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| **Title** | Improving methane production from Pennisetum hybrid by monitoring plant height and ensiling pretreatment |
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| **Publication date** | 2019-03-18 |
| **Original citation** | Zhang, Y., Li, L., Kang, X., Sun, Y., Yuan, Z., Xing, T. and Lin, R. (2019) 'Improving methane production from Pennisetum hybrid by monitoring plant height and ensiling pretreatment', Renewable Energy, 141, pp. 57-63. doi: 10.1016/j.renene.2019.03.084 |
| **Type of publication** | Article (peer-reviewed) |
| **Link to publisher's version** | http://www.sciencedirect.com/science/article/pii/S0960148119303891<br>http://dx.doi.org/10.1016/j.renene.2019.03.084<br>Access to the full text of the published version may require a subscription. |
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| **Embargo information** | Access to this article is restricted until 24 months after publication by request of the publisher. |
| **Embargo lift date** | 2021-03-18 |
| **Item downloaded from** | http://hdl.handle.net/10468/7857 |

# Improving methane production from *Pennisetum* hybrid by monitoring plant height and ensiling pretreatment

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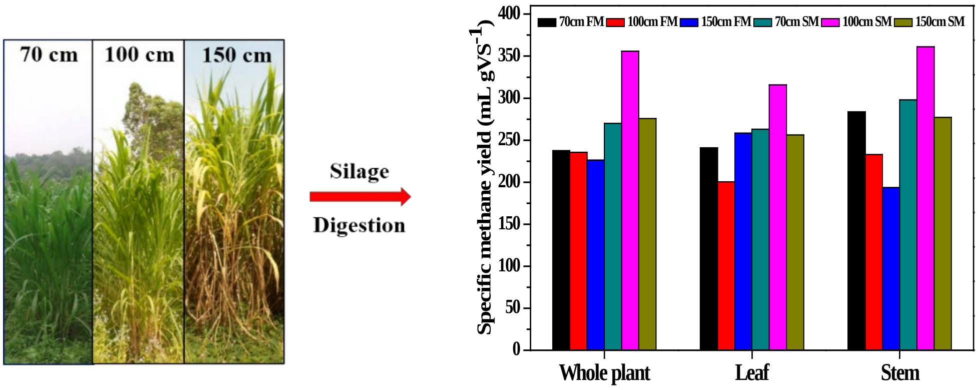
**Abstract:** The biomass of grass-based *Pennisetum* hybrid commonly use for abiogas production via anaerobic digestion. However, it is necessary to determine a method to optimize the plant harvest time for high biogas production. Moreover, ensiling of biomass in the presence of diverse microbes may offer a solution to improve biogas production. In this study, whole plant of *Pennisetum* biomass (including stems and leaves) was collected at different harvesting time (plant heights of 70, 100, 150 cm), and then comparatively assessed for further ensiling and biogas production. Compared to leaves, stems exhibited a significant linear relationship ($R^2 = 0.99$) with whole plants in terms of ensiling quality (i.e. pH and $NH_3$-N). Microbial analysis further revealed that *Lactobacillus* was the dominant bacterial genus during ensiling of stems and whole plants, with the highest relative abundance of 50.08% obtained at the height of 100 cm. Ensiling of biomass at a height of 100 cm achieved the best digestion performance, with the methane yields of 316 ± 20 mL/g VS for leaves, 361 ± 43 mL/g VS for stems, and 356 ± 28 mL/g VS for whole plants. A harvesting time at the plant height of 100 cm was the optimal from the silage quality and anaerobic digestion performance.

**Keywords:** *Pennisetum* hybrid biomass; plant height; ensiling; *Lactobacillus* bacteria; anaerobic digestion, methane.

## Graphical Abstract



## 1. Introduction

*Pennisetum* (subfamily: Panicoideae, tribe: Paniceae) is a genus of C4 grasses that are widely grown in Europe and Asia. [1] *Pennisetum* sp. is economically feasible and recommended as a promising feedstock for anaerobic digestion, due to its huge biomass yield and high organics content. [2-4] The annual *Pennisetum* biomass yield was reported as 88 MT/ha, 210 t/ha of which were produced in China. [5] The organic components of *Pennisetum* biomass are mainly composed of cellulose (40–60%) and hemicellulose (20–40%), which can be easily degraded and used in biological process.[1]

However, the use of *Pennisetum* biomass may not be straightforward. Plant harvest time is important for anaerobic digestion performance, because the chemical composition of grass varies with its growth stage. [6,7] For example, the specific methane yields of *Pennisetum* hybrid and switchgrass (*Panicum virgatum*) decreased from 280 to 119 mL/g VS, [8] and from 266–309 to 191–250 mL/g VS as crops matured.[,9] Lehtomaki et al.[10] observed that harvesting at a younger stage was optimal for Napier grass (*Pennisetum purpureum*) because it could achieve a higher specific methane yield, whereas marrow kale (*Brassica oleracea* var. *medullosa*) and Jerusalem artichoke (*helianthus tuberosus*) were optimal at a later harvest, which could obtain higher biomass yields. Dragoni et al. [11] reported that harvesting in September might be the most feasible option for *Phragmites australis*. Similarly, the optimal cutting time for *Miscanthus* was between September and October. [12] In addition, Surendra and Khanal [13] obtained a maximum methane yield of 219 ± 4.9 mL/g VS for *P. purpureum* harvested at 2 months old. Overall, the optimal harvest time varies by species, growth conditions, maturity stage, and planting area. Therefore, establishing a simple method to determine the harvest time is necessary to enhance methane production.

Furthermore, the rigid cell wall structures in biomass are strongly recalcitrant to microbial degradation. Therefore, it is critical to pretreat the *Pennisetum* hybrid to improve the specific methane yield. Compared to various pretreatments of biomass, ensiling is a commonly used technology that can destroy the structure of cellulose and hemicellulose, and preserve the nutrient component as effectively as possible. [14-18] High quality silage can recover 87‒98% of methane yield on the basis of methane potential of the biomass. [19] Vervaeren et al. [20] observed the process of silage could effectively improve anaerobic digestion performance with an increase 10.1‒14.7% of biogas production.

However, to the best of our knowledge, few researches were reported about combining the aspect of harvest time and ensiling pretreatment to enhance methane production from *Pennisetum* hybrid. Therefore, the present study aimed to (1) improve the silage quality and anaerobic digestion performance by comparing grass at different heights; (2) evaluate the leaf and stem parts in whole plant to study the primary influencing component of the silage process and conversion efficiency; and (3) conclude the feasibility of determining the optimal harvest time by monitoring plant height.

## 2. Methods

### 2.1 Grass materials and inoculum

The biomass, *Pennisetum* hybrid, was sown in Zengcheng district, Guangzhou, China. The *Pennisetum* hybrid planting spacing is 60 cm × 12 cm, and the planting area is 1000 $m^2$ (50 m × 20 m). Samples were collected at January 14, 2016, the corresponding grasses at heights of 70 cm, 100 cm, and 150 cm were selected for the study. 5-10 strains were randomly selected from the experimental base for each castration, leaving 10 cm for growth. Before processing the grass, the quality of fresh whole plant was weighted. For the comparison of the main factors for determining the silage quality and anaerobic digestion performance, some of the raw materials were separated and classified into leaves and stems, whereas other materials were classified as whole-plant samples.

The inoculum for the anaerobic digestion was obtained from continuously stirred tank reactors operated in the lab. The total solids (TS) and volatile solids (VS) contents of the inoculum were determined as 3.44% and 1.43%, respectively.

### 2.2 Experimental setup and procedure

The fresh materials were cut into small pieces of 2-3 cm, pulverized, and then stored at -20℃ in a refrigerator until further use. The silage materials were prepared in a plastic silo bag. For the ensiling process, about 200 g of fresh sample was placed in a bag, vacuumed-sealed, and then ensilaged at ambient temperature for 30 d. After ensiling processing, the silage samples were crushed and then stored at -20℃ in a refrigerator for spare. Each treatment was performed in triplicate.

The batch anaerobic digestion experiments were carried out using an automatic methane potential test system (AMPTS II, Bioprocess Control Sweden AB) at 35 ± 1 ℃., the total and working volume of reactor was 500 mL and 400 mL, respectively. In this process, 400 mL of inoculum were used, and the ensiling material was added based on the VS of substrate/inoculum ratio of 1. The experiments were performed in triplicate and were run for 30 d.

## 2.3 Analytical methods

The TS, VS, pH, total ammonia nitrogen concentration ($NH_3$-N), carbon (C), and nitrogen (N) analyses were performed according to previously published methods. [16] To determine the microbial community composition in silages of different materials, the collected samples were stored at -20℃ until the analysis. Microbial characterizations were based on the method of 16s rRNA high throughput sequencing. The microbial DNA was extracted, amplified, and analyzed according to a previously described method. [21]

## 2.4 Kinetic analysis

The modified Gompertz equation (Eq. (1)) was used for the kinetic analysis [22]:

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

where $M$, $P$, $R_m$, and $\lambda$ represent the cumulative methane yield (mL/g VS) at a given time, methane production potential (mL/g VS), maximum methane production rate (mL/g VS d), and lag phase (d), respectively.

## 3. Results and discussion

### 3.1 Chemical composition of the materials

*Pennisetum* hybrid as the feedstock for anaerobic digestion mainly includes the parts of stem and leaf, Table 1 presents the TS, VS, C, N, and C/N contents of the stem and leaf in the whole plant obtained at different conditions. Fresh and silage samples typically exhibited significant differences in terms of TS, VS, and N contents. Moreover, samples of different plant parts derived from various plant heights (i.e. 70, 100, and 150 cm) also contributed to different chemical compositions. For the fresh materials, the TS contents increased from 13.91 ± 1.09% to 23.11 ± 1.65% in the whole plant, from 18.13 ± 0.10% to 25.73 ± 1.08% in leaf, and from 11.97 ± 0.57% to 23.07 ± 0.03% in stem as the plant height increased. The increase in the TS and VS contents of *Pennisetum* hybrid showed a positive correlation with plant height. These results could be due to the total lignocellulose (including cellulose, hemicellulose, and lignin) content increased with crop maturity. [21] Moreover, leaf had the highest TS and VS contents, whereas stem had the lowest TS and VS contents in different samples of plant height. No significant difference was observed in the C contents among different biomass parts and heights; however, the highest N content was obtained in leaf and the lowest N content in stem. Correspondingly, the C/N values were higher in stem than those in leaf. Similar trends were previously observed by Erickson et al. [24] and Han et al., [25] who reported that the N concentration in sorghum leaf was higher than that in the stem. For the silage materials, the content of TS, VS, and N contents had a decrease compared to the fresh samples, whereas the corresponding C/N values showed an increase. Moreover, the N content in whole plant silage materials decreased from 0.98 ± 0.02% to 0.64 ± 0.01% with the plant height from 70 to 150 cm. The reason was that the process of ensiling could degrade carbohydrates and proteins into minor molecular such as volatile fatty acids (mainly including lactic acid, acetic acid, and propionic acid) and amino acids. [16] In addition, the lowest TS and VS contents were observed in the plant height of 100 cm with different plant parts. Similarly to the fresh materials, higher TS, VS, and N contents were observed in the leaf silage samples, corresponding to lower C/N values.

**Table 1.**

Figure 1 presents the pH values and $NH_3$-N concentrations in the silage samples of the stem, leaf, and whole plant. In agreement with the N contents of stem, lower $NH_3$-N concentrations of were obtained for stem silage samples. Meanwhile, lower pH values of 4.15–4.49 were observed in the stem silage samples. By contrast, the leaf silage samples had higher pH values of 4.73–5.54, which increased with plant height from 70 to 150 cm. In addition, the $NH_3$-N concentrations in whole plant silage materials decreased from 44.50 ± 0.64 mg/L to 14.00 ± 0.98 mg/L with the plant height from 70 to 150 cm. Nousiainen et al. [26] reported that a negative association was observed between the decreasing crude proteins contend and the certain stage of plant maturity. And the decreasing $NH_3$-N concentrations in the increasing heights of whole plant silage materials were similar to the results of ammonia nitrogen in the dairy cow fed silages harvested at four stages of grass maturity.[27] The pH values of the stem and whole-plant silage samples were similar to the so-called critical pH values (range: 4.10–4.45) for silage samples at the dry matter of 15–30%.[28] In order to understand the role of plant part in the silage samples, the correlations of pH values and $NH_3$-N concentration between the silages of stem, leaf, and whole-plant was analyzed in the Figure 2. In a comparison of the pH values among the silage samples, a positive linear relationship between stem and whole plant was observed, following the equation: $y= 7.8226-0.7983x$ ($R^2 = 0.9987$). However, a negative linear relationship between stem and whole plant was obtained by comparing the $NH_3$-N concentrations of silage samples, and the equation was $y= -3.5975+1.3736x$ ($R^2 = 0.9994$). Although the same linear relationship between leaf and whole plant was observed by comparing the pH value and $NH_3$-N concentration of silage samples, there were not significant linear correlation of pH ($R^2 =0.0805$) and $NH_3$-H ($R^2 = 0.3601$) between the leaf and whole plant. In addition, the stem accounted for over 60% of the content of fresh whole plant. Therefore, these results suggested that the part of stem had a greater effect than the leaf on the silage quality of the whole plant.

**Figure. 1**

**Figure. 2**

### 3.2 Bacterial community structure

Figure 3 presents the bacterial communities in the raw material and silage samples.

The dominant bacterial compositions at the levels of phyla and genera were similar among the fresh materials. The dominant bacteria were *Cyanobacteria/Chloroplast*, with a relative abundance of 71.03–94.86%, and the major genus was *Streptophyta*, with a relative abundance of 71.03–97.96%.

In the silage samples, a dramatic shift in the bacterial compositions at the phylum level was observed in comparison with those in the fresh materials. The relative abundance of *Cyanobacteria/Chloroplast* decreased to 0.72–28.27%, whereas *Firmicutes* (36.26–80.72%) and *Proteobacteria* (6.05–40.79%) became the dominant bacteria at the phylum level after ensiling. Remarkable differences in the relative abundance at the phylum level were observed among the stem, leaf, and whole-plant parts. Most sequences at the phylum level assigned to the genera *Streptophyta*, *Lactobacillus*, *Lactococcus*, *Raoultella*, *Enterobacter*, *Enterococcus*, *Leuconostoc*, *Serratia* and *Weissella*.

The most dominant at the phylum level was *Firmicutes*, and a higher relative abundance of *Firmicutes* was obtained in the stem and whole plant. Desirable functional bacteria in silage include *Lactobacillus, Enterococcus*, and *Lactococcus*, which are used widely as silage additives. [29] These bacteria belong to a major part of the lactic acid bacteria group, which could convert sugars to lactic acid. [30, 31] Since lactic acid was one of the main metabolic intermediates (VFAs) in process of anaerobic digestion, it could easily utilize by the acetogenic bacteria and methanogens. [32,33] For the stem silage samples, the relative abundance of *Lactobacillus* sp. ranged from 36.41% to 50.08%, reaching a maximum at a height of 100 cm, while the relative abundance of *Lactococcus* sp. decreased from 27.40% to 1.61% as height increased. This was coupled with an increase in the relative abundance of the genus *Enterococcus*. In the leaf silage samples, the relative abundance of *Lactobacillus* sp. ranged from 1.27% to 39.60%, reaching a maximum at a height of 150 cm, while the variations in the relative abundance of the genera of *Lactococcus* and *Enterococcus* were similar to those in the stem. In the whole plant, the dominant genera differed by height. For example, *Lactobacillus* was the primary genus at a height of 150 cm, while relative abundances of 37.62% (*Lactobacillus* and *Lactococcus*) and 46.70% (*Lactobacillus* and *Enterococcus*) were obtained for the silage samples at heights of 70 cm and 100 cm, respectively.

The other most abundant at the phylum level was *Proteobacteria* (6.05–40.79%), the genera of *Raoultella* and *Enterobacter* predominated in this phylum. The relative abundance of *Raoultella* in silage samples increased from 1.08% to 9.36% in stem and from 0.71% to 30.73% in leaf, while the relative abundance in the whole plant ranged from 2.42% to 24.52%. *Enterobacter* had a relative abundance of 0.55–30.57%. *Enterobacter* and *Raoultella* have been shown to be deleterious microorganisms during the ensiling process. [34, 35] Because these bacteria could largely consume sugars and other simple compounds in ensiling process [34, 35] it is not beneficial to produce more methane for anaerobic digestion. The lowest relative abundance of *Enterobacter* and *Raoultella* in whole pant samples was obtained at a height of 100 cm. Overall, the plant height of *Pennisetum* hybrid harvested at 100 cm for ensiling not only had the highest relative abundance of desirable functional bacteria (*Lactococcus*, *Lactobacillus* and *Enterococcus*), but also had the lowest relative abundance of deleterious bacteria (*Enterobacter* and *Raoultella*) for ensiling. Therefore, these results suggested that grass harvested at a plant height of 100 cm could improve the quality of silage.

**Figure. 3**

### 3.3 Anaerobic digestion performance

Figure 4 and Table 2 present the cumulative and specific methane yields of fresh and silage materials. For the fresh materials, the specific methane yields decreased from 238 ± 12 mL/g VS to 226 ± 8 mL/g VS for the whole plant and from 263 ± 5 mL/g VS to 194 ± 10 mL/g VS for stem as height increased. Meanwhile, the 80% cumulative methane yield was obtained at 9 d for the stem and whole plant at heights of 100 cm and 70 cm, respectively, but required 10–14 d for samples at a height of 150 cm. The specific methane yields of leaf ranged from 206 ± 5 mL/g VS to 258 ± 6 mL/g VS. Ensiling decreased the time required to obtain an 80% cumulative methane yield to 7–12 d for different parts of grass. Moreover, an increased specific methane yield was observed in the silage samples, and their specific methane yields were in the range of 263.17-298.04 mL/g VS, 315.75-361.25 mL/g VS, and 256.23-277.11 mL/g VS for the plant height of 70 cm, 100 cm, and 150 cm, respectively. The maximum specific methane yield of 316 ± 20 mL/g VS for leaf, 361 ± 43 mL/g VS for stem, 356 ± 28 mL/g VS for whole plant was obtained at a plant height of 100 cm. Since the lignocellulosic structure of *Pennisetum* hybrid was disrupted by the desirable functional bacteria in the process of ensiling, it could be efficiently converted into biogas by the microorganisms of anaerobic digestion. [32, 33] In addition, the samples harvested at plant height of 100 cm had a better silage quality by the bacterial community analysis. Similar specific methane yield results have been reported elsewhere. For example, the methane yields for tall fescue, cocksfoot, and reed canary grass were between 238 mL/g VS and 446 mL/g VS depending on N fertilization and harvest frequency. [36] Moreover, specific methane yields of 135 mL/g VS and 185 mL/g VS were reported for switchgrass and giant cane, respectively. [37, 38] The better performance of biogas production was observed in the silage samples of the plant height 100 cm for preferred bacteria community. These results suggested that harvesting plants at a height of 100 cm might be suitable for biogas production from the perspectives of silage quality and anaerobic digestion performance.

The regression analysis showed satisfactory overall agreements between the experimental data and the model, with high regression coefficients ($R^2 > 0.94$) (Table 2 and Figure 4). More methane production potential and higher maximum methane production rate were observed in the silage samples. Similar result was observed in anaerobic digestion of the silage *Pennisetum purpereum* with molasses-processed wastewater addition. [21] The stem, leaf, and whole plant from plants harvested at a height of 100 cm were associated with a higher methane production potential and maximum methane production rate compared with the silage samples harvested at heights of 70 cm and 150 cm. It indicated that the silage samples harvested at a height had a better anaerobic digestion performance than the other ensiling samples. These predicated results of the model were consistent with the specific anaerobic digestion performance of the silage samples harvested at the height of 100 cm. A negligible lag time (λ) was obtained for the fresh and silage samples. Allen et al. [39] reported the biochemical methane potential of hay grass varied from 156 mL/g VS to 433 mL/g VS for first cut baled silage, and the kinetics analysis showed the similar results of the methane production potential and lag time. According to the results of the specific methane yields and the bacterial community analysis in the ensiling samples, the optimal harvesting time at the plant height of 100 cm and the pretreatment of silage showed a positive effect on the anaerobic digestion performance of the energy grass.

**Table 2.**

**Figure. 4**

## 4. Conclusions

The height of *Pennisetum* hybrid at which it was harvested was demonstrated to have significant effects on silage quality and the subsequent anaerobic digestion. The results from silage quality of different materials concluded a linear relationship between the stem and whole plant. Microbial community analysis revealed that *Lactobacillus* was the dominant genus in stem silage, and reached the maximum at harvesting height of 100 cm. This suggested that the stem had a primary influence on the silage quality. The maximum specific methane yield was 356 ± 28 mL/g VS for the whole plant at a height of 100 cm, indicating that a harvesting height of 100 cm could be the optimal from the perspective of silage quality and biogas production.

## Acknowledgments

This collaborative Chinese Irish work was financially supported by the National Natural Science Foundation of China [grant number 51776208], the Strategic Priority Research Program of Chinese Academy of Sciences [grand number XDA21050400], the Science and Technology Planning Project of Guangdong Province [grant number 2017A050501049] and the Science and Technology Program of Guangzhou [grant number 201707010201]. Dr Richen Lin gratefully acknowledges the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 797259.

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**Table captions:**

**Table 1.** Characteristics of the fresh and silage materials of *Pennisetum* hybrid.

**Table 2.** Anaerobic digestion performance and kinetic parameters of the samples of *Pennisetum* hybrid.

**Figure captions:**

**Figure. 1.** The parameters determining the silage quality of different samples: (a) pH values, (b) $NH_3$-N concentrations.

**Figure. 2.** The correlations of (a) pH values and (b) $NH_3$-N concentration between the silages of stem, leaf, and whole-plant.

**Figure. 3.** Bacterial compositions at the (a) phylum and (b) genus level of the samples of *Pennisetum* hybrid. (Note: JX: Stem of fresh materials, YX: leaf of fresh materials, HX: whole plant of fresh materials; JQ: stem of silage samples, YQ: leaf of silage samples, HQ: whole plant of silage samples)

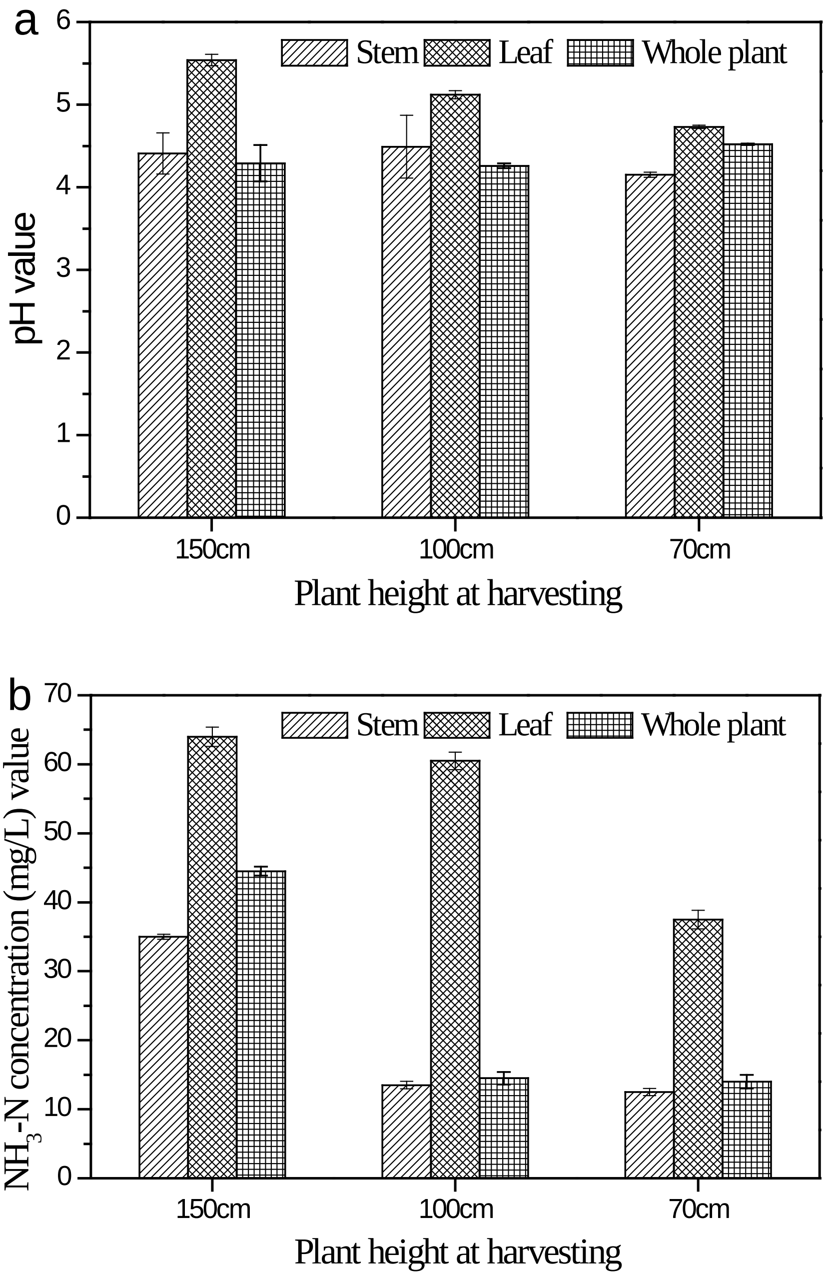
**Figure. 4**. Comparison of the cumulative biogas yields from the samples of *Pennisetum* hybrid.

**Table 1.** Characteristics of the fresh and silage materials of *Pennisetum* hybrid.

| | | | TS（%） | VS（%） | C（%） | N（%） | C/N |
|---|---|---|---|---|---|---|---|
| Fresh material | 70cm | Whole | 13.91±1.07 | 11.79±0.86 | 39.14±0.01 | 1.08±0.01 | 36.24±0.46 |
| | | Leaf | 18.13±0.09 | 15.44±0.30 | 40.44±0.14 | 1.43±0.01 | 28.28±0.18 |
| | | Stem | 11.97±0.57 | 10.51±.64 | 39.72±0.11 | 0.48±0.11 | 83.62±1.02 |
| | 100cm | Whole | 14.58±0.53 | 12.56±0.28 | 39.89±0.11 | 0.88±0.01 | 45.59±0.50 |
| | | Leaf | 18.37±0.46 | 16.11±0.50 | 40.61±0.08 | 1.50±0.01 | 27.16±0.07 |
| | | Stem | 11.92±0.49 | 10.56±0.61 | 39.83±0.10 | 0.51±0.00 | 78.10±0.19 |
| | 150cm | Whole | 23.11±1.65 | 20.29±2.34 | 40.98±0.09 | 1.03±0.01 | 39.79±0.46 |
| | | Leaf | 25.73±1.08 | 22.04±1.33 | 41.03±0.11 | 1.35±0.01 | 30.55±0.08 |
| | | Stem | 23.07±0.03 | 21.22±0.03 | 41.84±0.05 | 0.43±0.01 | 98.45±1.75 |
| Silage material | 70cm | Whole | 15.09±0.52 | 12.34±0.29 | 40.72±0.06 | 0.98±0.02 | 41.77±0.97 |
| | | Leaf | 18.82±0.25 | 15.77±0.48 | 40.83±0.22 | 1.34±0.01 | 30.58±0.00 |
| | | Stem | 11.79±0.27 | 10.00±0.32 | 40.74±0.06 | 0.54±0.01 | 75.46±2.09 |
| | 100cm | Whole | 13.72±0.35 | 11.55±0.34 | 39.59±0.04 | 0.92±0.02 | 43.28±0.96 |
| | | Leaf | 16.82±0.78 | 13.52±0.64 | 40.48±0.04 | 1.26±0.01 | 32.13±0.39 |
| | | Stem | 10.58±0.71 | 9.00±0.60 | 41.12±0.16 | 0.54±0.00 | 76.19±0.29 |
| | 150cm | Whole | 22.05±0.86 | 18.81±0.86 | 41.28±0.05 | 0.64±0.01 | 64.51±1.50 |
| | | Leaf | 28.52±0.56 | 23.83±0.81 | 41.00±0.16 | 1.41±0.05 | 29.20±0.92 |
| | | Stem | 18.32±3.31 | 16.04±3.47 | 41.79±0.05 | 0.36±0.01 | 117.73±2.21 |

**Table 2.** Anaerobic digestion performance and kinetic parameters of the samples of *Pennisetum* hybrid.

| Samples | | | Anaerobic digestion performance （mL/g VS） | Kinetic parameter | | | |
|---|---|---|---|---|---|---|---|
| | | | | P (mL/g VS) | $R_m$ (mL/g VS d) | Λ (d) | $R^2$ |
| Fresh material | 70 cm | Whole | 237.62 | 232.27 | 29.35 | 0.33 | 0.996 |
| | | Leaf | 240.90 | 235.87 | 29.87 | 0 | 0.995 |
| | | Stem | 283.60 | 273.97 | 33.15 | 0 | 0.988 |
| | 100 cm | Whole | 235.67 | 226.60 | 29.66 | 0 | 0.990 |
| | | Leaf | 200.40 | 197.12 | 20.69 | 0.10 | 0.998 |
| | | Stem | 232.85 | 224.00 | 30.38 | 0 | 0.988 |
| | 150 cm | Whole | 226.37 | 219.68 | 23.15 | 0 | 0.984 |
| | | Leaf | 258.34 | 249.32 | 31.28 | 0 | 0.987 |
| | | Stem | 193.70 | 194.04 | 13.31 | 0 | 0.988 |
| Silage material | 70 cm | Whole | 270.04 | 267.75 | 47.23 | 0.57 | 0.999 |
| | | Leaf | 263.17 | 259.43 | 35.26 | 0.45 | 0.998 |
| | | Stem | 298.04 | 293.52 | 46.45 | 0.34 | 0.997 |
| | 100 cm | Whole | 355.77 | 350.56 | 43.41 | 0.39 | 0.997 |
| | | Leaf | 315.75 | 312.90 | 40.74 | 0.82 | 0.999 |
| | | Stem | 361.25 | 353.73 | 46.25 | 0.11 | 0.993 |
| | 150 cm | Whole | 275.73 | 271.60 | 21.95 | 0 | 0.983 |
| | | Leaf | 256.23 | 248.56 | 29.63 | 0 | 0.982 |
| | | Stem | 277.11 | 271.72 | 23.13 | 0 | 0.981 |



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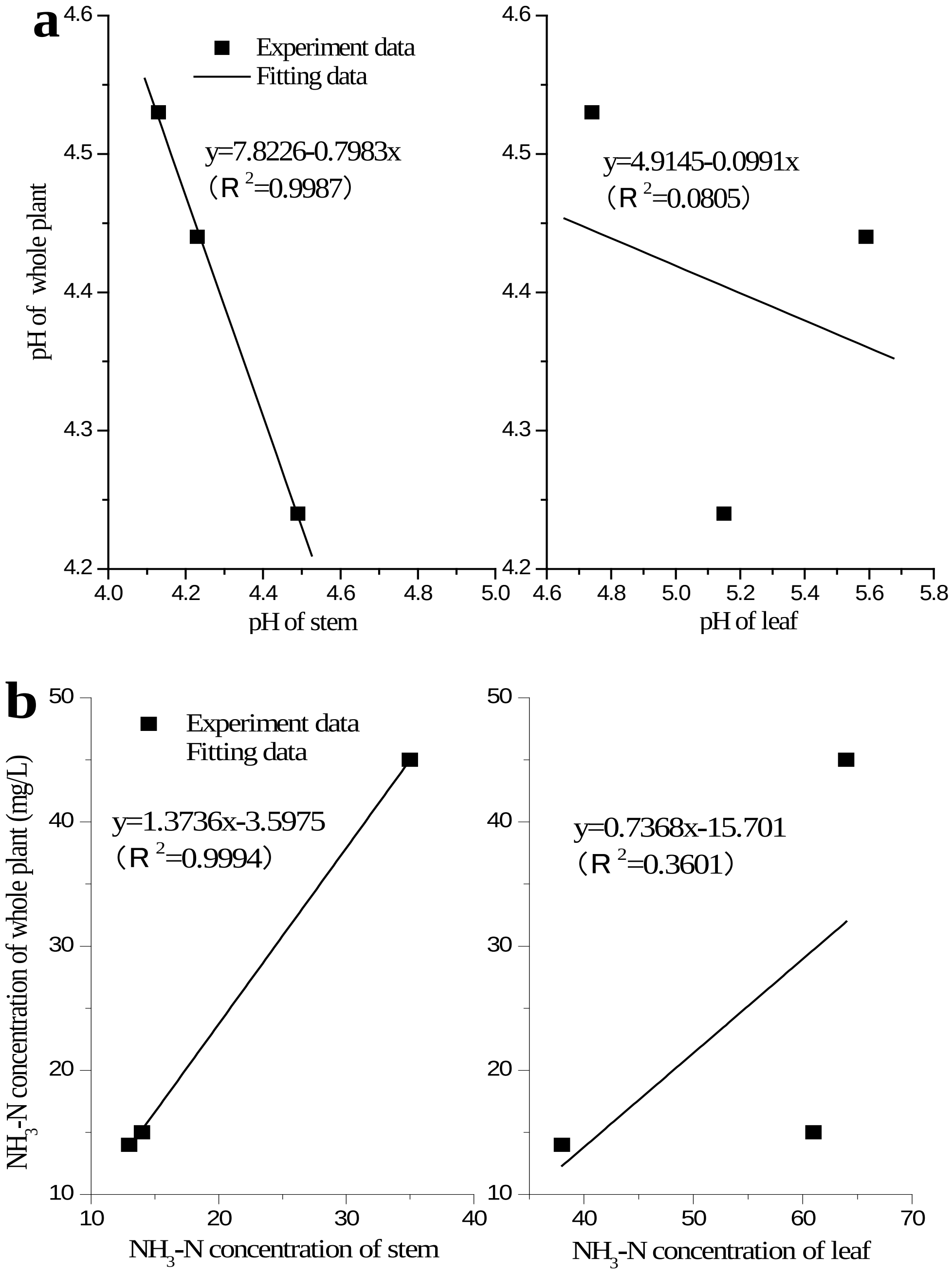

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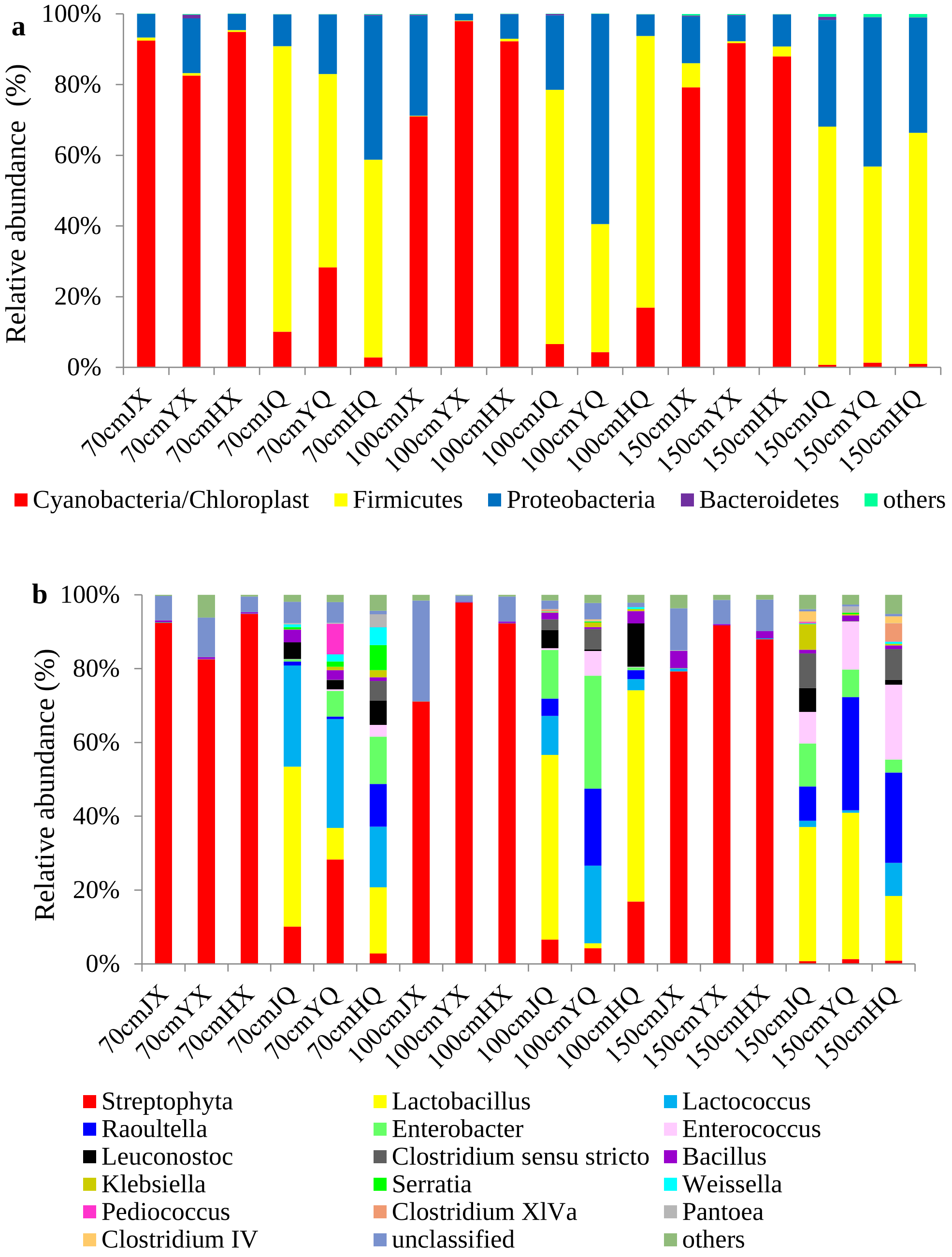

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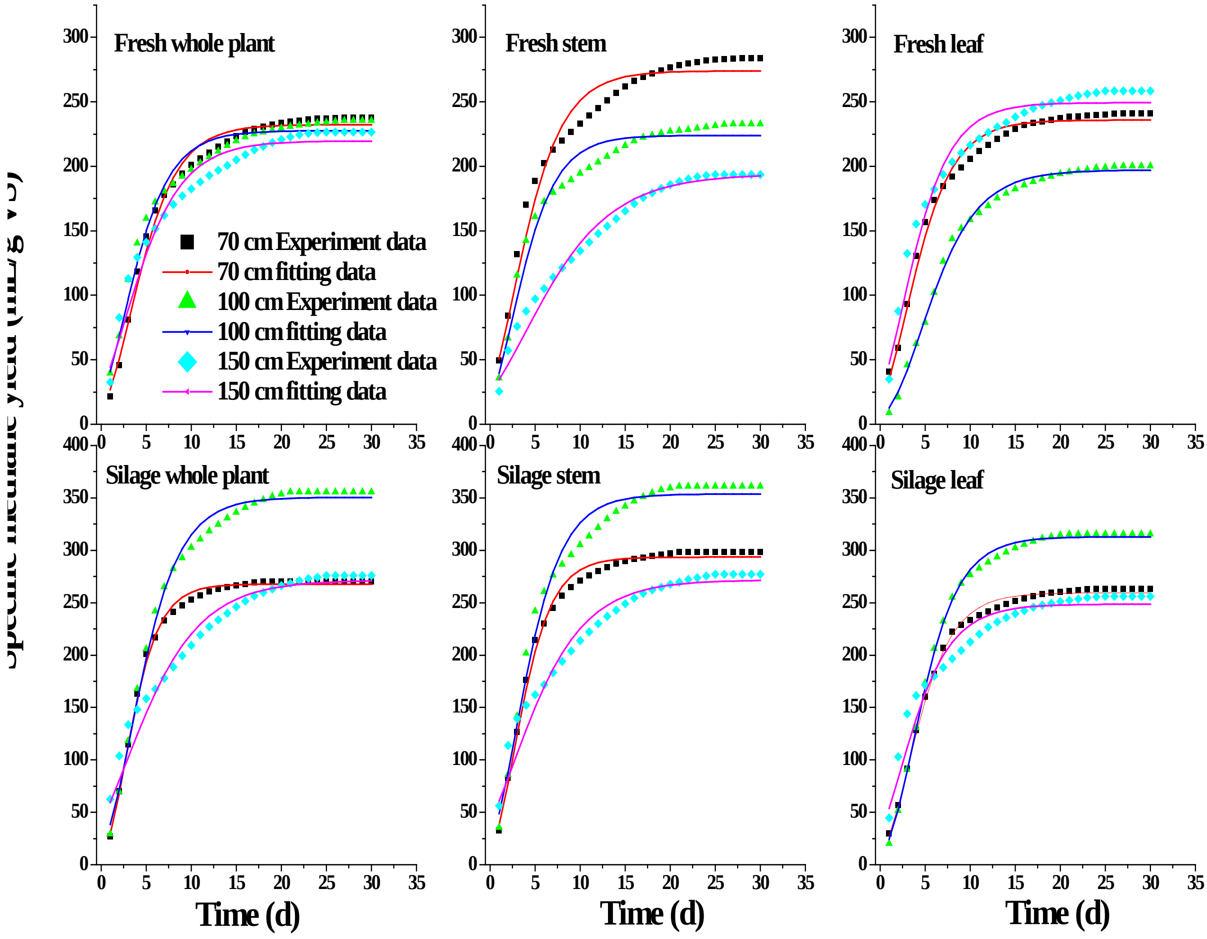

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