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Application of Rumen Microorganisms for Enhancing Biogas Production of Corn Straw and Livestock Manure in a Pilot-Scale Anaerobic Digestion System: Performance and Microbial Community Analysis

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Abstract: This study aimed to assess the feasibility of rumen microorganisms inoculated in a modified pilot-scale system for enhancing biogas production of (1) solely corn straw (CS) and (2) CS with livestock manure under different solid contents and mixture ratios. The biogas liquid was proven to pretreat CS at this scale. The digestion system was started up within 32 days at a retention time of 20 days. The rumen culture was found to have a positive response to the impact on temperature and pH. The optimal solid content of CS was detected to be 3%, resulting in a stable biogas yield of 395 L kg^{-1}·total solid $(TS)^{-1}$. A higher biogas yield of 400 L kg^{-1}·TS^{-1} – 420 L kg^{-1}·TS^{-1} was achieved at a solid content of 10% organic loading rate (OLR, 4.42 kg volatile solid (VS) m^{-3}·d^{-1}) in co-digestion systems with CS and livestock manure. The methane content could be maintained at about 60%. Hydrogenotrophic methanogens were dominated by *Methanobacterium* in the solely CS digestion system, and two methanogenetic pathways, including hydrogenotrophic and acetoclastic methanogens by *Methanosarcina* and *Methanobacterium*, co-occurred for methane production during the co-digestion of CS with pig manure (PM). This study indicates that rumen microbes could be utilized in a pilot-scale digestion system and that they greatly promoted the biogas yield.

Keywords: rumen microorganism; anaerobic digestion; pilot-scale test; corn straw; livestock manure

1. Introduction

Agricultural solid organic wastes (ASOWs), which are produced as a result of agricultural activities performed by human beings, have been confirmed as one of the main factors influencing the sustainable development of China. Among ASOWs, lignocellulosic biomass and livestock manure have been given wider attention due to their large yield, resource waste, and serious environmental pollution [1]. According to statistics, an estimated 598 million tons of crop straw were produced in 2014, including 472 million tons of collectable straw [2]. The yield of livestock manure reached 2.121 billion tons in 2011, which will continue to increase rapidly to 2.875 billion tons by 2020 and 3.743 billion tons by 2030 [3]. Crop straw, accounting for 38.9% of total biomass resources, is considered an ideal candidate for clean energy production [4], whereas the annual pig manure (PM) accumulation of 1.036 million tons caused 2.5 times the emissions as open straw burning in China due to improper treatment [5].

Several methods are currently used to treat and manage these wastes, such as anaerobic digestion, straw incorporation, aerobic composting, biomass power generation, pyrolysis, etc. [6–8]. Anaerobic digestion is one of the most widely used methods for its clean and positive production. Moreover,

the biogas produced from that amount of treated bio-waste could potentially solve the energy-crisis. Fundamental conditions, such as temperature and pH, are crucial for anaerobic degradation because they play a more important role in microbial metabolism, which would further influence the biogas yield [9]. It will be important to learn to control these two factors in a larger digestion system and to investigate the impact of temperature and pH on degradation performance. Furthermore, numerous studies have attempted to develop a feasible system with nitrogen-rich and carbon-rich materials as co-substrates to satisfy the applicable C/N ratio range of 20–30 [10].

Lignocellulosic biomass, consisting of cellulose, hemicelluloses, and lignin, is a naturally carbon-rich material. Restricted by its structure, including the crystalline cellulose, etc., a variety of pretreatment methods have been proposed and explored to increase the biogas yield of lignocellulosic biomass [11]. In a previous study, biogas liquid, as a residuum of anaerobic digestion, was adopted to pretreat maize straw in a laboratory-scale test, which achieved a similarly successful performance with other conventional physical and chemical methods. Because it is more economical and operational [12], the lignocellulosic waste pretreated by biogas liquid would have great potential for a wide application in anaerobic digestion projects if it can be verified in a larger-scale test.

Hydrolysis is the key process for achieving biological conversion of cellulosic materials. Rumen microorganisms in ruminants are a natural cellulose-degrading system, which are known to be able to effectively digest lignocellulosic biomass for a long period of time [13]. Not only in vivo but also in vitro, several culture resources have been applied as the inoculum for anaerobic bioconversion systems, demonstrating that rumen microorganisms are superior to other microbes for the hydrolysis and degradation of cellulosic substrates [14,15]. High yields of volatile fatty acids (VFAs) greater than 0.7 g $g^{-1}\cdot VS^{-1}$ with high degradation efficiency greater than 75% have been obtained [16,17]. In addition to bioconversion of cellulosic substrates, other systems, such as palm oil mill co-digested with rumen fluid or anaerobic acidification of papermill sludge, have shown a 90.3% COD removal rate and 62% neutral detergent fiber (NDF) degradation efficiency, respectively [18,19]. However, it is commonly neglected that 23–27% of the methane produced by human activities comes from ruminants on account of the 10^8–10^{10} g^{-1} methanogens in the rumen microbial ecosystem [20,21]. This indicates that rumen cultures not only have an ability to degrade cellulosic materials but also great potential to further convert VFAs into biogas. In this regard, research with respect to using rumen microorganisms as a single inoculum to produce methane and diversities of archaea have not been reported in a one-stage anaerobic digestion system for the bioconversion of ASOWs.

Moreover, it has not been demonstrated in any field whether rumen microorganisms are adoptable in pilot-scale production. Compared with lab-scale tests, pilot studies could not only identify potential practical problems in the research procedure, but also develop and test the adequacy of research instruments [22]. Thus, anaerobic digestion of corn straw (CS) and livestock manure by rumen microorganisms should be conducted to fulfill a range of important functions and provide valuable insights for other researchers.

This study aims to develop a high-efficiency and sustainable method for the application of rumen microorganisms in a pilot-scale anaerobic digestion system for enhancing the biogas production of CS and livestock manure. First, pilot-scale pretreatment of CS with biogas liquid was explored. After that, a system with solely CS and a system with CS and livestock manure were adopted to investigate (1) the optimal solid content of solely CS and (2) the effect of different substrates on the anaerobic digestion system. In this process, temperature, pH, method of starting up, VFAs production, biogas yield, etc., were examined to reveal the inherent relevance of these important factors for a better understanding of the ruminal digestion processes. Archaeal diversities and communities were developed to further reveal the generated mechanism of biogas. Finally, mass balance was made to evaluate the effectiveness of the conversion treatment process. The results from this study are expected to be helpful for designing and operating modified systems for the high-efficiency anaerobic digestion of ASOWs.

2. Results and Discussion

2.1. Pretreatment of CS with Biogas Liquid

The properties of pilot-scale pretreatment of CS by biogas liquid are discussed in the following section. Unlike the laboratory-scale test, during the initial period of pretreatment, the temperature increased rapidly to about 60 °C, followed by a gradual fall to 38 °C at the end of pretreatment. The results revealed that calorigenesis had been induced by the abundant microbial activity, where the microbes from the biogas liquid were widely distributed on the pretreated CS. Furthermore, the pretreated CS smelled like vinasse, indicating that some amount of alcohol had been synthesized. This production of alcohol from sugars or easily degradable materials during pretreatment was similar to the process of lignocellulosic forage pretreatment and bioalcohol production [23].

The extent of the relative crystallinity of the pretreated CS in the pilot-scale test was verified by powder X-ray diffraction patterns (Figure 1a,b). There was a decrease in the interplanar spacing of the d value as 2θ equal to 18 or 20, where the relative crystallinity of 59.4 ± 1.8% of the pilot-scale pretreated CS was similar to the laboratory-scale test of 57.5% but obviously lower than the untreated samples at 80%. After pretreatment, many obvious distinctions were detected between the untreated CS and the pretreated CS by FTIR spectra (Figure 1c,d). Specifically, the most effective changes for the following hydrolysis of lignocellulosic biomass were the weakness of absorbance at 1735 cm^{-1} due to the C=O stretch of the ester carbonyl group and the great intensity of the vibrations at 1335 cm^{-1} assigned to the O–H in-plane bending of lyocell and hydrolyzed lyocell [24,25]. This indicated that the connection structure between lignin and hemicelluloses had been destroyed and crystalline cellulose was converted into amorphous cellulose. It could be noted that the pilot-scale pretreatment test met expectations for accelerating the disruption of the structure of CS.

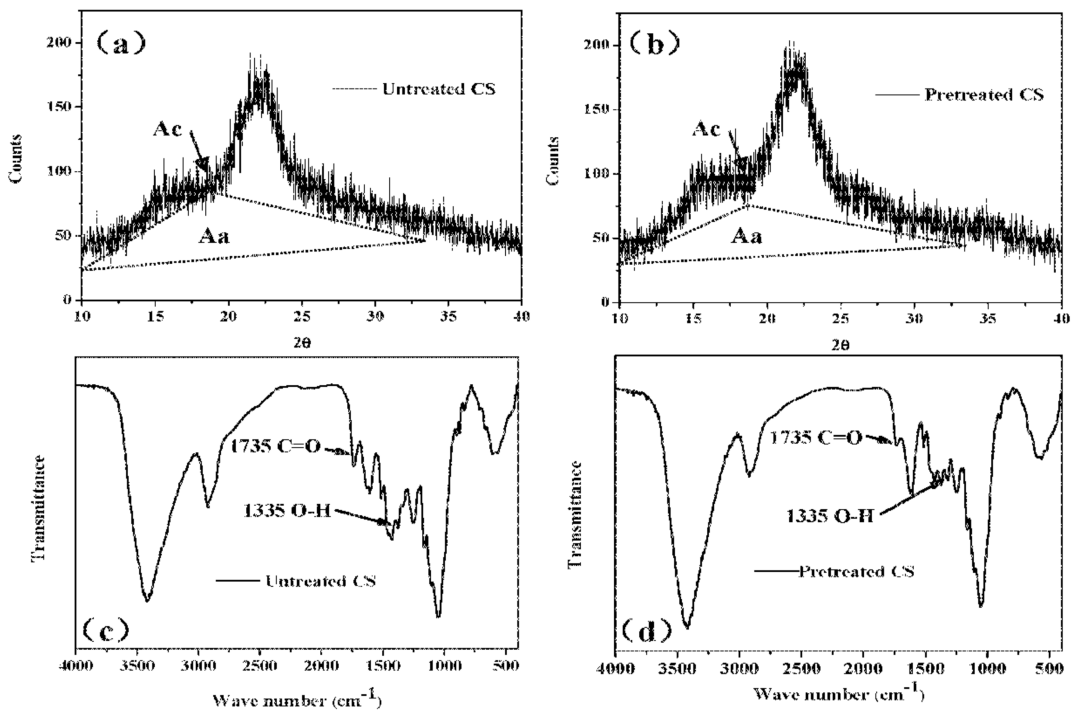

Figure 1. X-ray diffraction patterns and FTIR spectra of: (**a**,**c**) untreated corn straw (CS); and (**b**,**d**) pretreated CS with biogas liquid in the pilot-scale test. Ac and Aa represent the crystalline and amorphous portions of the X-ray diffractogram, respectively.

2.2. Temperature and pH Value

Figure S1 presents the temperature variation of the anaerobic digestion system. In the first three stages of the test, the electric tracing band was used to maintain the digestion temperature. However, the repetitive breakdown of this heating system resulted in a temperature fluctuation twice at 60 days and 85 days as a result of the weather turning cold suddenly. It was reported that use of biogas as a heat source is beneficial in terms of greenhouse gases and fossil fuel use [26]. In this case, the heating system was updated by using a biogas boiler for water heating together with rubber foam insulation material adhering to the external surface of the reactor. This modification was closer to the full-scale anaerobic digestion treatment plant [27], which could not only reduce the waste of energy but also lead to the stability of microbial metabolism.

The pH value of the biodegradation process was also explored (Figure S2). In stage I, 7.44 kg Na_2CO_3 and 19.7 kg $NaHCO_3$ were dissolved into the preheated substrate to avoid microbial acidosis, followed by the inoculation the rumen microorganisms. After adaption for a few days, the pH decreased to 6.1 within 7 days, during which the rumen microbial activity had recovered by achieving effective hydrolysis and acidogenesis. The pH value gradually stabilized at 6.8 and 6.9 in the end of stage I and stage II, respectively. It was demonstrated that the VFAs had been converted. Improving the solid content to 3%, the pH value decreased to 6.7 and stabilized at 6.5–6.6 in 80 days. Cellulose degradation increased with the pH from 6.0 to 7.5, whereas at a pH < 6.5, there was a negative effect on acidogenesis [28]. Therefore, the optimal solid content for the anaerobic digestion of carbon-rich materials was achieved at 3% without artificial factors. In consideration of the low C/N ratio of the CS, 2.6 kg of urea was added to the system to improve the nitrogen nutrient level of the digestion system. Nevertheless, unlike the fermentation cultures that could metabolize urea in rumen, the pH value increased up to 7.9 and still had to be adjusted with NaOH in stage IV at a solid content of 5% [29].

In stage V, livestock manure was co-digested with the CS. The pH value could not remain stable when 1% or 2% solid content of PM was mixed into the reactor. Specially, compared to the alkalescent cultures of the conventional anaerobic digestion systems, such as that in the literature by Zhou et al., at pH 7.01–7.25, a stable pH value was observed at 6.65 for the anaerobic digestion of both PM and CM by improving the solid content to 10% [30]. Regulating the ratio of substrates to 3:7, the pH value increased to 6.9 due to the high nitrogen content in CM. It was reported that a pH value of 6.5–8.2 was the optimal range for the growth of methanogens, and when the pH is below 6.6, the growth and methane productivity of methanogens is severely inhibited [31,32]. The lower pH value may be due to the fact that high-efficiency hydrolysis and acidogenesis occurred to degrade the high solid content CS with rumen microorganisms. It was demonstrated that these cultures were in accordance with the metabolic environment for cellulolytic microbes, acidogens, and methanogens.

2.3. VFAs Production

VFAs are a very useful index to reflect not only the microbial activity in the four phases of anaerobic digestion but also the comprehensive performance of degradation. Figure 2 presents the concentration of acetate, total volatile fatty acid (TVFA), the acetate/TVFA ratio, and the concentration of propionate, butyrate, and isobutyrate, respectively. During the initial days of starting up, acetate, propionate, and butyrate accumulation was observed up to 7560 $mg \cdot L^{-1}$, 500 $mg \cdot L^{-1}$, and 750 $mg \cdot L^{-1}$, respectively, followed by a sharp decline to 3600 $mg \cdot L^{-1}$, 220 $mg \cdot L^{-1}$, and 250 $mg \cdot L^{-1}$, respectively, at the end of stage I. The increase in VFAs indicated that the ruminal inoculum had adapted the environment to degrade the CS in vitro; meanwhile, the consumption for most VFAs proved that the methanogens had shown activity in utilization of VFAs.

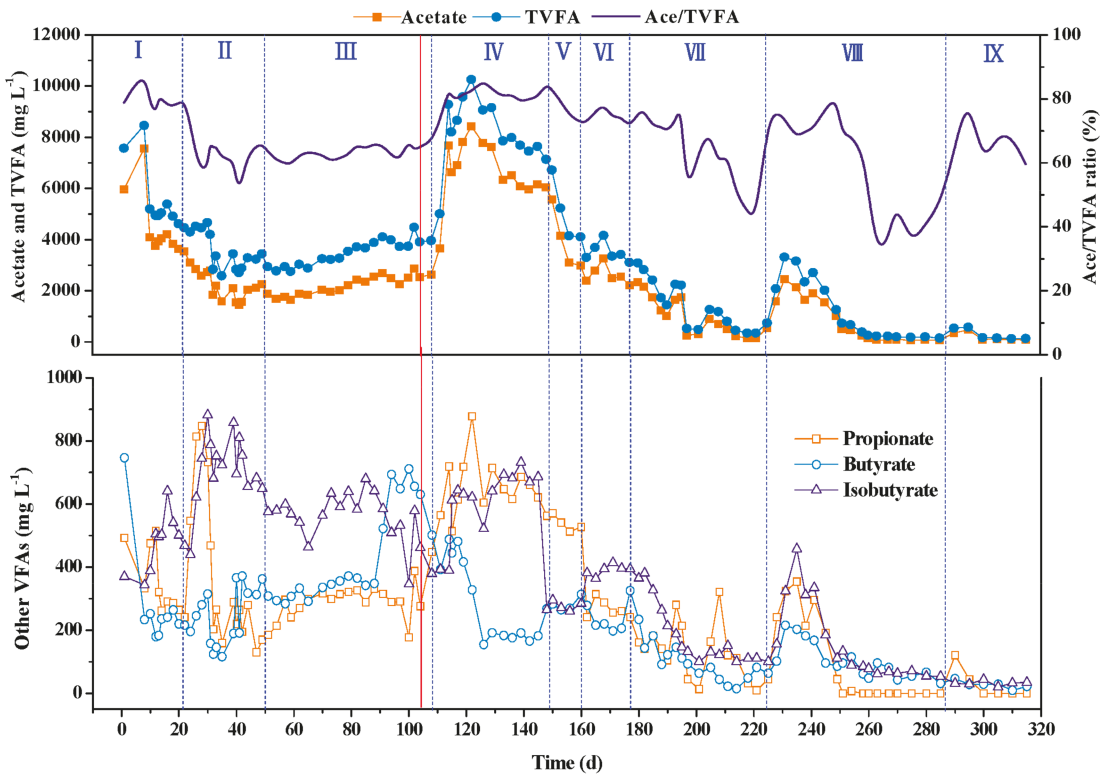

Figure 2. Concentrations of VFAs in the supernatant of anaerobic digestion. Ace = acetate.

The concentrations of VFAs, except isobutyrate, declined continuously in the process of stage II. Smooth growth of acetate, propionate, butyrate, and TVFA was obtained at 2000 mg·L^{-1}, 300 mg·L^{-1}, 350 mg·L^{-1}, and 3800 mg·L^{-1}, respectively by improving the solid content to 3%. The concentrations of VFAs were not affected by providing nitrogen to the digestion system. In order to investigate the performance on a higher solid content, 5% CS was added, which caused an obvious accumulation of VFAs, except butyrate. The concentrations of acetate and TVFA achieved at about 6200 mg·L^{-1} and 7500 mg·L^{-1}, respectively, at the end of stage IV were double and triple the concentrations of those with 3% solid content. Incorporating the tendency of the pH value, it could be verified that the VFAs could not be converted to methane completely in any metabolic pathway by methanogens, resulting in a maximum solid content of 3% for solely CS in the ruminal digestion system. Adequate branched-chain volatile fatty acids are required for the growth of most ruminal fiber-degrading microorganisms [33]. On the one hand, the opposite trend of isobutyrate during the first three stages may be due to the inhibition of microbial growth. On the other hand, high hydrogen partial pressure resulting from an accumulation of VFAs could decrease the conversion rate of long-chain fatty acids [34]. Overall, the activities of cellulolytic microbes and acidogens were not inhibited during any stage of part II, indicating that the micronutrients contained in the reactors and the C/N ratio could contend with such microbial survival conditions [35]. In other words, the accumulation of VFAs was mainly because of the low activity of the methanogens.

In stage V, part III, the livestock manure was added to the reactor, which formed a co-digestion system with the CS. A continuous decrease for all of the VFAs was detected with the addition of PM from 1% to 5% solid content. The concentrations of VFAs were similar to those with the 3% solid content CS digestion system in stage V and stage VI, indicating that the generated VFAs could not be entirely metabolized by the methanogens. The addition of PM was believed to provide buffer capacity and basicity to the anaerobic digestion system and improve the VFA consumption rate by methanogens [36]. In this context, the concentrations of VFAs, including acetate, propionate, butyrate, and isobutyrate, achieved at the end of stage VII were 150 mg·L^{-1}, 10 mg·L^{-1}, 150 mg·L^{-1},

and 100 mg·L^{-1}, respectively. This was in good agreement with the research by Kim et al. in which the reactor performance had achieved a steady state when the concentrations of VFAs were maintained at a lower level [37]. In conclusion, the solid content of this ruminal digestion system could be performed at 10% (i.e., a 1:1 mixing ratio of carbon-rich materials to nitrogen-rich materials).

CM was adopted to replace the PM to investigate the degradable properties of different manures in stage VIII. An apparent lack of adaptation was observed by the accumulation of VFAs. This process lasted for about 30 days, and then, the digestion system recovered to the previous status. Propionate was under the detection limit, and the concentrations of acetate, butyrate, and isobutyrate achieved were below 100 mg·L^{-1}, 60 mg·L^{-1}, and 50 mg·L^{-1}, respectively. Improving the solid content of CM to 7%, there was almost no fluctuation in the yield of VFAs. It could be deduced that this digestion system had an ability to resist the impact load.

Acetate is one of the main raw materials for synthesizing methane. Unlike fermentative culture in vivo, the acetate/TVFA ratio is more important than the acetate/propionate ration in a biogas production system [38]. It was demonstrated from the overall process of the pilot-scale test that when a phenomenon of inhibition of microbes or incomplete metabolism of intermediate products occurred during digestion, the acetate/TVFA ratio was above 0.6 in most cases. When the ruminal digestion system worked in good condition, the acetate/TVFA ratio decreased to nearly 0.4. This ratio may be considered an indirect index reflecting the properties of anaerobic digestion.

2.4. Biogas Yield

Figure 3 provides details of the total biogas yield and biogas per kilogram produced by the anaerobic digestion system. At the beginning of the inoculation and starting up, a relatively low yield of biogas was observed on account of insufficient methanogens in the original rumen microorganisms. The total biogas yield rapidly increased up to 410 L·d^{-1} on day 25 and then gradually stabilized at 500 L·d^{-1} on day 33. The biogas yield per kilogram was about 385 L kg^{-1}·TS^{-1}. Examining the biogas content at the beginning of degradation in Figure 4, it appeared that hydrogen was firstly detected in the reactor. The hydrogen liberated by hydrogenase enzymes arose from either phosphoroclastic reactions or nicotinamide adenine dinucleotide (NADH) during the drastic hydrolysis of CS by the rumen microorganisms [39]. Methane and carbon dioxide were detected almost at the same time on day 11. After a sharp increase from day 11 to day 25, the peak content of methane was achieved at 73.44%, followed by a gradual decline to about 60% on day 33. The variation tendency of methane was similar to the results of Zhao et al. [40], demonstrating that the acclimation of methanogens in the rumen microorganisms could be conducted within 25 days for biogas production. The content of carbon dioxide gradually rose up to about 40% on day 32. At this point, it could be identified from the stable biogas yield and biogas components that the ruminal pilot-scale anaerobic digestion system was operating successfully.

Remarkably, the production of biogas could be restored rapidly within 10 days after repairing the equipment in stage III, which implied that this improved system could resist the impact load of temperature. The final yields of total biogas and biogas per kilogram were 1550 L·d^{-1} and 395 L kg^{-1}·TS^{-1} at the end of stage III, respectively. A negligible impact on biogas production was realized after adding urea to the reactive system, whereas the biogas yield fell below 250 L·d^{-1} at a later improvement of the solid content of CS to 5%. Even though the biogas yield gradually recovered to 1494 L·d^{-1} and 230 L kg^{-1}·TS^{-1} on day 137, there was still a wide gap between the actual value and the predicted value. After that, no matter what feasible solutions were employed, such as adjusting the pH or supplying nitrogen nutrient, the biogas yield could not continue rising. It was found from the previous pH value that the ruminal digestion system had reached the ultimate solid content of CS at 3%. Further studies by Chandra et al. [41] proved that the high carbon content and low nitrogen content of CS led to a decrease in methanogenic activity, as also detected by the accumulation of VFAs at stage IV.

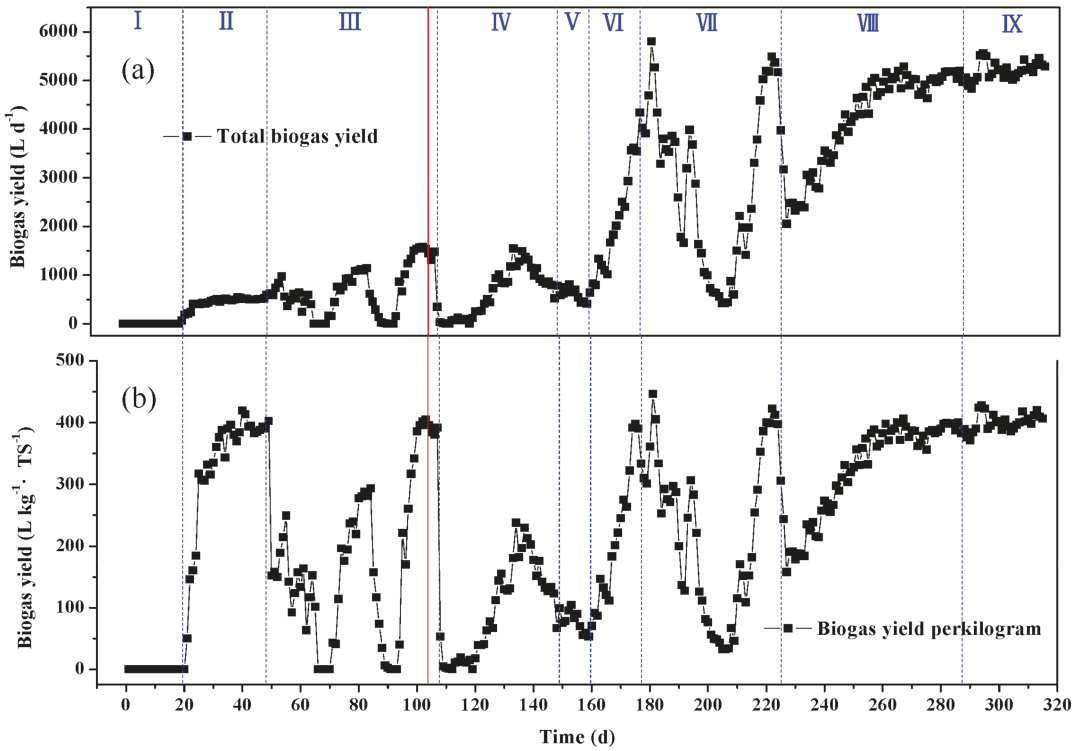

Figure 3. Biogas yield of the anaerobic digestion process in the pilot-scale test, (**a**) total biogas yield and (**b**) biogas yield per kilogram.

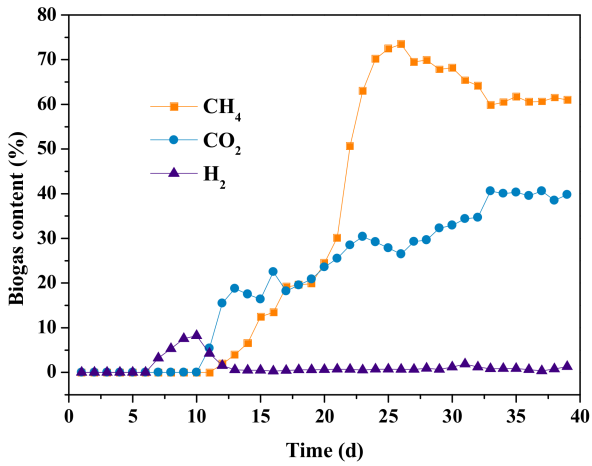

Figure 4. Biogas contents for the anaerobic digestion process in pilot-scale test.

In terms of a theory on ruminant nutrition, urea was used as the nitrogen source for improving the C/N ratio of this system [29]. Nonetheless, from the experimental results of gas production, the properties of anaerobic digestion were not significantly enhanced. The urea could be utilized by the rumen microorganisms, whereas most of the urea was converted into the alkalescent $(NH_4)_2CO_3$. Free ammonia nitrogen (FAN) was described as the primary cause of digester failure because of its direct inhibition of microbial activity [42]. However, according to an early study by Fernandes et al. [43], the highest FAN concentration was calculated at about 100 mg·L^{-1} in this period, which is far below the inhibitory levels. Additionally, the concentrations of accumulated VFAs, especially for propionate, had not reached the inhibitory concentrations, and the pH value was still at a suitable range for methanogenic metabolism [32,44]. Consequently, the inhibition of methanogens was not affected by

the simple superposition of various factors. How to improve the sole carbon-rich solid content in these ruminal digestion systems should be further studied. Despite these facts, the biogas yield from the anaerobic digestion of CS by the rumen microorganisms was still higher than other studies, such as where Yu et al. [45] compared six different digestates as inoculums and achieved a maximum biogas yield of 325.3 mL $g^{-1}\cdot VS^{-1}$ from digested dairy manure.

A sustained inhibition of anaerobic digestion was observed at the beginning of part II with the addition of PM. Subsequently, when the solid content of the process was improved to 7% (i.e., 3% PM and 5% CS), the biogas yield was then sharply increased to 3500 $L\cdot d^{-1}$ and 390 L $kg^{-1}\cdot TS^{-1}$, and methanogenic utilization of VFAs was re-established. The frequency of adjusting the pH was extended but still unable to work on self-adaption. With the further increase of the solid content of PM to 5%, unfortunately, a malfunction of the screw conveyor caused fluctuations of the biogas yield from the maximum 410 L $kg^{-1}\cdot TS^{-1}$ to the minimum 30 L $kg^{-1}\cdot TS^{-1}$ at least three times. After attempting to restore repeatedly, the screw conveyor was repaired on day 207, and then, the biogas yield rapidly rose to 415 L $kg^{-1}\cdot TS^{-1}$ within 13 days corresponding to a total biogas yield of 5200 $L\cdot d^{-1}$. In stage VIII, CM was used instead of PM. An obvious adaptation problem of the methanogens resulted in a 50% reduction of the biogas yield. Afterwards, it took about 30 days to recover the methanogenic activity and to observe the sustained production of biogas at 5100 $L\cdot d^{-1}$ and 400 L $kg^{-1}\cdot TS^{-1}$. Adjusting the mixing ratio of CM and CS to 7:3, there was not a conspicuous impact on the biogas yield as in a previous stage. On the contrary, a minor increase was detected at 5300 $L\cdot d^{-1}$ and 420 L $kg^{-1}\cdot TS^{-1}$. The biogas content was perturbed by the fluctuation of the system. However, it could recover to stable levels soon after being repaired, and then, the components of biogas including CH_4, CO_2, and H_2 could be maintained at 60%, 40%, and 0%, respectively.

Compared with the sole CS digestion system, anaerobic co-digestion of livestock manure and CS provided great enhancement not only of the biogas production properties but also of the solid content. These improvements may be due to two main factors. First, the solid waste from farm animals has been extensively investigated as an alternate source of concentrates to feed ruminants, with these wastes potentially supplying sufficient crude protein, nitrogen, and mineral element nutrition for ruminal cellulolytic microorganisms [46]. The pathogenic microorganisms and heavy metals in livestock manure have a negative effect on ruminal organs rather than rumen microorganisms [47]. Second, when using PM or CM as co-substrates, extra nitrogen including other nutrients could be provided to meet the needs of methanogens [10]. Mata-Alvarez et al. also published a critical review on anaerobic co-digestion achievements in recent years [10]. In comparison with the performance summarized in that article, the ruminal anaerobic digestion system had many advantages. First, the organic loading rate (OLR) of this study could be converted into more than 4.42 kg VS $m^{-3}\cdot d^{-1}$, which is higher than all the mesophilic co-digestion systems and closer to the maximum value of thermophilic co-digestion systems using cow manure and PM as the main substrate. The higher OLR reflected that the ruminal digestion system had excellent degradation efficiency and a rational retention time. Second, the mixture ratio of CS could reach 50% of the total solid (TS), which was relatively higher than other operations. It was noted that the factors restricting the large-scale addition of carbon-rich substrates to anaerobic digestion systems had been solved by this improved system. It potentially provides a sustainable technology for solving environmental pollution problems caused by straw, especially for developing countries. Finally, the most notable of these features was the biogas yield. Xie et al. [48] conducted a similar pilot-scale test of a continuous stirred tank reactor (CSTR), and they detected 251 mL CH_4 $g^{-1}\cdot VS^{-1}$ at an organic load rate of 1.74 kg VS $m^{-3}\cdot d^{-1}$ with a hydraulic retention time of 30 days. The methane yields obtained from the PM and CM co-digestion systems of this study were 276 L $kg^{-1}\cdot TS^{-1}$ and 267 L $kg^{-1}\cdot TS^{-1}$, respectively, which are relatively higher. Better performance in biogas production would bring more potential economic benefits, thus making large-scale application more beneficial.

2.5. Microbial Community Analysis

Unlike the original rumen fermentation system, most of the VFAs in this new established digester were converted to CH_4 and CO_2 by archaea rather than absorbed by the stomach wall. In this case, archaeal diversities and communities might be crucial to reveal the metabolic and generated mechanism of biogas. The operational taxonomic unit (OTU) numbers and alpha diversity, including ACE estimation, Chao1, coverage, and Shannon index were summarized in Table 1. All the coverage values were above 0.99, suggesting that most of the archaea were detected. The archaeal communities showed more complicated patterns in the co-digestion system than that in the original rumen archaea and the solely CS period, suggesting that both the digestion condition in vitro and the co-substrates had positive effects on richness of archaea.

Table 1. Richness and diversity indexes of archaeal communities under different conditions.

Sample	OTU	ACE	Chao1	Coverage	Shannon
Original rumen archaea	44	46	45	0.999608	0.97
Days 100 (solely CS)	74	82	83	0.999356	1.38
Days 220 (CS with pig manure (PM))	100	104	102	0.999335	2.4

As shown in Figure 5, the most abundant archaea in the genus level was *Methanobrevibacter* in the original rumen microorganisms, the relative abundance of which was 93.42%. Genus *Methanobrevibacter* is known to synthesize CO_2 and H_2 to CH_4 in ruminal fermentation systems, indicating that hydrogenotrophic pathway was dominant [49].

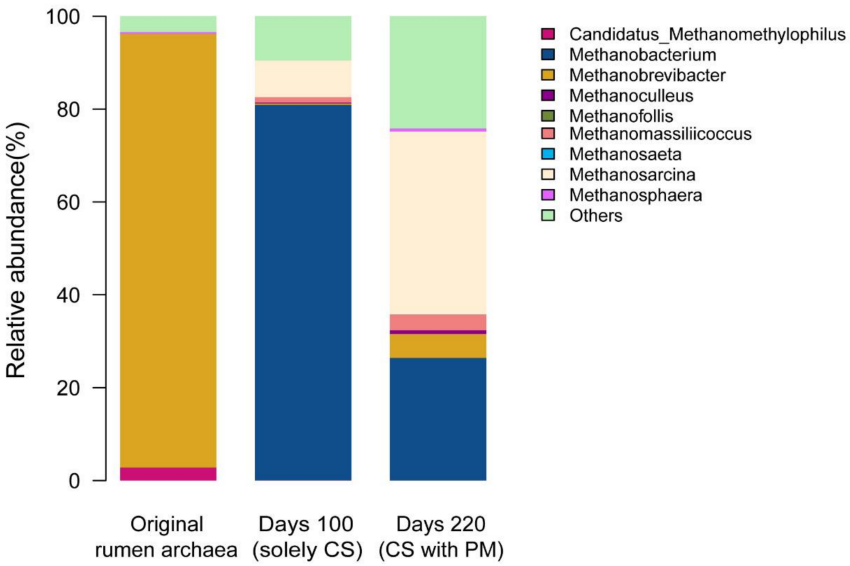

Figure 5. Composition of the archaeal community at the genus level.

Five genera of methanogens, *Methanobacterium* (80.91%), *Methanosarcina* (7.87%), and *Methanomassiliicoccus* (1.17%) were found in the solely CS digestion system. Compared with the original culture, the dominant hydrogenotrophic methanogenesis was shifted from *Methanobrevibacter* to *Methanobacterium*. This might be related to its higher VFAs content, in which VFAs concentrations of 50 mg·L^{-1} or higher would inhibit *Methanobrevibacter* [50]. This finding was surprising because the dominant genus *Methanobacterium* has been described to grow primarily at mesophilic temperatures (37–45 °C) [51]. A sharp increase in the relative abundance of the hydrogenotrophic methanogen *Methanobacterium* might be induced by the accumulation of VFAs presented in Figure 2, which would cause an increase in H_2 partial pressure [52]. This result was consistent with the findings

by Yan et al. [53], who reported that hydrogenotrophic methanogens were dominant at the stabilization stage in the solid-state anaerobic digestion of rice straw. Furthermore, the relative abundance of *Methanosarcina* indicated the acetoclastic pathway existing in this system.

In a later stage, acetoclastic methanogen, *Methanosarcina*, accounted for 39.35%, of the populations, and hydrogenotrophic methanogens, *Methanobacterium* and *Methanobrevibacter*, accounted for 26.42% and 5.15%, respectively, suggesting that two methanogenetic pathways co-occurred for methane production during the co-digestion of CS with PM. It is known that hydrogenotrophic methanogens are most common in the digestion of agricultural waste [54]. In contrast, we found more abundant acetoclastic methanogens than hydrogenotrophic methanogens. This discrepancy might be explained by two aspects. First, the decreased concentrations of VFAs caused by the co-digestion system seemed to reduce H_2 partial pressure and resulted in a decrease of the relative abundance of *Methanobacterium*. Second, the dominance of *Methanosarcina* is, however, in line with previous studies of manure-based digesters [55–57]. High abundance of this methanogen has been suggested to be caused by its relatively high growth rate and ability to tolerate conditions inhibitory to other methanogens, such as presence of ammonia [58].

2.6. Physical Form and SEM Images of Substrates

From an appearance point of view, the digestate was brown rather than black, which was due to a high mixture ratio of CS. After anaerobic digestion, the particle size of CS in the digestate decreased obviously, and the pulverized CS was degraded from 0.5–1 cm to indiscernible minced material. The SEM images in Figure 6 demonstrated that the compact structure of the fibers had been swollen and destroyed by the amounts of bacteria in association with fungus [59,60], indicating that many ruminal microbes were still viable by these stages. These procedures of adherence and degradation were similar to the metabolic system in rumen, further confirming the positive effect of rumen microorganisms on the pilot-scale anaerobic digestion [13]. In addition, a similar smell was noted between the digestate and rumen fluid, which showed from a side view that it had remarkable homology in vivo to the newly conducted pilot-scale system.

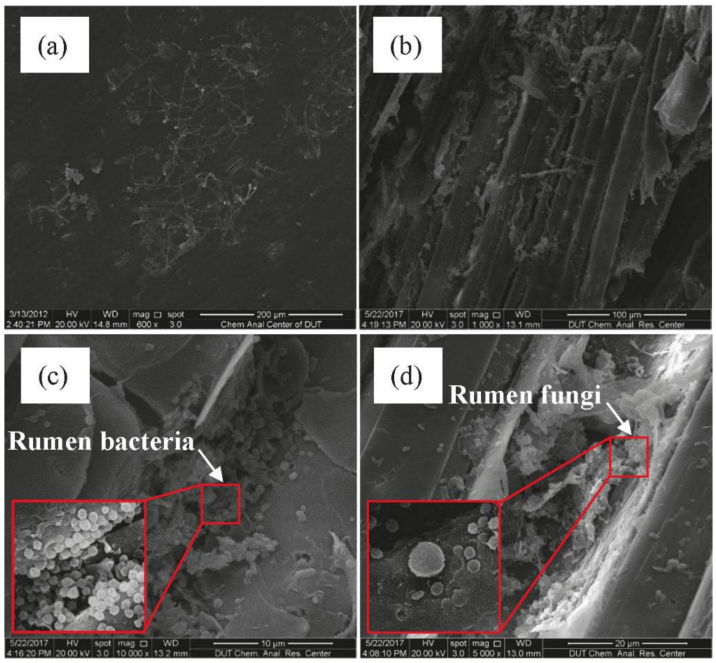

Figure 6. SEM images of: (**a**) undigested CS, (**b**) digested CS, (**c**,**d**) rumen microorganisms' adhesion on the surface of CS.

2.7. Mass Balance

Figure 7 shows the mass balances determined for the two typical and steady sections including stage III (solely CS at 3% solid content) and stage VII (1:1 mixture ratio of CS with PM at 10% solid content). As shown in Figure 7a, for the TS and VS mass balance, the input of solely CS was mostly converted into biogas, which achieved 72.56% and 77.35% removal rates, respectively. For the water, balance, the main pathway of the moisture in the feedstock included biogas liquid, biogas residue and gas–water separator. Biogas was used for the lamp and stove, resulting in 51.6% consumption. The mass balance and final fate of the product from the two sections were slightly different. The residual materials of the co-digestion system originated from two substrates, including the pretreated CS and PM, resulted in an improvement of the TS and VS removal rate to 76.38% and 79.33%. Due to the increase of biogas yield, the consumption rate declined to 46.88%.

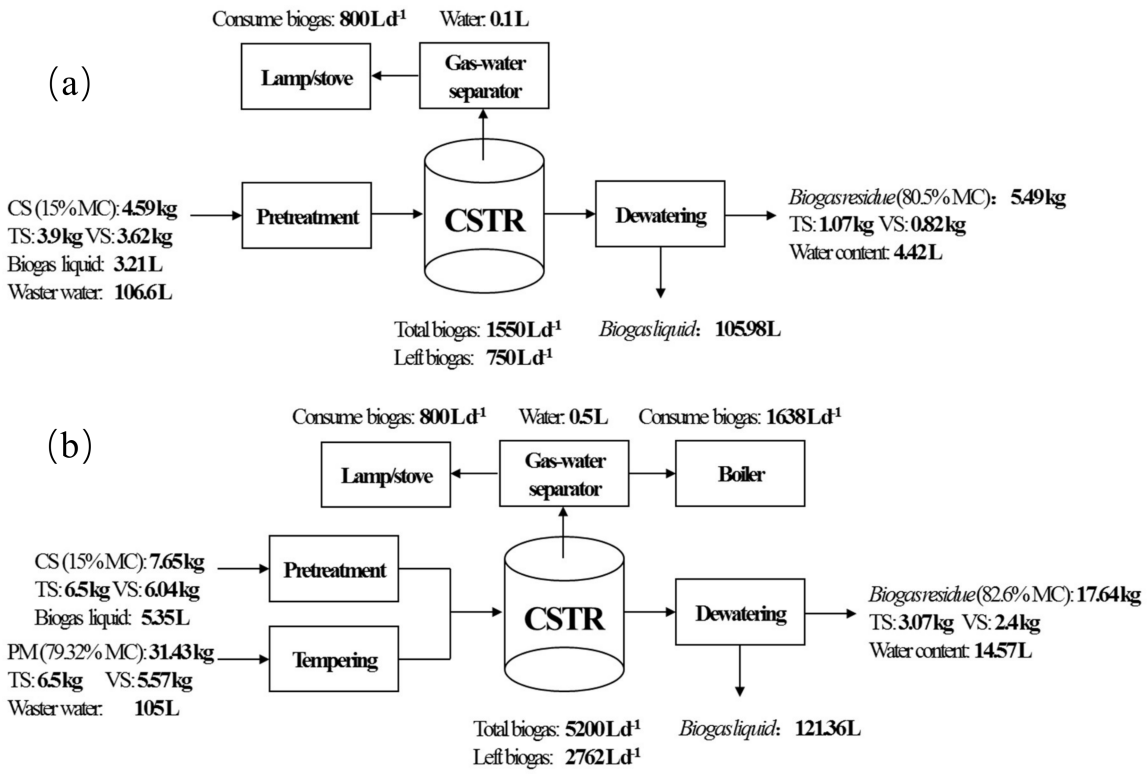

Figure 7. Mass balance of two sections, (**a**) stage III, solely CS at 3% solid content and (**b**) stage VII, 1:1 mixture ratio of CS with PM at 10% solid content (Moisture content, MC).

Furthermore, based on the calculation of removed VS, the biogas yield could be transformed into 553.57 L $kg^{-1} \cdot VS^{-1}$ removed and 564.6 L $kg^{-1} \cdot VS^{-1}$ removed. The similar conversion rate indicated that the rumen microorganisms had the same ability of degradation in both the solely CS digestion system and the co-digestion system. However, the accumulation of VFAs in stage III might imply that (1) the rumen microbes in co-digestion system needed more energy for self-growth or metabolism; and/or (2) a small quantity of intermediate products was not converted into biogas completed.

3. Materials and Methods

3.1. Inoculum and Substrates

Inoculum obtained from fresh bovine rumen in a slaughterhouse was poured into six pails (50 L, PVC) filled with N_2 for the anaerobic digestion system. This was conveyed to the pilot-scale test base immediately as the ruminal inoculum for starting up the anaerobic digestion reactor.

The naturally dried CS used as the carbon-rich material was collected from a farm near the test base. After being smashed to 0.5–1 cm pieces by a pulverizer, the CS was pretreated with biogas liquid coinciding with the method of Jin et al. in [12]. The nitrogen-rich materials were mainly composed of livestock manure, such as PM and CM, and they were obtained from a large-scale raising farm. The properties of the CS, PM, and CM are listed in Table 2.

Table 2. Characteristics of CS, PM, and chicken manure (CM).

Parameter	CS [b]	PM [b]	CM [b]
pH	-	7.89 ± 0.03	7.73 ± 0.01
Moisture content (%)	15 ± 2.2%	79.32 ± 0.05	73.36 ± 0.03
Volatile solid [a] (%)	92.90 ± 0.32	85.62 ± 0.08	80.67 ± 0.12
Total organic carbon [a] (%)	51.88 ± 0.82	43.57 ± 0.38	39.9 ± 0.29
NH_4^+–N (%)	-	0.85 ± 0.02	1.39 ± 0.05
Kjeldahlnitrogen [a] (%)	0.61 ± 0.13	3.28 ± 0.34	5.33 ± 0.42
C/N ratio	85.05 ± 0.48	13.28 ± 0.28	7.49 ± 0.36

[a] Dry weight basis. [b] Means ± S.E. (N = 3).

3.2. Experimental and Incubation Conditions

The pilot-scale experimental base had a total area of 2000 m^2. There was one mainstream and two sidestreams in this treatment system, including an anaerobic digestion unit, a pretreatment unit, and a biogas comprehensive utilization unit. The flow diagram and the schematic diagram of the technical system are shown in Figure 8.

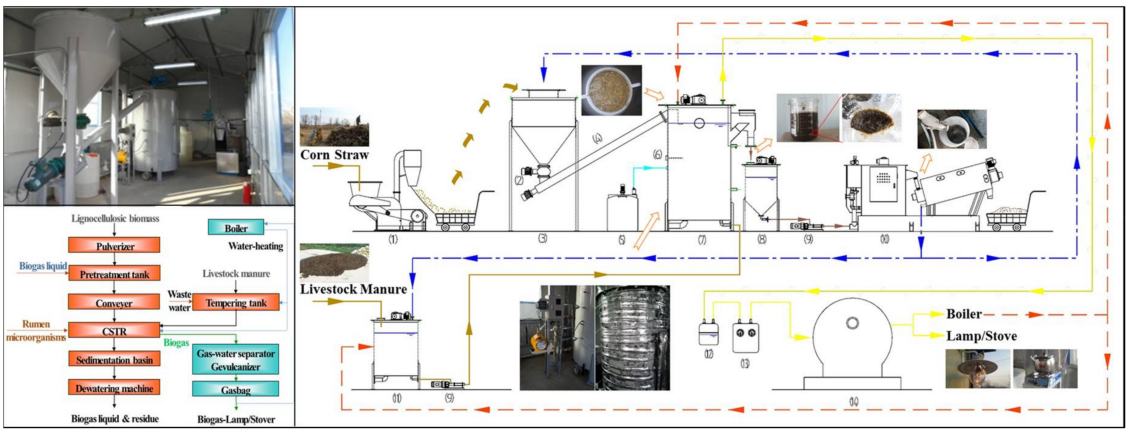

Figure 8. The flow and schematic diagram of the technical system including: (**1**) pulverizer, (**2**) unloader, (**3**) pretreatment tank, (**4**) conveyer, (**5**) pH adjustment bucket, (**6**) pH meter, (**7**) CSTR, (**8**) sedimentation basin, (**9**) screw pump, (**10**) screw type sludge dewatering machine, (**11**) tempering tank, (**12**) gas–water separator, (**13**) devulcanizer, and (**14**) gasbag. Pathway of substrates (——), digestate (——), pH adjustment (——), biogas (——), biogas liquid (— · —), and water-heating (——).

Above all, the smashed CS was mixed with biogas liquid in a tank for improved digestibility. After pretreatment, the CS was accurately weighed, and these materials were used as the carbon-rich substrate for the anaerobic digestion system. Separately, feeding substrates with different densities could significantly lower the risk of pipeline block. A screw conveyor system with a hopper, an unloader, and a conveyer was installed on the top of the anaerobic reactor for adding the CS into the tank directly. After desilting and regulating the water content of the livestock manure, the mixture was pumped from the tempering tank into the bottom of the reactor by a screw pump.

Subsequently, a CSTR, equipped with an online monitoring and controlling system for pH and temperature, was constructed at a volume of 3 m^3 for anaerobic digestion in this test. The biogas overflowing from the top of the reactor under the ordinary pressure passed through a gas–water separator and a devulcanizer and then was temporarily stored in a 3 m^3 gasbag. Some of the facilities were adopted for comprehensive utilization of the biogas, such as a biogas lamp for illumination, a gas stove for cooking, and a boiler for heat addition. The screw type sludge dewatering machine was connected with a sedimentation basin to separate the digestate into biogas liquid and biogas residue.

The working volume of the CSTR was 2.6 m^3, and 0.26 m^3 of ruminal inoculum with 2.1 m^3 preheated livestock wastewater (10% v/v) was loaded into the reactor. After that, the batch stirred blender was turned on according to the index of working time at 15 min h^{-1} and a stirring speed at 60 rpm. The initial pH was adjusted to 7.0 ± 0.1, while based on the experimental requirements, the pH was maintained above 6.5 by an automatic controller with 10% NaOH. A water-heating pipe assisted by an electric tracing band was wrapped around the external wall of the reactor and tempering tank to maintain the temperature at 39 ± 1 °C. The retention time was 20 days. The anaerobic digestion test was divided into three parts including nine stages to comprehensively investigate the properties of inoculating and starting up, as well as CS as the sole substrate and mixed substrates by rumen cultures. The experimental design is listed in Table 3.

Table 3. Experimental design for the pilot-scale anaerobic digestion test.

Experimental Procedure	Experimental Stage	Solid Content			Remarks
		CS	PM	CM	
Part I	Stage I	1%	-	-	Inoculating and starting up
Part II	Stage II	1%	-	-	Anaerobic digestion of the CS
	Stage III	3%	-	-	CS associating with urea addition
	Stage IV	5%	-	-	Limiting solid content of solely carbon-rich substrate
Part III	Stage V	5%	1%	-	Anaerobic co-digestion of CM with livestock manures
	Stage VI	5%	2%	-	
	Stage VII	5%	5%	-	
	Stage VIII	5%	-	5%	
	Stage IX	3%	-	7%	

3.3. Analytical Methods

The temperature and pH during the digesting process were detected by automatic sensors (NB2-ICSS-18G-12, OMEGA; PP-100A, InPro 3250, Weihong, Mettler, Zurich, Switzerland). An anticorrosion wet gas flow meter (LMF-2, Kesion, Qingdao, China) was applied to monitor the biogas yield of the anaerobic digestion. Biogas yield expressed in L $kg^{-1}\cdot TS^{-1}$ was calculated as the volume of biogas produced per kg of the feedstock TS added to the digester. Biogas components such as CH_4, H_2, and CO_2 were analyzed by a portable gas detector (ZR-3110, Junray, Qingdao, China).

Powder X-ray diffraction diagrams (XRD, D/max-2400, Rigaku, Tokyo, Japan) were employed to estimate the relative crystallinity of the pretreated CS [61]. The functional groups of pretreated maize straw were characterized by a FTIR spectrometer (EQUINOX55, Bruker, Karlsruhe, Germany). The morphological characteristics of the digested substrates were observed using scanning electron microscopy (SEM, QUANTA 450, FEI, Hillsboro, FL, USA). After being washed several times with phosphate buffer, the samples were fixed with 3.0% glutaraldehyde for 24 h at 4 °C. Subsequently, the experimental samples were dehydrated through a graded series of tertiary ethanol solutions (from 50% to 100%). Finally, the samples were evacuated by vacuum drying and gold-coated by a sputter.

Total solids (TS), moisture content (MC), and volatile solids (VS) were analyzed according to standard methods [62]. The digested samples were transferred to the laboratory as soon as possible in a cold closet. After centrifuging the samples at 12,000 rpm for 10 min (CT14TD, Techcomp, Shanghai, China) and filtering with a 0.45-μm membrane, the concentrations of VFAs,

including acetate, propionate, butyrate, and isobutyrate, in the samples were determined by a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and a 30 m × 0.53 mm × 0.1 μm fused-silica capillary column (DB-FFAP). The temperature of the injector and detector were maintained at 250 °C and 300 °C, respectively. The initial temperature of the oven was 70 °C for 3 min, followed by a ramp of 20 °C min^{-1} for 5.5 min, and the final temperature was 180 °C for 6 min. High-purity nitrogen was used as the carrier gas with a flow rate of 32.8 mL min^{-1}. The VFA content was evaluated with calibration curves.

The digestate samples were collected on days 100 and 220 to determine the archaeal communities at both the solely CS and mixed substrates digestion phases, respectively. The samples, including original rumen archaea, were frozen at −80 °C immediately. Genomic DNA was extracted from each sample with an E.Z.N.A Soil DNA kit (OMEGA, Atlanta, GA, USA) according to the manufacturer's instructions. PCR amplification was performed using specifically synthesized primers with a barcode of Arch519F (5′-CAGCCGCCGCGGTAA-3′) and Arch915R (5′-CAGCCGCCGCGGTAA-3′) in a PCR thermal cycler Dice (BioRad Co. Ltd., Berkeley, CA, USA) using the following program: 2 min of denaturation at 95 °C, followed by 27 cycles of 30 s at 95 °C (denaturation), 30 s for annealing at 55 °C and 1 min at 72 °C (elongation), with a final extension at 72 °C for 10 min. After amplification, the PCR products were purified, quantified, and then pooled at equal concentrations. Finally, the PCR products were sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for high-throughput sequencing using paired-end sequencing with an Illumina MiSeq PE300 platform (San Diego, CA, USA).

For quality-control purpose, any sequences that contained mismatches and ambiguous reads (N) in primers were removed. After deleting unqualified sequences, total of 11,276, 18,623, and 12,027 high-quality 16S rRNA gene sequences were obtained for each sample. These clean, non-continuous sequences were then clustered by the complete linkage clustering method in the QIIME pipeline. OTUs were classified using 97% identity of the 16S rRNA gene sequence as a cutoff, and an OTUs table was generated for each sample and used for statistical analysis. Richness estimators of Chao1, ACE, and Shannon index were calculated by MOTHUR. Taxonomic classification at the genus level was performed using the Ribosome Database project (RDP) algorithm to classify the representative sequences of each OTU.

4. Conclusions

A pilot-scale anaerobic digestion system was successfully developed to demonstrate the biogas yield enhancement by rumen microorganisms in the bioconversion of solely pretreated CS and pretreated CS with livestock manures. Pretreatment of CS by biogas liquid could be adopted at this scale. The rumen culture had a positive response to the impact of temperature and pH. There was almost no promotion of the solely CS digestion system after adding urea. The ruminal pilot-scale digestion reactor was successfully started up within 32 days at a retention time of 20 days. The optimal solid content of solely CS was 3%, which resulted in a biogas yield of 395 L kg^{-1}·TS^{-1}. The addition of livestock manure was helpful for improving the comprehensive performance of the ruminal digestion system. The components of biogas including CH_4, CO_2, and H_2 could be maintained at about 60%, 40%, and 0%. Microbial community analysis demonstrated that hydrogenotrophic methanogens dominated in the solely CS digestion system. Hydrogenotrophic and acetoclastic methanogens co-occurred for methane production during the co-digestion of CS with PM. SEM images proved the effect of the rumen microorganisms. Mass balance revealed the internal relevance of the conversion treatment process. The experimental results demonstrated that the newly established ruminal anaerobic digestion system has great potential in the sustainable and comprehensive utilization of ASOWs.

Supplementary Materials: E-supplementary data of this work can be found in online version of the paper. The following are available online at http://www.mdpi.com/1996-1073/11/4/920/s1, Figure S1: Temperature variation of the anaerobic digestion system, Figure S2: pH value of the anaerobic digestion system.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ju, X.; Zhang, F.; Bao, X.; Römheld, V.; Roelcke, M. Utilization and management of organic wastes in Chinese agriculture: past, present and perspectives. *Sci. China Ser. C: Life Sci.* **2005**, *48*, 965–979.
2. Li, H.; Cao, Y.; Wang, X.; Ge, X.; Li, B.; Jin, C. Evaluation on the production of food crop straw in China from 2006 to 2014. *BioEnergy Res.* **2017**, *10*, 949–957. [CrossRef]
3. Ning, Z.; Ji, M. Changes and outlook about production amount of livestock and poultry manure in China. *Agric. Outlook* **2014**, *1*, 46–48.
4. Zhang, X.; Zhai, M.; Wang, Y.; Gao, Y.; Zhao, H.; Zhou, X.; Gao, J. Research on biomass energy utilization in Chinese rural area: Challenges and opportunities. *Emerald Group Publ. Ltd.* **2016**, 257–281. [CrossRef]
5. Zhang, L.; Liu, Y.; Hao, L. Contributions of open crop straw burning emissions to $PM_{2.5}$ concentrations in China. *Environ. Res. Lett.* **2016**, *11*. [CrossRef]
6. Prapinagsorn, W.; Sittijunda, S.; Reungsang, A.; Sciubba, E. Co-digestion of napier grass and its silage with cow dung for methane production. *Energies* **2017**, *10*, 1654. [CrossRef]
7. Powlson, D.S.; Riche, A.B.; Coleman, K.; Glendining, M.J.; Whitmore, A.P. Carbon sequestration in European soils through straw incorporation: Limitations and alternatives. *Waste Manag.* **2008**, *28*, 741–746. [CrossRef] [PubMed]
8. Yang, X.; Wang, H.; Strong, P.J.; Xu, S.; Liu, S.; Lu, K.; Sheng, K.; Guo, J.; Che, L.; He, Z.; et al. Thermal properties of biochars derived from waste biomass generated by agricultural and forestry sectors. *Energies* **2017**, *10*, 469. [CrossRef]
9. Mao, C.; Feng, Y.; Wang, X.; Ren, G. Review on research achievements of biogas from anaerobic digestion. *Renew. Sustain. Energy Rev.* **2015**, *45*, 540–555. [CrossRef]
10. Mata-Alvarez, J.; Dosta, J.; Romero-Güiza, M.S.; Fonoll, X.; Peces, M.; Astals, S. A critical review on anaerobic co-digestion achievements between 2010 and 2013. *Renew. Sustain. Energy Rev.* **2014**, *36*, 412–427. [CrossRef]
11. Brodeur, G.; Yau, E.; Badal, K.; Collier, J.; Ramachandran, K.B.; Ramakrishnan, S. Chemical and physicochemical pretreatment of lignocellulosic biomass: A review. *Enzym. Res.* **2011**, *4999*, 787532. [CrossRef] [PubMed]
12. Jin, W.Y.; Xu, X.C.; Gao, Y.; Yang, F.L.; Wang, G. Anaerobic fermentation of biogas liquid pretreated maize straw by rumen microorganisms in vitro. *Bioresour. Technol.* **2014**, *153*, 8–14. [CrossRef] [PubMed]
13. Hungate, R.E. *The Rumen and Its Microbes*; Academic Press Inc.: London, UK, 1966; pp. 466–525.
14. Li, N.; Yang, F.; Xiao, H.; Zhang, J.; Ping, Q. Effect of feedstock concentration on biogas production by inoculating rumen microorganisms in biomass solid waste. *Appl. Biochem. Biotech.* **2017**, 1–13. [CrossRef] [PubMed]
15. Yue, Z.; Wang, J.; Liu, X.; Yu, H. Comparison of rumen microorganism and digester sludge dominated anaerobic digestion processes for aquatic plants. *Renew. Energy* **2012**, *46*, 255–258. [CrossRef]
16. Yue, Z.; Yu, H.; Wang, Z. Anaerobic digestion of cattail with rumen culture in the presence of heavy metals. *Bioresour. Technol.* **2007**, *98*, 781–786. [CrossRef] [PubMed]
17. Hu, Z.H.; Yu, H.Q. Application of rumen microorganisms for enhanced anaerobic fermentation of corn stover. *Process Biochem.* **2005**, *40*, 2371–2377. [CrossRef]
18. Alrawi, R.A.; Ahmad, A.; Ismail, N.; Kadir, M.O.A. Anaerobic co-digestion of palm oil mill effluent with rumen fluid as a co-substrate. *Desalination* **2011**, *269*, 50–57. [CrossRef]
19. Gijzen, H.J.; Derikx, P.J.; Vogels, G.D. Application of rumen microorganisms for a high rate anaerobic digestion of papermill sludge. *Biol. Wastes* **1990**, *32*, 169–179. [CrossRef]
20. Change, O.C. Intergovernmental Panel on Climate Change. World Meteorological Organization, 2007. Available online: http://www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4_syr_full_report.pdf (accessed on 12–17 November 2007).

21. Mosoni, P.; Martin, C.; Forano, E.; Morgavi, D.P. Long-term defaunation increases the abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep. *J. Anim. Sci.* **2011**, *89*, 783. [CrossRef] [PubMed]
22. Van, T.E.; Hundley, V. The importance of pilot studies. *Nurs. Stand.* **2010**, *16*, 33.
23. Chundawat, S.P.S.; Balan, V.; Sousa, L.D.C.; Dale, B.E. *Bioalcohol Production: Biochemical Conversion of Lignocellulosic Biomass*; Woodhead Publishing Limited: Cambridge, UK, 2010.
24. Carrillo, F.; Colom, X.; Sunol, J.J.; Saurina, J. Structural FTIR analysis and thermal characterisation of lyocell and viscose-type fibres. *Eur. Polym. J.* **2004**, *40*, 2229–2234. [CrossRef]
25. Phillipson, K.; Hay, J.N.; Jenkins, M.J. Thermal analysis FTIR spectroscopy of poly (ε-caprolactone). *Thermochim. Acta* **2014**, *595*, 74–82. [CrossRef]
26. Mezzullo, W.G.; Mcmanus, M.C.; Hammond, G.P. Life cycle assessment of a small-scale anaerobic digestion plant from cattle waste. *Appl. Energy* **2013**, *102*, 657–664. [CrossRef]
27. Lindorfer, H.; Braun, R.; Kirchmayr, R. Self-heating of anaerobic digesters using energy crops. *Water Sci. Technol.* **2006**, *53*, 159–166. [CrossRef] [PubMed]
28. Hu, Z.; Yu, H.; Zhu, R. Influence of particle size and pH on anaerobic degradation of cellulose by ruminal microbes. *Int. Biodeter. Biodeg.* **2005**, *55*, 233–238. [CrossRef]
29. Dixon, R.M.; Dixon, R.M. Effects of addition of urea to a low nitrogen diet on the rumen digestion of a range of roughages. *Aust. J. Agric. Res.* **1999**, *50*, 1091–1098. [CrossRef]
30. Zhou, J.; Yang, J.; Yu, Q.; Yong, X.; Xie, X.; Zhang, L.; Wei, P.; Jia, H. Different organic loading rates on the biogas production during the anaerobic digestion of rice straw: A pilot study. *Bioresour. Technol.* **2017**, *244*, 865–871. [CrossRef] [PubMed]
31. Lee, D.H.; Behera, S.K.; Kim, J.W.; Park, H.S. Methane production potential of leachate generated from Korean food waste recycling facilities: A lab-scale study. *Waste Manag.* **2009**, *29*, 876–882. [CrossRef] [PubMed]
32. Zhang, P.; Chen, Y.; Zhou, Q. Waste activated sludge hydrolysis and short-chain fatty acids accumulation under mesophilic and thermophilic conditions: Effect of pH. *Water Res.* **2009**, *43*, 3735–3742. [CrossRef] [PubMed]
33. Liu, Q.; Wang, C.; Pei, C.X.; Li, H.Y.; Wang, Y.X.; Zhang, S.L.; Zhang, Y.L.; He, J.P.; Wang, H.; Yang, W.Z. Effects of isovalerate supplementation on microbial status and rumen enzyme profile in steers fed on corn stover based diet. *Livest. Sci.* **2014**, *161*, 60–68. [CrossRef]
34. Schmidt, J.E.; Ahring, B.K. Effects of hydrogen and formate on the degradation of propionate and butyrate in thermophilic granules from an upflow anaerobic sludge blanket reactor. *Appl. Environ. Microbiol.* **1993**, *59*, 2546–2551.
35. Baek, G.; Kim, J.; Kim, J.; Lee, C. Role and potential of direct interspecies electron transfer in anaerobic digestion. *Energies* **2018**, *11*, 107. [CrossRef]
36. Yangin-Gomec, C.; Ozturk, I. Effect of maize silage addition on biomethane recovery from mesophilic co-digestion of chicken and cattle manure to suppress ammonia inhibition. *Energy Convers. Manag.* **2013**, *71*, 92–100. [CrossRef]
37. Kim, M.; Ahn, Y.H.; Speece, R.E. Comparative process stability and efficiency of anaerobic digestion: Mesophilic vs. Thermophilic. *Water Res.* **2002**, *36*, 4369. [CrossRef]
38. Russell, J.B. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. *J. Dairy Sci.* **1998**, *81*, 3222–3230. [CrossRef]
39. Hegarty, R.S.; Gerdes, R. Hydrogen production and transfer in the rumen. *Recent Adv. Anim. Nutr. Aust.* **1999**, *12*, 37–44.
40. Zhao, B.; Yue, Z.; Ni, B.; Mu, Y.; Yu, H.; Harada, H. Modeling anaerobic digestion of aquatic plants by rumen cultures: Cattail as an example. *Water Res.* **2009**, *43*, 2047–2055. [CrossRef] [PubMed]
41. Chandra, R.; Takeuchi, H.; Hasegawa, T. Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. *Renew. Sustain. Energy Rev.* **2012**, *16*, 1462–1476. [CrossRef]
42. Moestedt, J.; Påledal, S.N.; Schnürer, A.; Nordell, E. Biogas production from thin stillage on an industrial scale—Experience and optimisation. *Energies* **2013**, *6*, 5642–5655. [CrossRef]
43. Fernandes, T.V.; Keesman, K.J.; Zeeman, G.; van Lier, J.B. Effect of ammonia on the anaerobic hydrolysis of cellulose and tributyrin. *Biomass Bioenergy* **2012**, *47*, 316–323. [CrossRef]

44. Wang, Y.; Zhang, Y.; Wang, J.; Meng, L. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. *Biomass Bioenergy* **2009**, *33*, 848–853. [CrossRef]
45. Yu, G.; Chen, X.; Liu, Z.; Zhou, X.; Zhang, Y. Effect of inoculum sources on the anaerobic digestion of rice straw. *Bioresour. Technol.* **2014**, *158*, 149–155.
46. Muller, Z.O. *Feed from Animal Wastes: Feeding Manual*; Food and Agriculture Organization of the United Nations: Rome, Italy, 1982.
47. Bhattacharya, A.N.; Taylor, J.C. Recycling animal waste as a feedstuff: A review. *J. Anim. Sci.* **1975**, *41*, 1438–1457. [CrossRef]
48. Xie, S.; Lawlor, P.G.; Frost, P.; Dennehy, C.D.; Hu, Z.; Zhan, X. A pilot scale study on synergistic effects of co-digestion of pig manure and grass silage. *Int. Biodeter. Biodeg.* **2017**, *123*, 244–250. [CrossRef]
49. Miller, T.L. *Methanobrevibacter*; William, B.W., Fred, R., Eds.; Wiley Online Library: Hoboken, NJ, USA, 2015.
50. Henderson, C. The effects of fatty acids on pure cultures of rumen bacteria. *J. Agr. Sci.* **1973**, *81*, 107–112. [CrossRef]
51. David, R.B.; Richard, W.C. *Bergey's Manual of Systematic Bacteriology: Volume One: Archaea and the Deeply Branching and Phototrophic Bacteria*; Springer Science & Business Media: Berlin, Germany, 2010.
52. Goberna, M.; Gadermaier, M.; García, C.; Wett, B.; Insam, H. Adaptation of methanogenic communities to the cofermentation of cattle excreta and olive mill wastes at 37 °C and 55 °C. *Appl. Environ. Microb.* **2010**, *76*, 6564–6571. [CrossRef] [PubMed]
53. Yan, Z.; Song, Z.; Li, D.; Yuan, Y.; Liu, X.; Zheng, T. The effects of initial substrate concentration, C/N ratio, and temperature on solid-state anaerobic digestion from composting rice straw. *Bioresour. Technol.* **2015**, *177*, 266–273. [CrossRef] [PubMed]
54. Ziganshin, A.M.; Liebetrau, J.; Pröter, J.; Kleinsteuber, S. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 5161–5174. [CrossRef] [PubMed]
55. St-Pierre, B.; Wright, A.D.G. Comparative metagenomic analysis of bacterial populations in three full-scale mesophilic anaerobic manure digesters. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 2709–2717. [CrossRef] [PubMed]
56. Demirel, B.; Scherer, P. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. *Rev. Environ. Sci. Biotechnol.* **2008**, *7*, 173–190. [CrossRef]
57. Karakashev, D.; Batstone, D.J.; Angelidaki, I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl. Environ. Microbiol.* **2005**, *71*, 331–338. [CrossRef] [PubMed]
58. De Vrieze, J.; Hennebel, T.; Boon, N.; Verstraete, W. Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. *Bioresour. Technol.* **2012**, *112*, 1–9. [CrossRef] [PubMed]
59. Akin, D.E. Ultrastructure of rumen bacterial attachment to forage cell walls. *Appl. Environ. Microbiol.* **1976**, *31*, 562–568. [PubMed]
60. Rezaeian, M.; Beakes, G.W.; Parker, D.S. Methods for the isolation, culture and assessment of the status of anaerobic rumen chytrids in both in vitro and in vivo systems. *Mycol. Res.* **2004**, *108*, 1215–1226. [CrossRef] [PubMed]
61. Segal, L.; Creely, J.J.; Martin, A.E., Jr.; Conrad, C.M. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Text. Res. J.* **1959**, *29*, 786–794. [CrossRef]
62. Apha, A.W. *Standard Methods for the Examination of Water and Wastewater*, 19th ed.; American Public Health Association: Washington, DC, USA, 1995.