Anaerobic Co-digestion of Grass, Alfalfa, and Red Clover for Methane Production and the Kinetic Analysis

Wentao Li,* Baohua Chai, Yan Lu, and Meijing Wang

The residual of perennials in landscape trimming and agricultural interval planting are produced massively, and they can provide an innovative way to increase anaerobic digestion efficiency *via* co-digestion process. In this study, the bio-methane potential (BMP) of different perennial crops (grass, alfalfa, and red clover) in various feedstock concentrations based on volatile solid (VS) and the kinetic analysis of the co-digestion process were investigated. The results showed that grass and legumes reached the highest methane yield at 5 VS/L and 20 VS/L, respectively. Co-digestion of grass and perennials had better methane production of 338 mL/g VS, which is 9.1% higher than mono-digestion. Further analysis demonstrated that VS removal efficiency of mono-legumes digestion was below 60%, while co-digestion of grass and legumes can improve VS removal efficiency dramatically. Volatile fatty acids (VFAs) and ammonia in the digestate accumulated at 40 VS_{added}/L. Additionally, kinetic analysis was employed to predict and evaluate the performance of anaerobic digestion, with the Cone model showing the best fitting curve.

DOI: 10.15376/biores.18.1.1742-1756

Keywords: Co-digestion; Perennials; Legumes; BMP; Kinetic analysis

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INTRODUCTION

Perennials are a very important biomass resource in spare land and landscape trimming processes. Legumes perennials, by way of fixing free nitrogen to ionic form, can hold soil moisture, improve soil quality, and soil fertility, which are commonly applied to cultivated in saline-alkali soils (Das *et al.* 2018). With the source of perennials being wide and available, their biomass has the potential to provide cheap and stable clean energy for modern society.

However, anaerobic digestion of lignocellulose-rich feedstocks, such as energy crops and perennials, faces a serious challenge, because these materials are often hard to degrade and arouse serious acidic inhibition, such that they fail to operate continuously (Qi *et al.* 2021). One of the main barriers lies in their complex structure, with lignin and cellulose twisted and hemicellulose being interspersed among it (Abraham *et al.* 2020). Hence, necessary pretreatment is employed to break down these recalcitrant structures and improve bio-degradation efficiency. Feedstocks with small particle size and large contact surface are attractive to microorganisms that will live in the feedstock and release decomposition enzymes (Tsapekos *et al.* 2017). According to Moset *et al.* (2017), grass pretreated with excoriating, swatting, and chopping have different bio-methane potential production (BMP), with excoriated grass performing the best. Therefore, an appropriate pretreatment is essential for estimating certain materials' BMP.

Chemical composition is another critical factor that affects anaerobic digestion of perennials. Although cellulose, hemicellulose, and lignin account for most of the chemical proportions in all perennials, they can vary from species to species, and even differ from season to season. Sun *et al.* (2019) reported that the BMP of macroalgae had a positive relationship with hemicellulose content, while it was negatively correlated with lignin content. Meanwhile, the perennials harvested in spring and summer also had a profound impact on their chemical composition, with higher hemicellulose content and, hence, better and higher biogas production in anaerobic processing.

Due to relatively high lignocellulose content compared with animal manures, perennials usually have higher carbon/nitrogen ratios, which means that acidic inhibition is easier to happen in mono-digestion of perennials with excessive concentrations. Therefore, co-digestion with nitrogen rich materials is the common method to solve this problem. Grass and cattle manure co-digestion showed a very good improvement in methane production, acquiring the most net energy (Moset *et al.* 2017). Wang *et al.* (2022) reported that co-digestion of grass and pretreated sewage sludge under thermophilic condition, based on C/N ratio of 10, acquired highest methane production, and dissolution of organics improved from 25% to 33.6%. However, animal manure and perennials are not always available at the same spot, and transporting this feedstock to the biogas plant would be cost-consuming. In addition, perennials are seasonal, which means that keeping a constant C/N with manure is difficult in perennials shortage period. Legumes, known for their rich nutrition, are often used as animal forage with abundant nitrogen. Therefore, co-digestion of grass, which is poorer in nitrogen content, and legumes would be an innovation act in this field.

Measuring bio-methane production of certain materials by fitting the data to different kinetic equations is a common method. The fitted parameters obtained from these equations can predict and evaluate the performance of anaerobic digestion (Nguyen *et al.* 2019). According to Pardilhó *et al.* (2022), the maximum methane production rate of co-digestion meadow grass and cattle manure predicted by modified Gomperz model, had improved by 114% compared with mono-digestion of grass. Mono-digestion of riverbank grass in different feedstock and inoculum ratio had obviously various lag time, which was predicted by first order kinetic model (Wang *et al.* 2020). Hence, the degree of inhibition resulting from high feedstock concentration can be depicted by appropriate kinetic model equations. Although the cumulative methane production from co-digestion of perennials and animal manure predicted by different kinetic models has been studied by most researchers (Dai *et al.* 2016; Thanarasu *et al.* 2019; Millati *et al.* 2020), no relevant investigations concentrate on the co-digestion of different perennials using kinetic models.

In this study, different perennials, normal grass, and legumes plants produced in the same season were collected and used for BMP tests in thermophilic conditions. Then, in order to improve methane production in relative high feedstock concentration, co-digestion of perennials in various of concentrations was employed as a strategy to adjust C/N ratio and enhance necessary nutrition in anaerobic digestion compared with mono-digestion of grass and legumes. Main components of perennials were determined in the initial step of the experiment, and intermediates generated in the anaerobic process were measured to evaluate inhibition degree caused by overload feedstock concentration. Kinetic models, such as Modified Gomperz model, the First kinetic model, the Cone model, and the Logistics model, were applied to predict and assess cumulative methane production of all mono- and co-digestion circumstances. Then the parameters calculated by these kinetic models, such as lag phase time λ, the first order hydrolysis rate constant k, and daily maximum methane production rate R_{max}, were estimated the inhibition levels and predicted BMP of each perennial.

The aim of this study is to (1) investigate the BMP of each perennial; (2) explore the different BMPs in various feedstock concentration; (3) compare the difference of mono- and co-digestion methods in methane production; and (4) evaluate and assess the inhibition levels caused by overload feedstock concentration.

EXPERIMENTAL

Perennials and Inoculums

The perennials used in this experiment originated from embankments along the sides of the Weihe River in Shaanxi (108.08°E, 34.24°N), China. These perennials were cultivated for feeding animals and harvested in mid-October. Two main species of legume, including alfalfa and red clover, and two main spices of grass, including cock's foot and tall fescue, were collected around this district. The perennials were spread on the ground with thickness around 5 cm and air dried under natural conditions with a temperature of about 30 °C and relative humidity less than 30%. A plastic cloth was prepared to prevent the perennials from being rained on. Airing was done for about 7 days to remove extra moisture until the weight of dry perennials was unchanged. Then the material was crushed and milled into powder, under 10 mesh sieves (maximum pore diameter <1.7 mm). Inoculum was obtained from a nearby biogas plant, which used swine and cattle manure as main feedstock and operated at mesophilic condition. The physio-chemical characteristics of the perennials and inoculum are summarized in Table 1. The sampling, collecting, and storing of experimental materials, including perennials and inoculum, all followed laboratory rules.

Table 1. Physio-chemical Characteristics of the Perennials and Inoculum

Parameters	TS (%)*	VS (%)**	Hemi-cellulose(mg/g)	Cellulose (mg/g)	ADL*** (mg/g)	Total Nitrogen (mg/g)
Grass****	93	81.0	221	246	24	4.59
Alfalfa	91	85.1	117	343	85	7.6
Red Clover	94	84.2	71	353	42	5.96
Inoculum	3.7	2.6	-	-	-	2.67

* Total solids
** Volatile solids, Based on wet weight
*** Acid detergent lignin
**** Mixture of cock's foot and tall fescue (1:2)

Experimental Design

The diagram and main parameters of batch vials used in this experiment are shown in Fig. 1. The total volume of each vial was 1124 mL, including 964 mL headspace and 160 mL of substrates. The substrates were a mixture of perennial samples, inoculum, and deionized water. The perennial sample were divided into 5 groups according to local harvest statistics, which were cock's foot + tall fescue (1:2) also called grass group, alfalfa group, red clover group, red clover + grass (3:7) called mixture 1 group, alfalfa + grass

(2:8) called mixture 2 group. In order to determine the most appropriate organic loading rate for BMP of each sample, these perennials were added into batch vials in amount of 5, 10, 20, 40 g VS/L, respectively (Angelidaki *et al.* 2009). In addition, a control group that only contained inoculum was set to calculate extra methane generated from the inoculum. More details about adding amount and groups dividing are depicted in Table 2.

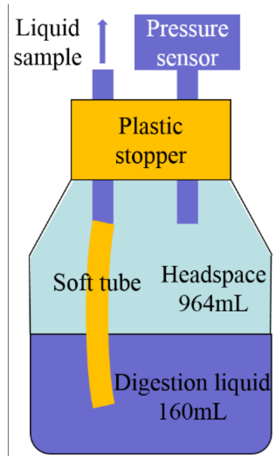

Fig. 1. The diagram and main parameters of batch vials

Table 2. Improvement of BMP by Co-digestion in 20 g VS/L

Substrate	Practical BMP (mL)	Rule of Mixtures (mL) *	Improvement (%)
Grass	320.01	-	-
Alfalfa	269.05	-	-
Red Clover	276.87	-	-
Mixture 1	322.32	307.07	4.97
Mixture 2	337.96	309.82	9.08
* Rule of Mixtures was the calculation based on the mono-digestion BMP ** Improvement = (Practical BMP- Rule of Mixtures)/ Rule of Mixtures ×100%			

The BMP process was conducted by incubating the aluminum bottles (1000 mL) at 55 ± 1 °C for 18 days (Filer *et al.* 2019). BMP was determined by using methane volume divide the removing amount of the volatile solid.

The whole anaerobic process was carried in a constant temperature incubator, set at 55 ± 1 °C for 18 days and shaking for 5 min every 8 h at a rate of 90 rpm. In order to create a strict anaerobic environment, all vials were flushed continuously with CO_2/N_2 (20/80% by v/v) in headspace for at least 5 min. Then a thick butyl rubber stopper was used to seal the mouth of the vial. A pressure sensor with a needle detector was pierced into the stopper, such that the headspace was connected to a gas chamber. After acquiring gas pressure data every day, which could transform to gas volume automatically according to the Ideal Gas Law, the gas stored in the headspace would be released and returned to standard atmosphere pressure through the gas outlet. A gas collection bag was connected to the other side of the gas outlet to sample gas, and then gas composition would be analyzed. Eventually, every experiment group was done in triplicate to calculate statistical errors.

Equations

Four different kinetic models were applied in this experiment to evaluate and predict methane production yield of each combined group. The modified Gomperz model (Eq. 1), the first order kinetic model (Eq. 2), the Cone model (Eq. 3), and the Logistics model (Eq. 4) were all selected to fit experimental data and provide necessary parameters that were critical to assess BMP of each perennials and its potential inhibition conditions. The fitted curve and data dots were calculated and plotted by Origin 7.5 (OriginLab Corporation, USA). The specific form of these equations was as follows:

The modified Gomperz model:

$$M(t) = M_{max} \times exp\left(-exp\left(R_{max} \times e \times (\lambda - t) \ / \ M_{max} + 1\right)\right) \tag{1}$$

The first order kinetic model:

$$M(t) = M_{max} \times \left(1 - exp(-k \times t)\right) \tag{2}$$

The Cone model:

$$M(t) = M_{max} \ / \ \left(1 + (k \times t)^{-n}\right) \tag{3}$$

The Logistics model:

$$M(t) = M_{max} \ / \ \left(1 + exp\left(4 \times R_{max} \times (\lambda - t) \ / \ M_{max} + 2\right)\right) \tag{4}$$

In these equations, $M(t)$ is the cumulative methane production yield at specific time, "t" (mL/g VS_{added}); M_{max} is the methane production yield of a given feedstock in mL/g VS_{added}; R_{max} is the daily maximum methane production rate (mL/g VS_{added}×day); e is the natural logarithm constant, taken as 2.718 here; λ is lag phase time (day), which is considered that little methane is produced during this period; t (day)is the experimental carrying time, also the methane production time; k is the first order hydrolysis rate constant in (day^{-1}) that is assumed stable during the complete anaerobic process; and n is the shape feature.

Statistical indicators, such as Goodness in fitness (R^2) and the Akaike information criterion (AIC) test, which was examined by several previous papers for evaluating the fitting curve (Donoso-Bravo *et al*. 2011b; Li *et al*. 2018; Nguyen *et al.* 2019), were employed to determine the most appropriate model.

When $N/K \geq 40$,

$$AIC = N \times ln(RSS / N) + 2K \tag{5}$$

When $N/K < 40$,

$$AIC = N \times ln(RSS / N) + 2K + 2K(K+1)/(N-K-1) \tag{6}$$

where N is the number of measured data dots; RSS is residual sum of squares; and K is the estimated parameter number of fitting curves.

Analytical Methods

The composition of biogas was analyzed by a gas chromatography (GC-2014, Shimadzu, Japan) aided with a thermal conductivity detector (TCD) (heater 150 °C, helium flow: 10 mL/min). The temperatures of the gas entrance and column oven were kept at 100 and 80 °C, respectively. Volatile fatty acids (VFAs) were detected using gas chromatography (GC-2014, Shimadzu, Japan) facilitated with flame ionization detector (FID) (heater 250 °C, nitrogen flow: 30 mL/min). The size of the chromatographic column was Φ 30 m × 0.82 mm. The temperature of the column oven was set in an automatic heating procedure, which began heating at 80 ℃, with a ramp rate of 10 ℃/min, reached 150 ℃ and was kept there for 6 min. The pH value was measured by using a digital pH meter (PB-10, sartorius, China) during the experiment. The liquid samples were treated by high speed refrigerated centrifuge (HC-3018R, Zonkia Scientific Instruments Co. Ltd., China) at 4 ℃ and 15000 rpm for 20 min. Nitrogen- ammonia concentrations, TS and VS were all determined by the standard method of American Public Health Association (Walter 1998). The contents of hemicellulose, cellulose and lignin were determined by fiber extractor (ANKOM A220, USA), and were calculated according to Van Soest method.

Statistical Analysis

The methane production of the samples was performed in three repetitions, and the variance of the resulting values was analyzed by using one-way (ANOVA). SPSS v.18.0 software was used for statistical analysis executed to compare the least significance difference (LSD) at P = 0.05 values.

RESULTS AND DISCUSSION

Daily Methane Production of Perennials in Thermophilic Conditions

The amount of VS added had a profound effect on daily methane production, while daily methane production of all experimental groups showed the same tendency (Fig. 2). The daily methane production dropped instantly within 2 to 3 days and gradually declined until the end of the experiment. This is because along with the experimental operation time, the available substrate became depleted. When soluble nutrition that was generated at the start of the experiment was used up, the daily methane production decreased sharply, owing to not-easily-available substrate. The cellulose, which is a major proportion of perennials, was difficult to degrade due to lack of cellulolytic bacteria at the start of the experiment. Along with operation time, these cellulolytic bacteria continued to increase, and the soluble substrate began to rise, which provided the methanogenesis process with nutrition. Hence, rich cellulose substrates, such as alfalfa and red clover, had a slight second peak in 8 to 11 days, which was in accordant with previous research (Wang *et al.* 2020). The slight 3rd peak in Alfalfa and the Mixture may be attributed to the fact that the high N content in Alfalfa could result in the ammonium inhibition on the methane production as AD process going. Subsequently, the ammonium inhibition was relieved on day 15 to 17 to help the methane production by generating a slight 3rd peak.

Overall, the daily methane production increased with the growing VS_{added} amount, and the concentration of 40 gVS_{added}/L acquired the highest daily methane production of all five groups. This is because higher concentration of VS contains more biodegradable nutrition, which can convert methane eventually and this result is also accordant with previous research (Wang *et al.* 2020).

Compared with the peak time 7 to 8 days of perennials in mesophilic conditions in a previous study (Wu *et al.* 2023), the daily methane production of all groups in this experiment reached their peaks at 2 to 3 days in thermophilic condition. This may contribute to high activity of microorganisms and, therefore, may produce more enzymes to accelerate hydrolysis and methanogenesis process (Gao *et al.* 2022). In addition, the size of perennials used in this experiment also played an important role in daily methane production. Tsapekos *et al.* (2017) found that after mechanical smashing pretreatment, the methane yield of grass increased dramatically. It was concluded that reducing the size of feedstock could increase the specific surface area and bio-reaction spots, which was critical to combine enzymes and microorganisms and accelerate bio-chemical reaction. The particle sizes of perennials in this study were all less than 1.7 mm. Not only did this result in higher specific surface area, but it also generated more soluble and easily-bio-decomposed substrate, such as hemicellulose contained in perennials, which stimulated methanogenesis process and enabled the system to reach the daily methane peak earlier. Therefore, rich hemicellulosic substrate, in this study grass, had the highest peak of daily methane production. Notably, in all groups, along with the increasing VS_{added} amount, the rise of daily methane production was not precisely proportional. That means that although the VS added amount reached 40 g VS_{added}/L, which is fourfold higher than 5 g VS_{added}/L group, the methane production of 40 g VS_{added}/L was not fourfold in comparison to 5 g VS_{added}/L. Consequently, inhibition existed in high substrate concentration groups caused lower methane production.

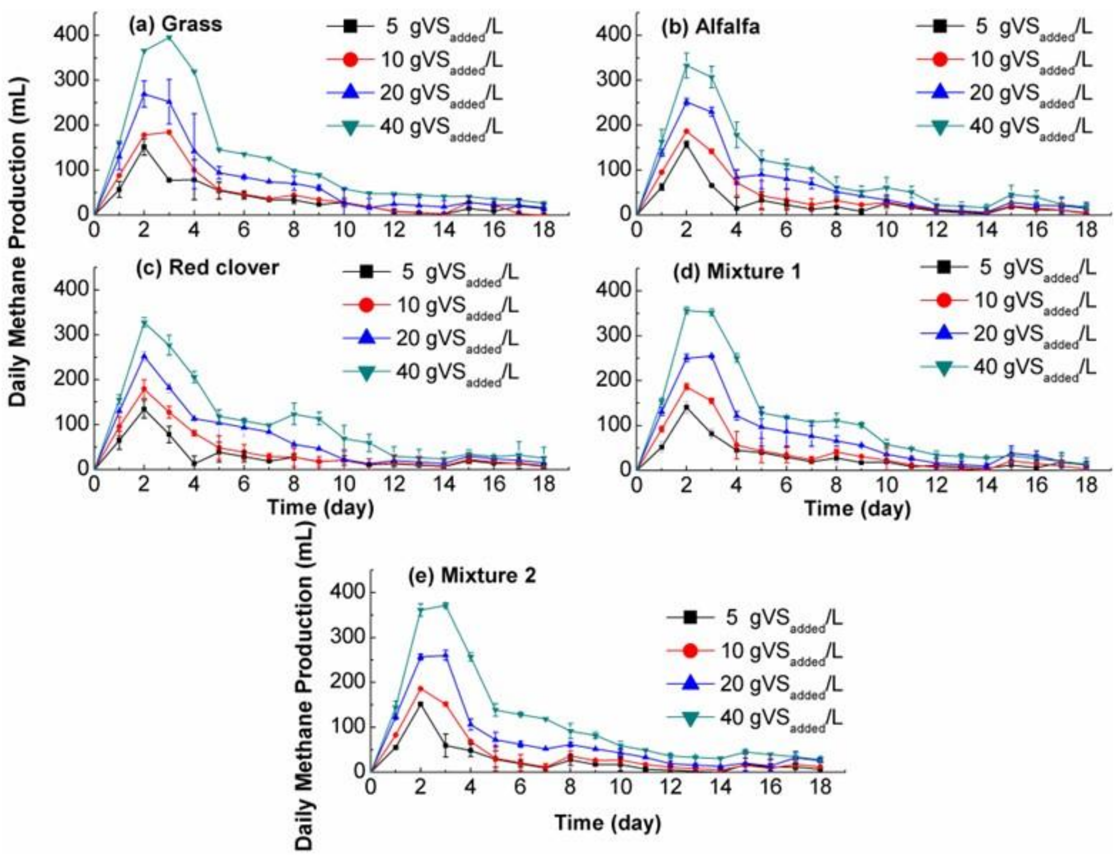

Fig. 2. Daily methane production from anaerobic digestion of perennials

Digestate Properties

One of the most effective methods to evaluate anaerobic digestion (AD) stability is to investigate the quantity of the ultimate products of the AD process and the intermediate metabolic products present in the liquid phase, such as VFAs. The VFAs concentration reached their peak at day one of all experimental groups (Fig. 3). This was because in thermophilic condition the activity of microorganisms was in relatively high level, especially that of organic pyrolysis bacteria; therefore, a huge amount of VFAs was accumulated (Wu *et al.* 2023). Owing to the formation of acetic acid that was the direct available substrate for methanogenesis organisms, relatively high daily methane production also was observed from day one to day 4, when acetic acid concentration was above 200 to 300 mg/L. As is shown in Fig. 3, except for the 40 g VS_{added}/L group, the VFAs concentration declined sharply after they reached the peak. This was in accordance with Gao *et al.* (2022), who found that the VFAs concentration of Napier grass thermophilic anaerobic digestion had the same tendency. On the other hand, 40 g VSadded/L group kept the relatively high VFAs concentration and high propionic acid/acetic acid ratio (Fig. 3). According to Wongwilaiwalin *et al.* (2018), high propionic acid/acetic acid ratio for long time was the primer factor that caused anaerobic acidic inhibition and reactor failure. Apparently, when VS concentration reached 40 g VS_{added}/L, a constant VFAs accumulation was found, and it may have caused inhibition to some extent. In addition, this explained that daily methane production of 40 g VS_{added}/L group was not strictly fourfold in comparison to the 5 g VS_{added}/L group. However, VFAs concentration remained stable in the next days, which can be attributed to the comparatively low substrate concentration in this experiment. And pyrolysis bacteria and methanogenesis archaea achieved dynamic balance eventually (Wongwilaiwalin *et al.* 2018).

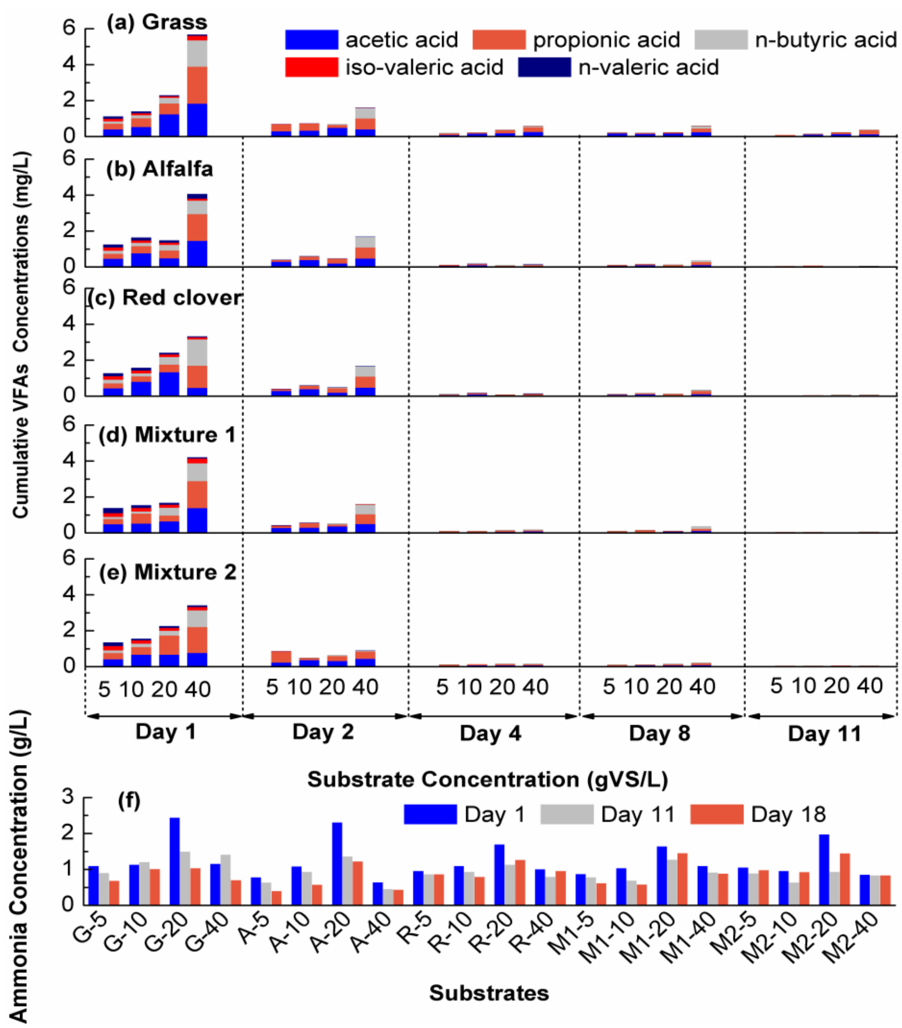

Fig. 3. The development of VFAs and Ammonia concentration during AD

From the VFAs peak value perspective, the grass group was higher than legumes and mixture groups. This was because grass contained more hemicellulose than legumes, and it was easy to decompose and converted into VFAs rapidly. Whereas cellulose was the main component for legumes, the VFAs concentration in legume groups were a little lower than grass group. Because cellulosic bacteria need a longer incubation to reach maximum activity and release enough cellulolytic enzyme (Qi *et al.* 2021), these cellulose-rich substrate had slight VFAs accumulation, hence, they affected little acidic inhibition.

The ammonia concentration of all experimental groups maintained the appropriate range during the middle and end of the experiment (Fig. 3). According to Yellezuome *et al.* (2022), the methanogenesis process was inhibited when the total ammonia concentration was beyond 1.7 g/L. It seemed that only 40 g VS_{added}/L mono digested group was inhibited by ammonia accumulation at the start of the experiment, which all exceeded 2 g/L. Simultaneously; the mono digested 40 g VS_{added}/L group had obvious accumulation of VFAs on the first day. Although the nitrogen content in legumes was far less than animal manure used in anaerobic digestion (Pardilhó *et al.* 2022), a noticeable ammonia accumulation was observed in this experiment. This may support a conclusion that VFAs accumulation boosts the buildup of ammonia, which was also confirmed by previous papers (Ajayi-Banji and Rahman 2022). In addition, the average of ammonia concentration of grass was lower than in the case of legumes, showing that nitrogen content in raw substrate had an important effect on ammonia concentration. According to Qi *et al.* (2021), the failure of mono anaerobic digestion of silage grass attributed to rapidly declining pH, while a suitable ammonia concentration not only provides microorganisms with necessary nutrition, but it also offers anaerobic systems an essential buffer to keep the process stable and efficiency. Therefore, the mixture of grass and legumes showed a perfect scenario, sustaining ammonia concentration in an appropriate range of all stages, which also validated that the co-digestion made the process better and smoother.

The aim of applying the AD technique is mainly to convert organic parts of biowastes into biogas. Therefore, the removal efficiency of organic wastes was adopted as the main indicator for evaluating the AD performance. In this study, the AD efficiency was mainly assessed by VS removal rate. Figure 4 showed the VS removal efficiency of mono and mixture perennials anaerobic digestion. It was clearly indicated that the VS removal efficiency of grass and mixture was almost above 60%, which was apparently higher than that of legumes groups that only range from 40% to 60%. Considering the composition of substrate (Table 1), it found that cellulose-rich substrate had lower VS removal efficiency than those of hemicellulose rich substrates. Besides, mixing hemicellulose rich grass and cellulose-nitrogen rich legumes wound hugely improve the VS removal efficiency overall. This result was consistent with Hagos *et al.* (2017), who found that co-digestion would enhance VS removal efficiency for cellulose rich feedstock, during which the activity of some selective cellulolytic enzymes had improved. Therefore, the mixing of grass and legumes in an anaerobic environment may promote and activate some metabolic regulatory genes that are not expressed during mono legume anaerobic digestion. Additionally, the VS concentration of anaerobic digestion had an impact on VS removal efficiency. Except for the alfalfa group, the VS removal efficiency reached maximum when VS concentration was 20 g VS_{added}/L. The finding was also confirmed by Körber *et al.* (2022), who suggested the most appropriate TS concentration for energy crops anaerobic digestion was 3%, which was approximately equivalent to this study. According to Sun *et al.* (2019), pyrolysis bacteria and methanogenesis archaea would adhere to the surface of cellulose rich substrate and form symbiosis, as long as the VS concentration maintained the proper range.

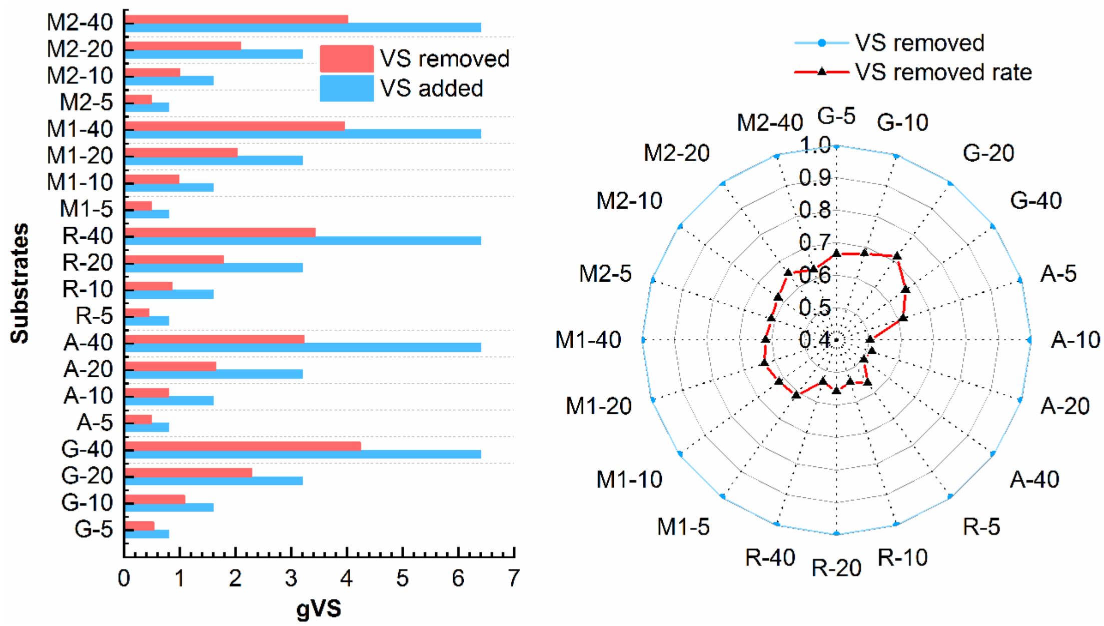

Fig. 4. VS removal rate during AD

BMP and Kinetic Analysis of Cumulative Methane Yield

The BMP of different perennials varied with different substrate concentrations (Fig. 5).

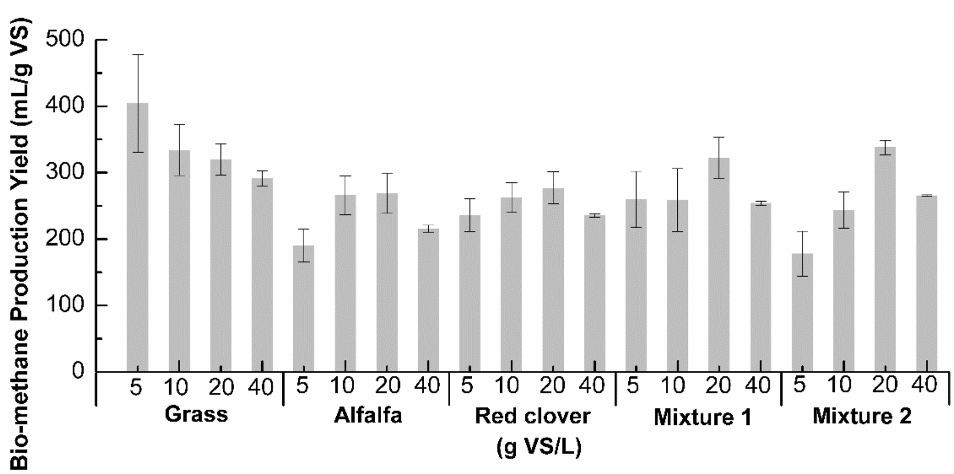

Fig. 5. BMP at different feedstock concentrations

The BMP of the grass group gradually decreased along with the increasing substrate addition, with peak at 5 g VS_{added}/L for 404.64 mL/g VS_{added}. However, the BMP of legumes seemed a little different, since it reached the highest BMP at 20 g VS_{added}/L. In a real biogas plant, high solid concentration and high BMP would increase efficiency and make a profit. Therefore, lifting the feedstock concentration while attaining relatively high BMP was worth being investigated. In this study, co-digestion was employed to achieve comparatively high BMP in 20 g VS_{added}/L. This may be attributed to the diverse composition of each kind of perennial. The legumes contained more nitrogen with lack of hemicellulose, whereas grass comprised more hemicellulose, which was easily degraded and lacked nitrogen nutrition to some extent.

Kinetic analysis was applied to describe tendency of methane or biogas production and predict the ultimate cumulative methane production, as has been considered in many of previous papers (Körber *et al.* 2022; Pardilhó *et al.* 2022). Through investigating the parameters given by these selected kinetic equations, some microorganism activities can be revealed and explained along with the changing trend of intermediate products and metabolic pathways (Donoso-Bravo *et al.* 2011a). Due to different kinetic equations that had distinguished parameters, which can be seen in the same process in various ways, using different kinetic curves was necessary.

The cumulative methane yield and process parameters of all five groups in 20 g VS_{added}/L are depicted in Fig. 6 and Table 2, respectively.

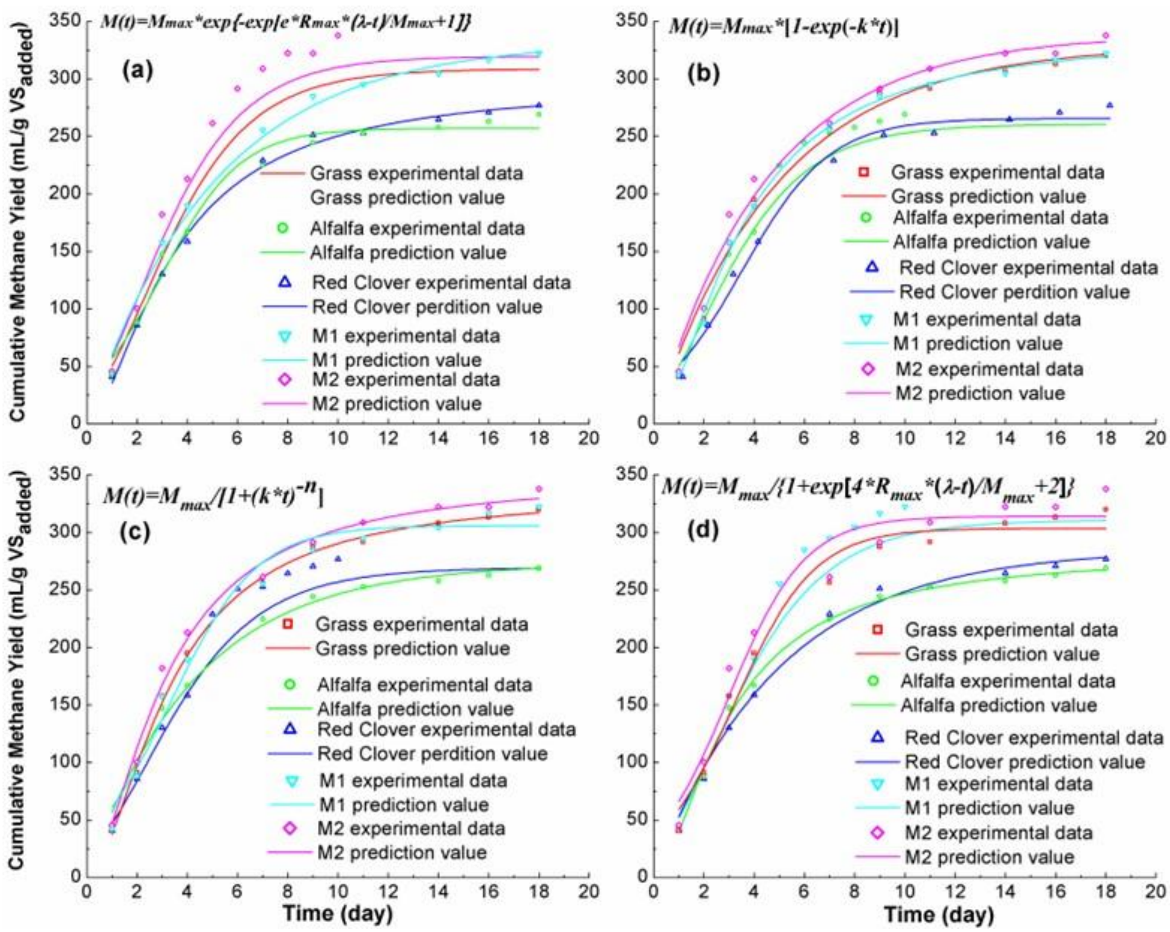

Fig. 6. Experiment data and fitting curves of cumulative methane yield

Visually, these curves could be divided into two clusters. The top ones were M2, M1, and grass group, which acquired more cumulative methane yield through the process, whereas legumes alfalfa and red clover group achieved less BMP. This result was in agreement with daily methane production discussed before, with the higher BMP group having the higher methane peak. Generally, fitting curves with higher R^2 but lower AIC manifested the better performance and credibility (Li *et al.* 2018). Therefore, the Cone model, with calculating the maximum R^2 and lowest AIC, had the best fitting condition (Table 2).

For ultimate maximum methane yield M_{max}, the results of the modified Gomperz and Logistic models were both lower than practical BMP for 0.17% to 5.31%, whereas the ultimate BMP should be higher than practical ones because of a limited operational time. Hence, the M_{max} obtained from First order and Cone models were more convincing. Lag time (λ) represented the anaerobic set-up time in some measures, which demonstrated the methane producing speed of certain substrate (Donoso-Bravo *et al.* 2010). Because the methane production was directly related to the activity of microorganisms living in anaerobic environment, λ was also an indicator for inhibition cumulation. Noticeably, the λ calculated by the modified Gomperz and Logistic models were negative except for the G20 group, which was usually ignored and considered zero in previous studies (Li *et al.* 2018), indicating that the readily bio-degraded soluble material in liquid phase was rapidly consumed by microorganisms. All the perennials were meshed into tiny particles, and this pretreatment could cause the nutrient concentrations to rise quickly. On the contrary, the λ of G20 displayed that mono-digestion of grass in 20 g VS_{added}/L had already been inhibited slightly at the start of the test. From the VFAs concentration, changing analysis as discussed above, this outcome may be attributed to the imbalance of propionic acid and acetic acid as well as excess total VFAs concentration. Daily maximum methane production rate (R_{max}) denoted the efficiency of anaerobic digestion, with higher R_{max}, the methane production rate could be faster. That meant that, in real biogas plant operation, much more methane production would be acquired in a given time. Both modified Gomperz and Logistics models showed that M2-20 had the highest R_{max}, indicating that co-digestion improved the efficiency of anaerobic digestion of legumes. The first order hydrolysis rate constant k was 0.2 to 0.23 (no units) for the First order kinetic model and 0.29 to 0.33 for the Cone model. Despite the difference between the two models, the k value of all five groups changed regularly. With A20 and M2 group having higher k value than others, it assumed that alfalfa, which accounted for 100% and 20% of A20 and M2-20, was easy to be pyrolyzed. The variety of k values may depend on the compositions of these substrates. Similar results were also proved by Dandikas *et al.* (2014).

CONCLUSIONS

In this study, the biomethane potential (BMP) of three different perennials and the mixtures in various feedstock concentrations were determined. The grass group had the highest methane yield of 405 mL/g of volatile solids addition (VS_{added}) at 5 g VS_{added}/L, whereas the alfalfa and red clover groups reached their highest methane yield of 269 and 277 mL/g VS_{added} at 20 g VS_{added}/L, respectively. The increasing feedstock concentration at lower range (5 to 20 g VS_{added}/L) resulted in the increase of the cumulative methane production. When feedstock concentration rose higher to 40 VS_{added}/L, however, the cumulative methane production decreased due to acidic conditions and ammonia. The VS removal efficiency of mono-digestion of legumes was 60% lower than grass and co-digestion of grass and legumes. Kinetic analysis of cumulative methane yield in 20 g VS_{added}/L showed that co-digestion of 20% alfalfa and 80% grass (M2) group reached the maximum at 338 mL/g VS_{added}, with improving VS removal efficiency of 9.08% in comparison to mono-digestion. In addition, according to the coefficient of determination R^2 and the Akaike information criterion (AIC), the Cone model had the best fitting performance. The results suggested that the appropriate co-digestion of perennials can improve methane production by lowering acidic and ammonium inhibition. The conclusions generated in this work can supply a co-digestion strategy for anaerobic digestion of lignocellulose fermentation. However, in-depth study on materials conversion and microbial interaction mechanism of lignocellulose fermentation is needed in future research.

ACKNOWLEDGMENTS

The authors are grateful for the support of Science and Technology Project of China Electric Power Construction Group Co., Ltd. (DJ-ZDXM-2018-29), The Fifth "Young Talents Support Project" of Chinese Society for Electrical Engineering (JLB-2020-181) and The Young Talent Project of “Xi'an Talent Program” (XAYCQN21002).

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Article submitted: November 7, 2022; Peer review completed: December 15, 2022; Revised version received and accepted: January 3, 2023; Published: January 17, 2023.
DOI: 10.15376/biores.18.1.1742-1756