semi-continuous AD of chicken manure. On the contrary, an appropriate recirculation ratio of liquid digestate was favorable for promoting the digester performance (Nordberg *et al.* 2007). Wu *et al.* (2018) found that 30% LDR of food waste significantly improved the system alkalinity, which was important for maintaining an optimum pH for methanogens. The possible reason for the biogas production being enhanced by 30% LDR was that the increased concentration of NH_4^+-N resulting from the LDR was still lower than the threshold value. The concentrations of VFAs firstly increased and then decreased by 30% LDR, possibly due to the increased population of hydrolysis and fermentation microorganisms (Gulhane *et al.* 2017). The analysis of cyclic biochemical methane potential (BMP) indicated that the proper proportion of LDR could stimulate methanogen activity and increase biogas production. LDR ratio of food waste should not exceed 50% during long-term operation of AD process (Shahriari *et al.* 2012). Estevez *et al.* (2014) discovered that 50% LDR of cow manure did not cause inhibition of microbial activity and the methane production was increased by 16%. Compared with food waste and animal manure, straw as substrate has the disadvantages of single nutrient composition and being nutrient-deficient (Nges *et al.* 2015). Therefore, a higher recirculation ratio not only supplements more nutrients but also maintains the equilibrium of the microbial community. Li *et al.* (2018) reported that 60–75% LDR of straw didn't inhibit production of biogas and methane production was increased by 2.3%. Overall, 50% LDR of corn straw seems preferred for AD.

LDR leads to the accumulation of organic and inorganic compounds and changes in bacterial biomass, which will result in changes in the fermentation environment, thus affecting the enzyme activities (Nordberg *et al.* 2007). In the AD process, extracellular enzymes play a major role in the hydrolysis phase (Xin *et al.* 2018); the dehydrogenase is closely related with the process of oxidative phosphorylation, and can reflect

the microbial activity (Yu *et al.* 2019); coenzyme F_{420} is one of the methanogen's peculiar coenzymes, which could be used as a monitoring indicator of methanogenic archaea activity (Cheng *et al.* 2007). Despite a lot of research about the influence of LDR on NH_4^+-N and VFAs being reported in the last few decades, the influence of LDR on enzyme activities is seldom reported to our knowledge.

The objectives of this research were (1) to investigate the influence of 50% LDR on system stability, biogas production characteristics and enzyme activities during the AD of corn straw. (2) to explore the dynamic relationship between enzyme activities and daily biogas/methane production and correlation between metabolic intermediates and enzyme activities. (3) to provide reference for improving the quality and efficiency of AD fed with crop straw by means of biological enzymes.

MATERIALS AND METHODS

Feedstock and inoculum

The corn straw samples were obtained from the rural biogas technology innovation base of the Northwest A&F University, and were air-dried and then ground into 3–5 mm particles by a grinder (DFY-1000, Beijing, China). The inoculum was obtained from a long-term operating mesophilic anaerobic digestor of pig manure in our lab and was put without any pre-treatment into fermentation systems. The characteristics of the corn straw and inoculum samples are listed in Table 1.

Experimental set-up

Two identical CSTRs were employed for AD tests with a total volume of 10 L and a working volume of 8 L, the hydraulic retention time (HRT) and organic loading rate (OLR) were set at 48 days and 2 g VS·L^{-1}·d^{-1}, respectively. For both CSTRs, the initial TS concentration and inoculation rate were designed as 10% (W/V) and 25% (in total dry weight), respectively. The fermentation temperature of both CSTRs were controlled at 37 ± 1 °C with a water bath jacket. Then, distilled water was added to 8 L before CSTRs starting. Sodium bicarbonate solution of 4 mol·L^{-1} was used to adjust the initial pH value of about 5.4 to about 7 and after then the pH value was not adjusted. The stirring of both CSTRs were set at a speed of 30 rpm for 10 min every 2 h. After adaptation of 11 days, both CSTRs were operated in semi-continuous mode, by which the feed-stock was fed once every 3 days and then biogas and methane production were recorded in normalized volume according to the state equation of ideal gas. The day when the semi-continuous mode was introduced was recorded as the first day of the experiment. The liquid digestate of 500 mL was expelled every 3 days in both CSTRs, and then filtered by a sifter with 0.85 mm meshes. 15 g corn straw mixed with the diluted filtrate of 500 mL (filtrate and water 1:1 volume ratio) was imported in the 50% liquid digestate recirculation reactor (labeled as $Reactor_{LDR}$). The control reactor (labeled as $Reactor_{CK}$) without the filtrate of the liquid digestate was checked through feeding in 15 g corn straw mixed with water of 500 mL. The semi-continuous fermentation was monitored for 31 days. The mixed digestate was collected at discharging and centrifuged at 8,000 g for 20 min (HC-3018R). Liquid supernatant (accounted for 90% of the mixed digestate) was removed and used to measure enzyme activities, alkalinity concentration (AC), VFAs and NH_4^+-N concentration and the remaining digestate was used to measure final TS and VS contents (Lei *et al.* 2010). In order to maintain the consistency of measurement conditions, initial TS and VS contents were measured at feeding. Specifically, the influent of cornstalk was fed into the digester and the reactor was mixed immediately. And then the fully mixed slurry was collected and liquid supernatant (accounted for 90% of the mixed slurry) was removed by centrifuging at 8,000 g for 20 min (HC-3018R), and the pellet was used to measured initial TS and VS contents.

Table 1 | Characteristics of corn straw and inoculum

Parameter	Corn straw Average	Inoculum Average
TS (%)	90.0 ± 0.1[a]	27.0 ± 0.1
VS (%)	83.0 ± 0.3[a]	17.0 ± 0.2
C (%)[b]	42.8 ± 0.4	11.3 ± 0.8
N (%)[b]	0.75 ± 0.10	0.77 ± 0.40

Data presented as means ± SD ($n = 3$).
TS and VS represents total solids and volatile solid, respectively.
[a]Based on the air-dried matter (w/w).
[b]The total carbon (C) and nitrogen (N) contents were based on the oven-dried matter (w/w).

Analytical methods

Total alkalinity concentration (TAC), TS and VS were measured via the standard methods of APHA (2005). C, N contents were measured by an element analyzer (Italy Euro Vector, EA3000). pH value was determined by a pH meter (Hach Company, HQ40d-pHC101). NH_4^+-N was

measured by rapid determination of ammonium nitrogen analyzer (5B–6D, LH-technology, Beijing, China). VFAs was analyzed using a gas chromatograph (6890N, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and capillary column type 19091N-133 (30 m·0.25 mm·0.25 mm). Temperatures of the detector and injector were 270 °C and 250 °C, respectively. The column had an initial temperature of 80 °C (5-min hold time) then was ramped up to 220 °C (3-min hold time) with the rate of 10 °C·min^{-1}. Assays of cellulase, xylanase and dehydrogenase activities were conducted as described by Guan (1983). Coenzyme F_{420} activity was determined by ultraviolet spectrophotometry (Wu 1984). Biogas production was measured every day by using the drainage method and the parallel connection with the biogas bag. Biogas compositions were measured and recorded by a portable biogas analyzer (Geotechnical Instruments Ltd, BM3785, UK).

Statistical analysis

Every result in this paper was recorded as the average value of a triplicate. Microsoft Excel 2010 software was used to analyze the standard deviations and statistical differences of the data and to make the diagrams in the paper. Pearson correlation coefficient analysis was used to determine the correlations between parameters (metabolic intermediates, daily biogas/methane production and enzyme activities) in the software of SPSS (Version 22, USA). Statistical significance is defined with $P < 0.05$ and $P < 0.01$ is very significant.

RESULTS AND DISCUSSION

Influence of LDR on daily biogas and methane productions

The changes in daily biogas and methane production are shown in Figure 1. The daily biogas and methane productions in $Reactor_{CK}$ increased continuously to the maximum value of 8,650 mL·d^{-1} and 390 mL CH_4·g^{-1} VS_{added}·d^{-1}, respectively, on day 7. During days 8–13, the daily biogas and methane productions in $Reactor_{CK}$ were maintained in a relatively low and stable range. On day 14, the daily biogas and methane productions in $Reactor_{CK}$ showed a second peak value of 5,200 mL·d^{-1} and 219 mL CH_4·g^{-1} VS_{added}·d^{-1}, respectively, which was 5 days earlier than $Reactor_{LDR}$. During the initial 7 days, the tendency of daily biogas and methane productions in $Reactor_{LDR}$ was like to that in $Reactor_{CK}$ with a similar increase range. After 7 days, the daily biogas and methane productions in $Reactor_{LDR}$ increased continuously to the maximum value of 12,980 mL·d^{-1} and 567 mL CH_4·g^{-1} VS_{added}·d^{-1}, respectively, on day 8. In the following days, the advantage of $Reactor_{LDR}$ compared to $Reactor_{CK}$ was maintained in terms of daily biogas and methane production. After the appearance of maximum daily biogas and methane production, a similar stable stage presented in the $Reactor_{LDR}$ during days 9–16. Even an obvious decrease in the daily biogas and methane production was observed during days 16–18. This duration (days 9–18) was probably an adaptive phase for the system from the batch mode to the semi-continuous mode. Interestingly, it was found that during days 19–31, peaks of daily biogas and methane productions appeared every three days in $Reactor_{LDR}$ but not in $Reactor_{CK}$, which was very consistent with the operation interval of LDR. Therefore, it was concluded that there was a tight linkage between the biogas production and LDR.

LDR of 50% promoted the biogas production characteristics. The maximum volume biogas production rate in $Reactor_{LDR}$ was 33.3% higher than that in $Reactor_{CK}$. The average biogas and methane production in $Reactor_{LDR}$ was 7,891 mL·d^{-1} and 347 mL CH_4·g^{-1} VS_{added}·d^{-1} respectively, which was 58.4% and 61.5% higher than that in $Reactor_{CK}$, respectively. In addition, biogas production is directly related to the degree of substrate degradation. The TS and VS reduction rate was 30.1% and 42.1% in $Reactor_{CK}$, respectively, for which the values were 35.2% and 54.2% in $Reactor_{LDR}$, respectively (Table 2). The removal rate of VS could be used to determine the degree of substrate degradation (Peng *et al.* 2016). LDR improved the substrate degradation, which contributed to the better biogas production. Similar results were reported by Peng *et al.* (2016), that the solid removal efficiencies were high during the LDR and correspondingly, the biogas production also improved. Estevez *et al.* (2014) found that methane production increased by 16% with 50% LDR during the anerobic co-digestion of cow manure with Salix. Honghui (2018) found that biogas production increased by 29.84% with 50% LDR compared to the control reactor fed with food waste. The enhancing effect of recirculation on biogas production observed in this study was higher than most reported data. Compared with animal manure and food waste, corn straw as raw material had a single nutrient composition and was nutrient-deficient. Therefore, the metabolic intermediates recycled into the fermentation system along with liquid digestate were more effective, which not

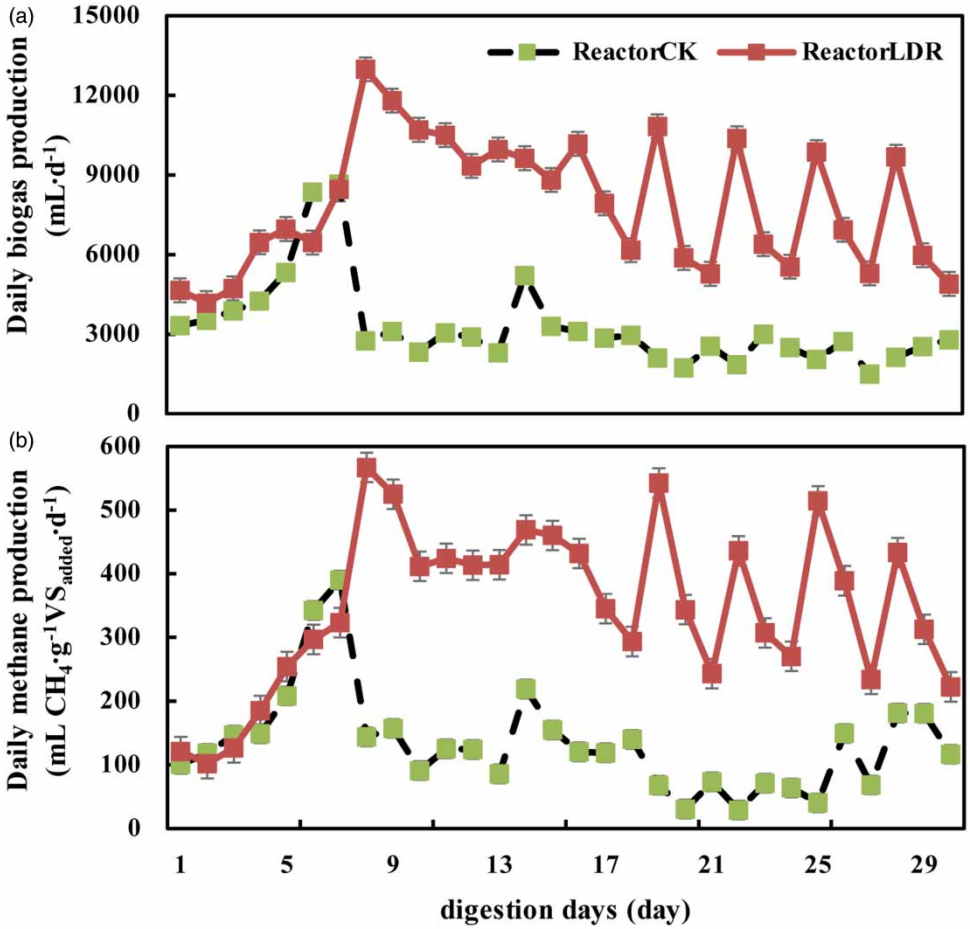

Figure 1 | Daily biogas and methane production variations during anaerobic digestion.

only supplements nutrients but also makes it difficult to destroy the balance of the system.

Influence of LDR on system stability

The changes of pH value, NH_4^+-N and TVFAs are shown in Figure 2. It can be seen that the pH values of both reactors decreased obviously in the first four days, then increased, and finally tended to be stable in the range of 6.4 to 7.6 (Figure 2(a)). The rapid hydrolysis and acidification of organic matter at the initial stage leads to the production and accumulation of VFAs. Therefore, pH values continued to decline, reaching the lowest value on day 4. The accumulation of NH_4^+-N in the fermentation system can also regulate the pH value to some extent (Ni *et al.* 2017). The accumulation of NH_4^+-N occurred at 4 to 13 days and 7 to 10 days in $Reactor_{CK}$ and $Reactor_{LDR}$, respectively (Figure 2(b)) and correspondingly, the pH also rose. On day 7, the pH value of $Reactor_{CK}$ and $Reactor_{LDR}$ increased to 7.18 and 7.27, respectively, and then gradually tended to be stable. The changing tendency of pH values was basically consistent in both reactors, with the pH of $Reactor_{LDR}$ a little higher than that of $Reactor_{CK}$ as a whole, especially during days 10 to 16 and after 25 days. In the AD process, NH_4^+-N was an important indicator. On one hand, microbes consume NH_4^+-N as the nitrogen source for their own growth and metabolism. On the other hand, NH_4^+-N can also regulate the pH value of the fermentation system to some extent. However, when the concentration of NH_4^+-N is too high or too low, the stability of the fermentation system will be affected (Ni *et al.* 2017). On the whole, the concentration of NH_4^+-N in both reactors decreased slowly in the first 31 days. Specifically, the concentration of NH_4^+-N decreased from 825 $mg \cdot L^{-1}$ on the first day to 182 $mg \cdot L^{-1}$ on the 31st day and from 1,472.5 $mg \cdot L^{-1}$ on the first day to 335.5 $mg \cdot L^{-1}$ on the 31st day in $Reactor_{CK}$ and $Reactor_{LDR}$, respectively (Figure 2(b)). The reasons for the decreasing NH_4^+-N content are probably that the

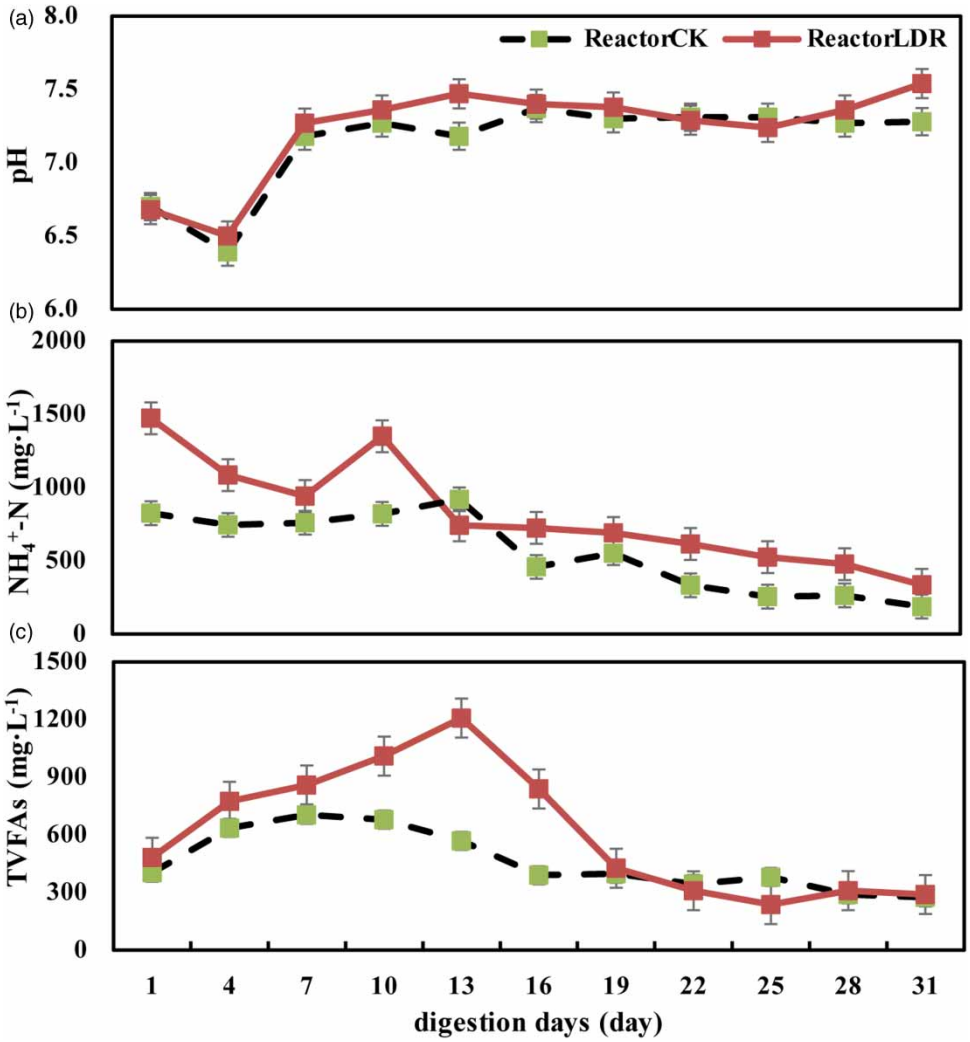

Figure 2 | pH value, ammonia nitrogen (NH_4^+-N) and total volatile fatty acids (TVFAs) variations during anaerobic digestion.

Table 2 | The TS and VS reduction rates

	$Reactor_{CK}$		$Reactor_{LDR}$	
Parameters	**Initial**	**Final**	**Initial**	**Final**
TS (%)	8.8 ± 0.2	6.1 ± 0.4	8.7 ± 0.5	5.6 ± 0.6
VS (%)	7.3 ± 0.9	4.2 ± 0.3	6.9 ± 0.3	3.2 ± 0.8
TS reduction (%)[a]	30.1 ± 0.7		35.2 ± 0.4	
VS reduction (%)[a]	42.1 ± 0.1		54.2 ± 0.6	

Date presented as means ± SD ($n = 3$).
[a]The reduction rates of TS and VS were determined by 100% · ($TS_{initial}$ -TS_{final})/$TS_{initial}$ and 100% · ($VS_{initial}$-VS_{final})/$VS_{initial}$, respectively.

digestate was discharged with NH_4^+-N and corn straw as a nitrogen-deficient feedstock was introduced with additional water; in other words, the influent of cornstalk with much less nitrogen content diluted the mixed slurry in the digesters, which resulted in the decrease of NH_4^+-N content (Peng *et al.* 2016). Our results deviated from the observations of Hu *et al.* (2014) regarding that the NH_4^+-N concentration accumulated with time. Possibly because of that, the OLR was different. Both studies used cornstalk as the raw material, but the OLR in Hu's research was 3.5 kg VS·m^{-3}·d^{-1}, which is higher than our study's OLR of 2 g VS·L^{-1}·d^{-1}. Similar results were reported by Wu *et al.* (2018) with food waste as raw material when the OLR was 2 g VS·L^{-1}·d^{-1}, the concentration of NH_4^+-N decreased with time. Nevertheless, it is the opposite of when the OLR increased. Kwietniewska & Tys (2014) reported that the activity of methanogens decreased by 10% when the concentration of NH_4^+-N was between 1,670 and 3,720 mg·L^{-1}, and there were no adverse effects below this limit.

Although the concentration of NH_4^+-N was obviously increased with 50% LDR compared to the control reactor, it still remained below the limit of being toxic to the methanogens. On the contrary, the accumulation of VFAs was partly caused by LDR. Nevertheless, the content of NH_4^+-N in $Reactor_{LDR}$ was higher than that in $Reactor_{CK}$. Therefore, the accumulation of NH_4^+-N resulting from LDR was possibly beneficial to the system stability, which was reflected in the higher biogas production.

The accumulation of TVFAs in the fermentation system appeared from day 1 to 16 and 1 to 13 in $Reactor_{LDR}$ and $Reactor_{CK}$, respectively. And the maximum concentration of 1,208 $mg{\cdot}L^{-1}$ and 704 $mg{\cdot}L^{-1}$ was observed on day 13 and on day 7 in $Reactor_{LDR}$ and $Reactor_{CK}$, respectively (Figure 2(c)), which was far lower than the threshold of 5,000 $mg{\cdot}L^{-1}$ proposed by Gan *et al.* (2008) to inhibit the production of VFAs and the hydrolysis of substrates. After that, the TVFAs gradually decreased to below the initial level in both fermentation systems, indicating that the production rates of VFAs by acid-producing bacteria was less than the consumption rates by methanogens (Zhang *et al.* 2017). In the current study, the accumulation of TVFAs was obvious and short in duration in $Reactor_{LDR}$ with the concentration of TVFAs increasing at first and then decreasing. This result deviated from the observations of Peng *et al.* (2016) regarding the TVFAs concentration increasing with time, possibly owing to the different recirculation ratio (Wu *et al.* 2018). Acetic, propionic and butyric acids are dominant VFAs in the AD process. The changes of specific VFAs are shown in Figure 3. As one of the VFAs, acetic acid is considered as the key intermediate (Gulhane *et al.* 2017). However, a concentration of acetic acid above 1.5 $g{\cdot}L^{-1}$ will inhibit cellulase activity and thus inhibit the hydrolysis process (Romsaiyud *et al.* 2009). And a high concentration of propionic acid and butyric acid will be toxic to methanogens. When the concentration of propionic acid reached 15 $g{\cdot}L^{-1}$ or the concentration of butyric acid reached 3.5 $g{\cdot}L^{-1}$, methanogen activity would be inhibited (Dogan *et al.* 2005). The concentration of acetic and butyric acids increased initially and then decreased with time in both reactors. The maximum concentration of acetic and butyric acids was 345 $mg{\cdot}L^{-1}$ and 242 $mg{\cdot}L^{-1}$ in $Reactor_{LDR}$, respectively, and 328 $mg{\cdot}L^{-1}$ and 178 $mg{\cdot}L^{-1}$ in $Reactor_{CK}$, respectively (Table 3). The propionic acid concentration varied in the range of 172.5 to 256.8 $mg{\cdot}L^{-1}$ in $Reactor_{CK}$, with a slow rate of continuous accumulation. The accumulation of propionic acid occurred in the first 16 days then decreased to below the initial level in $Reactor_{LDR}$, with a maximum concentration of 778 $mg{\cdot}L^{-1}$. Therefore, the 50% LDR caused the accumulation of propionic acid to some extent. Nevertheless, considering the biogas production in $Reactor_{LDR}$ was higher than that in $Reactor_{CK}$, it indicated that the system was still vigorous to recover from the accumulation of propionic acid. Rather than the VFAs concentrations, the VFAs/AC ratio is more suggested for use to evaluate the stability of AD system. When the VFAs/AC ratio was greater than 0.8, the stability of the fermentation system decreased (Li *et al.* 2018). With and without recirculation, the TVFAs/TAC ratio were 0.22 ~ 0.78 and 0.32 ~ 0.65, respectively (Table 3), which were much lower than the reported values, indicating that both systems had high stability.

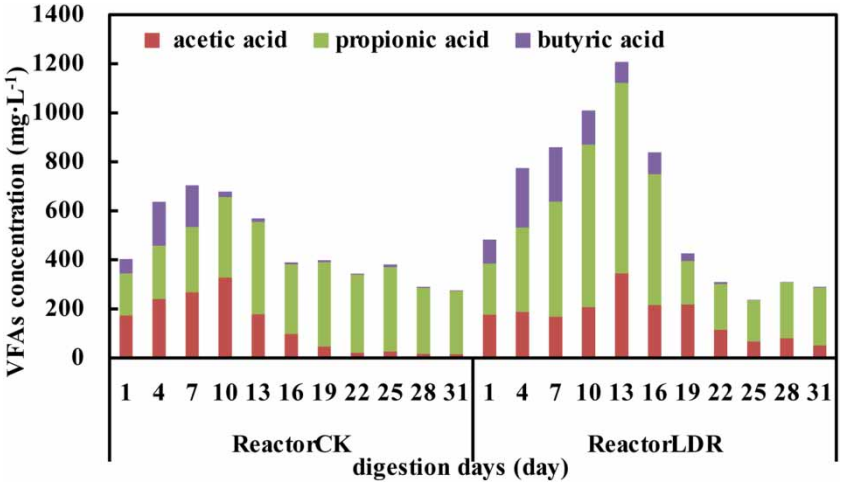

Figure 3 | Volatile fatty acids (VFAs) concentration variations during anaerobic digestion.

Table 3 | Liquid digestate characteristics and nutrients after anaerobic digestion of corn straw

	$Reactor_{CK}$		$Reactor_{LDR}$	
Parameters	**Max**	**Min**	**Max**	**Min**
pH	7.4 ± 0.4	6.4 ± 0.1	7.5 ± 0.4	6.5 ± 0.3
TVFAs ($mg{\cdot}L^{-1}$)	901.4 ± 0.1	289.5 ± 0.3	1,472.5 ± 0.9	325.5 ± 0.1
Acetic acid ($mg{\cdot}L^{-1}$)	328.4 ± 0.3	15.8 ± 0.4	344.9 ± 0.3	51.3 ± 1.2
Propionic acid ($mg{\cdot}L^{-1}$)	378.2 ± 0.5	172.5 ± 0.2	778.1 ± 0.1	168.5 ± 0.4
Butyric acid ($mg{\cdot}L^{-1}$)	177.9 ± 0.8	2.25 ± 0.6	242.3 ± 0.4	1.89 ± 0.7
NH_4^+-N ($mg{\cdot}L^{-1}$)	920.5 ± 0.4	188.2 ± 0.4	1,472.5 ± 0.7	335.5 ± 1.3
TAC ($mg\text{-}CaCO_3\ L^{-1}$)	1,384.6 ± 0.2	901.5 ± 0.3	1,876.8 ± 0.3	1,483.0 ± 0.5
TVFAs/ TAC ratio	0.65	0.32	0.78	0.22

Date presented as means ± SD ($n = 3$).

Influence of LDR on enzyme activities

Cellulase activity

The variations of cellulase activity are shown in Figure 4(a). On the whole, cellulase activity in $Reactor_{LDR}$ was higher than that in $Reactor_{CK}$. The possible reason was that the undecomposed cellulose and cellulose-decomposing microorganisms that remained in the liquid digestate were returned to the digestion system with the recirculation process (Li *et al.* 2018). Cellulase activity in the two reactors, both variations increased firstly and then decreased. Cellulase activity in $Reactor_{CK}$ presented a peak value of 0.32 $mg{\cdot}g^{-1}{\cdot}h^{-1}$ on day 4, and then stabilized after day 22. Cellulase activity in $Reactor_{LDR}$ presented a peak value of 0.51 $mg{\cdot}g^{-1}{\cdot}h^{-1}$ on day 4, and then stabilized after day 25. Luo & Wong (2019) reported the ability of LDR in extracting metabolic intermediates. However, a high concentration of metabolic intermediates in the fermentation system would result in the inhibition of hydrolase. Romsaiyud *et al.* (2009) found that when the mass concentration of acetic acid exceeded 1.5 $g{\cdot}L^{-1}$, cellulase activity would be inhibited. The maximum concentration of acetic acid was 345 $mg{\cdot}L^{-1}$, which was achieved on day 4 and was far below the threshold (Table 3). Interestingly, cellulase activity reached the maximum also on day 4, indicating that the accumulation of VFAs did not reach the threshold value of inhibiting cellulase activity. The enzymatic reaction rate is significantly affected by the substrate concentration (Zhang *et al.* 2019). Therefore, changes of TS and VS contents also contributed to the variations of cellulase activity in the AD process (Table 2).

Xylanase activity

The variations of xylanase activity are shown in Figure 4(b). Xylanase activity in $Reactor_{CK}$ presented a peak value of 0.27 $mg{\cdot}g^{-1}{\cdot}h^{-1}$ on day 7, then began to fall rapidly, stabilized after day 13. Xylanase activity in $Reactor_{LDR}$ presented a peak value of 0.29 $mg{\cdot}g^{-1}{\cdot}h^{-1}$ on day 7, then began to decline rapidly; the decline trend became gentle after day 13. On the whole, xylanase activity in $Reactor_{LDR}$ was higher than $Reactor_{CK}$, especially after day 13. Nissilä *et al.* (2012) detected that *Ruminofilibacter xylanolyticum* could utilize xylan in the AD system with corn and wheat straw as raw materials. The possible reason was that the xylan was degraded exhaustedly, in the later phase the low content of xylan became the limiting factor for xylanase activity. However, LDR reintroduced the unhydrolyzed xylan and xylan-decomposing microorganisms into the digestion system and promoted the xylanase activity significantly in $Reactor_{LDR}$ after day 13.

Dehydrogenase activity

The variations of dehydrogenase activity are shown in Figure 4(c). The dehydrogenase activity in two reactors both increased firstly and then decreased, and afterward tended to be stable. On day 7, dehydrogenase activity in $Reactor_{CK}$ and $Reactor_{LDR}$ reached the peak value in both reactors with 4.71 $mL{\cdot}g^{-1}{\cdot}h^{-1}$ and 4.88 $mL{\cdot}g^{-1}{\cdot}h^{-1}$, respectively. Interestingly, the hydrolytic enzyme activities also reached the peak on day 7, the accumulation of VFAs seems able to stimulate the dehydrogenase activity of microorganisms. With the consumption of substrates, the activity of dehydrogenase decreased and then tended to be stable. On the whole,

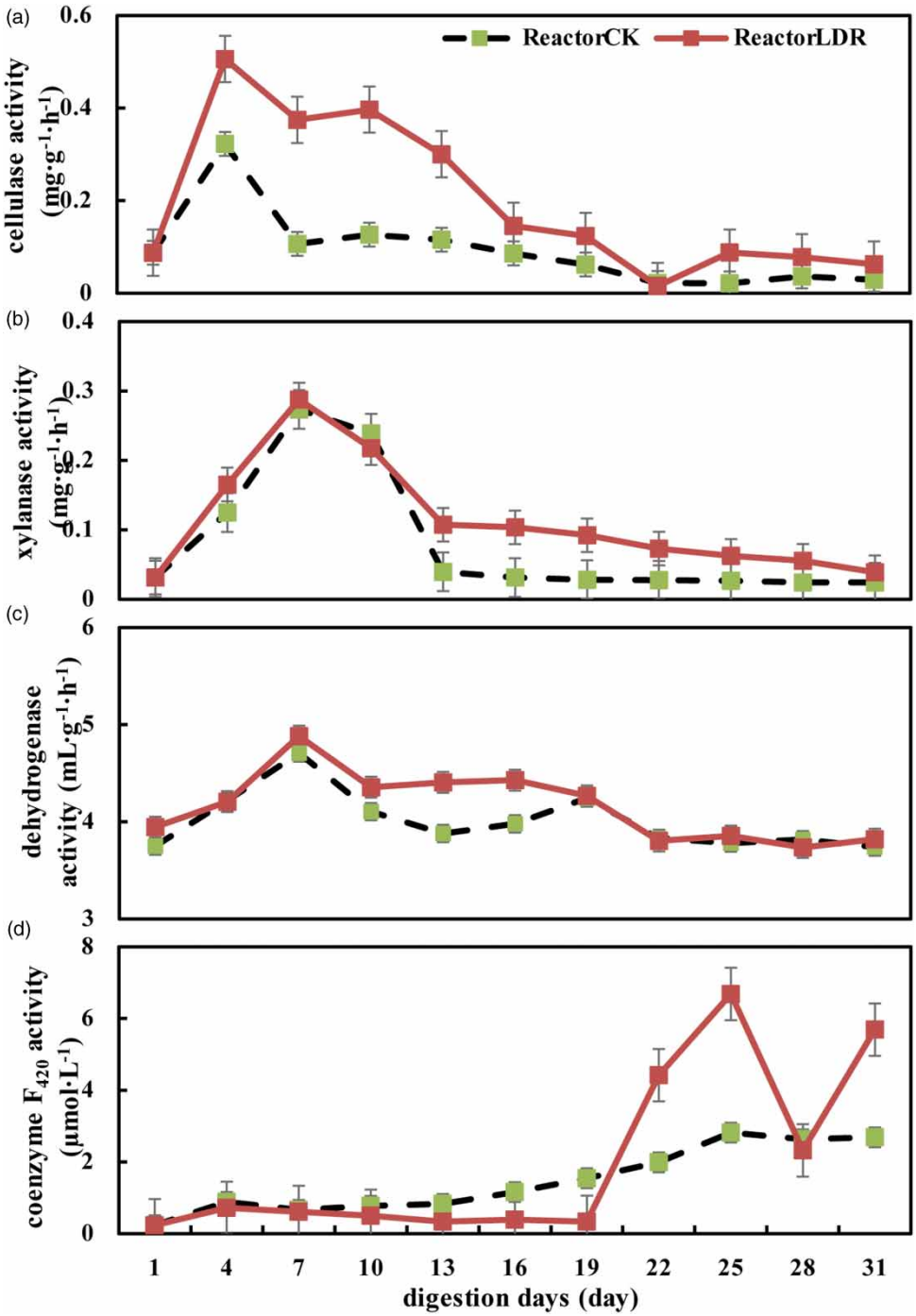

Figure 4 | Cellulase, xylanase, dehydrogenase and coenzyme F_{420} activity variations during anaerobic digestion.

dehydrogenase activity in $Reactor_{LDR}$ was higher than that $Reactor_{CK}$, especially from day 7 to day 19. Benitez *et al.* (1999) found that the activity of dehydrogenase decreased when available organic compounds decreased. Therefore, the accumulation of TVFAs in the fermentation system with LDR from day 1 to day 19 (Figure 2(c)) possibly stimulated the activity of dehydrogenase.

Coenzyme F_{420} activity

The variation of coenzyme F_{420} activity is presented in Figure 4(d). Coenzyme F_{420} is one of the unique coenzymes of methanogens and the activity of coenzyme F_{420} is usually regarded as the activity indicator of methanogens in the AD process (Dong *et al.* 2010). Coenzyme F_{420} activity in

$Reactor_{CK}$ increased slowly in the first 25 days and reached the peak value of 2.82 μmol·L^{-1} on day 25. During the first four days, coenzyme F_{420} activity in $Reactor_{LDR}$ was very close to that in $Reactor_{CK}$. But from day 4 to day 19, coenzyme F_{420} activity in $Reactor_{LDR}$ decreased gently and became lower than that in $Reactor_{CK}$. On day 19, coenzyme F_{420} activity in $Reactor_{LDR}$ began to increased sharply and reached the maximum peak value of 6.69 μmol·L^{-1} on day 25, subsequently declined to 2.63 μmol·L^{-1}, and then recovered to 5.69 μmol·L^{-1} on day 31. It was indicated that activity of coenzyme F_{420} was promoted by LDR after day 19. Similar results were reported by Wu *et al.* (2018), that liquid digestion recirculation improved the alkalinity of the reactor, so that maintained the optimal pH value of the methanogens.

Correlation between metabolic intermediates and enzyme activities

One of the advantages of producing biogas using corn straw as raw material is the high carbon content, while the disadvantage is low nitrogen content (Table 1). On the whole, the concentration of NH_4^+-N decreased slowly with time, but the accumulation of TVFAs was obvious in fermentation systems (Figure 2). The influence of TVFAs on enzyme activities was greater than that of NH_4^+-N in a suitable pH environment. Pearson correlation coefficient analysis revealed that there was not a good correlation between NH_4^+-N and enzyme activities in the two reactors ($P > 0.05$, Table 4). A significant correlation existed between the TVFAs concentration and enzyme activities in the two reactors ($P < 0.01$ or $P < 0.05$, Table 4). Distinctly, there was a tight linkage between the variation of TVFAs concentration and the variations of enzyme activities. With or without recirculation, the concentration of TVFAs was always positively correlated with cellulase, xylanase and dehydrogenase activities, while was negatively correlated with coenzyme F_{420} activity. The enhanced decomposition of organic matter resulted from the high activities of hydrolase led to the accumulation of VFAs, which in turn provided a sufficient substrate for dehydrogenase during AD. Therefore, the concentration of TVFAs was positively correlated with the activities of hydrolase and dehydrogenase. Coenzyme F_{420} activity is closely related to the concentration of TVFAs. In $Reactor_{LDR}$, the concentration of TVFAs increased rapidly in the first 4 days. Interestingly, the coenzyme F_{420} activity also reached the peak value on day 4. After that, the concentration of TVFAs changed from 1,006 mg·L^{-1} on the fourth day to 950 mg·L^{-1} on the 16th day, during which the coenzyme F_{420} activity decreased continuously. It was revealed that the coenzyme F_{420} activity was inhibited by the accumulation of VFAs. On the contrary, the maximum concentration of TVFAs in $Reactor_{CK}$ reached on the fourth day was less than 950 mg·L^{-1} and the coenzyme F_{420} activity increased consistently with time. It was concluded that the TVFAs concentration of less than 950 mg·L^{-1} was likely to be the threshold of inhibiting the coenzyme F_{420} activity.

Correlation between enzyme activities and daily biogas/methane production

Pearson correlation coefficient analysis revealed that there was a very significant correlation between hydrolase activities and daily biogas production in $Reactor_{CK}$ ($P < 0.01$, Table 5). There was not a good correlation between hydrolase activities and daily biogas production in $Reactor_{LDR}$ ($P > 0.05$, Table 5), possibly due to that the peak of daily biogas production was delayed with LDR. Cellulase activity in the two reactors both reached the peak on day 4. While daily biogas production in $Reactor_{CK}$ and $Reactor_{LDR}$ reached the peak on day 7, and 10, respectively. Both

Table 4 | Correlation between metabolic intermediates and enzyme activities

	$Reactor_{CK}$		$Reactor_{LDR}$	
Parameter	**VFAs**	**NH_4^+-N**	**VFAs**	**NH_4^+-N**
Cellulase	0.789 ± 0.04**	0.511 ± 0.05	0.809 ± 0.03**	0.587 ± 0.06
Xylanase	0.847 ± 0.01**	0.529 ± 0.09	0.684 ± 0.02*	0.558 ± 0.08
Dehydrogenase	0.726 ± 0.01*	0.456 ± 0.16	0.822 ± 0.02**	0.507 ± 0.11
Coenzyme F_{420}	−0.697 ± 0.02*	0.487 ± 0.01	−0.762 ± 0.06**	0.525 ± 0.03

*The correlation is significant at the 0.05 level.
**The correlation is very significant at the 0.01 level.

Table 5 | Correlation between enzyme activities and daily biogas /methane production

	$Reactor_{CK}$		$Reactor_{LDR}$	
Parameter	**Daily biogas production**	**Daily methane production**	**Daily biogas production**	**Daily methane production**
Cellulase	0.293 ± 0.03	/	0.411 ± 0.02	/
Xylanase	0.740 ± 0.01**	/	0.460 ± 0.03	/
Dehydrogenase	0.840 ± 0.04**	/	0.529 ± 0.05	/
Coenzyme F_{420}	/	0.263 ± 0.05	/	0.127 ± 0.02

**The correlation is very significant at the 0.01 level.

xylanase activity and daily biogas production in $Reactor_{CK}$ reached the peak on the day 7. Xylanase activity and daily biogas production in $Reactor_{LDR}$ reached the peak on day 7 and 10, respectively. Interestingly, the peak of daily biogas production was delayed to the activities of hydrolytic enzymes. This finding is consistent with the phenomenon reported by Juanjuan *et al.* (2010) that the peak value of cellulase and xylanase activities were slightly ahead of the peak value of biogas production. Hydrolysis is the rate-limiting step of AD, the hydrolytic bacteria transform the organic matter into short-chain VFAs, which were then utilized by acid-producing bacteria and methane-producing archaea to produce biogas (Shin *et al.* 2001; Wu *et al.* 2018). Therefore, the peak of hydrolase activities was earlier than the peak of the daily biogas production. Pearson correlation coefficient analysis revealed that there was a very significant correlation between dehydrogenase activity and daily biogas production in $Reactor_{CK}$ ($P < 0.01$, Table 5). There was not a good correlation between dehydrogenase activity and daily biogas production in $Reactor_{LDR}$ ($P > 0.05$, Table 5), possibly due to that the peak of daily biogas production was delayed with LDR. Both dehydrogenase activity and daily biogas production in $Reactor_{CK}$ reached the peak on day 7. This finding is consistent with the phenomenon reported by Juanjuan *et al.* (2010) that the peak of dehydrogenase activity was synchronous with the peak of biogas production. Dehydrogenase activity and daily biogas production in $Reactor_{LDR}$ reached the peak on day 7 and 10, respectively. LDR led to the peak of daily biogas production appearing later than that of dehydrogenase activity. The dehydrogenase activity and daily biogas production in $Reactor_{LDR}$ were more stable after day 22.

The variations of coenzyme F_{420} activity in the two reactors was not well correlated with the variations of daily methane production ($P > 0.05$, Table 5), this was consistent with the research conclusion of Kida *et al.* (2001), but inconsistent with the result reported by Cheng *et al.* (2007), who found that the activity of coenzyme F_{420} was positively correlated with methane production during the anaerobic biodegradation of the organic components of municipal solid waste. The divergent result was possibly related with the methanogenic pathway: Alex *et al.* (1990); Braks *et al.* (1994) reported that coenzyme F_{420} has been found in both aceticlastic methanogens and hydrogenotrophic methanogens. This coenzyme F_{420} binds to hydrogenase to participate in the production of methane from H_2-CO_2 in hydrogenotrophic methanogens (Thauer *et al.* 1993). Nevertheless, the role of coenzyme F_{420} in aceticlastic methanogens is unclear and the content of coenzyme F_{420} in aceticlastic methanogens is much lower than that of those in hydrogenotrophic methanogens (Kida *et al.* 2001). Therefore, coenzyme F_{420} was a good monitoring indicator of methanogenic archaea activity when hydrogenotrophic methanogen dominated. And the predomination of aceticlastic methanogens was likely to result in the low correlation between the coenzyme F_{420} and methane production.

CONCLUSIONS

50% LDR can accelerate the degradation of substrate and maintain stable operation of a fermentation system. Therefore, 50% LDR showed promising results in producing biogas and methane from corn straw. During the trial, although the LDR resulted in the accumulation of VFAs, the concentration of VFAs was smaller than the threshold of inhibiting the biogas production, cellulase, xylanase and dehydrogenase activities. However, coenzyme F_{420} activity was inhibited when the concentration of TVFAs exceeded 950 $mg \cdot L^{-1}$. Furthermore, the analysis of the correlation between enzyme activities and daily biogas/methane production can provide a reference for improving the quality and efficiency of the AD fed with crop straw by means of

biological enzymes. The dynamic tendency of daily biogas production very much resembled some enzyme activities delay. However, the variations of coenzyme F_{420} activity was inconsistent with the variations of daily methane production. Therefore, the characterization of methanogenic archaea revealed by coenzyme F_{420} activity needs to be further studied.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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