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Co-digestion of tobacco waste with different agricultural biomass feedstocks and the inhibition of tobacco viruses by anaerobic digestion

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HIGHLIGHTS

- The methane yield of tobacco stalk was 0.163 m^3 CH_4·kg VS^{-1}.
- Cucumber Mosaic Virus can be inactivated by mesophilic anaerobic digestion.
- Tobacco Mosaic Virus can be inactivated by thermophilic anaerobic digestion.

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ABSTRACT

Tobacco is widely planted across the world especially in China, which means that a large amount of tobacco waste needs to be treated. This study investigated the biogas fermentation of tobacco stalks co-digested with different biomass feedstocks and the inactivation of Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV) by anaerobic digestion. Results showed that the maximum methane yield of tobacco stalks at 35 °C was 0.163 m^3 CH_4·kg VS^{-1}, which was from the co-digestion of tobacco stalks, wheat stalks and pig manure. The largest VS removal rate of tobacco stalks was 59.10%. Proven by indicator paper stripe, half-leaf lesion and RT-PCR, CMV could be inactivated by mesophilic and thermophilic anaerobic digestion, whereas TMV could be only inactivated by thermophilic anaerobic digestion over 20 days. These results suggested that using tobacco stalks as feedstock for anaerobic digestion and applying the digested residue and slurry to Solanaceae crop land are feasible.

1. Introduction

China is the world's largest tobacco producer and consumer, and contributes about 40% of the world's total tobacco production (Eriksen et al., 2015). This considerable production of tobacco leads to large quantities of tobacco waste (tobacco stem and discarded tobacco leaf), which is harmful to the environment and is generated during the cultivation and manufacturing processes (Meng et al., 2014; Zhong et al., 2010; Zi et al., 2013). The conventional methods for dealing with tobacco waste are direct burning or reusing it as organic fertilizer after composting (Kopčić et al., 2014; Yang et al., 2012b). Moreover, some chemical compounds, such as nicotine and solanesol, which are used as pesticides, can also be extracted from tobacco waste (Piotrowska-Cyplik et al., 2009). Over the last few years, more attention has been paid to the thermo-chemical conversion of tobacco waste for energy production using processes such as pyrolysis or co-combustion with other fuels (Li et al., 2011; Yang et al., 2012b; Zhang et al., 2013).

In contrast to the treatment methods above, anaerobic digestion is a more renewable and sustainable solution. Most biomass types can be used as feedstock in an anaerobic digester, including animal manure, crop residues, municipal waste, aquatic biomass etc. (Wellinger et al., 2013). The cellulose contents in tobacco waste can vary between 43.4% and 68.4% (Ye et al., 2013b), which is good for anaerobic digestion. Meher et al. (1995) obtained a biogas yield of 0.169–0.282 m^3 kg TS^{-1} of tobacco waste using a semi-continuous experiment at temperatures between 23.5 °C and 36.0 °C. González-González et al. (2014, 2013) obtained a methane yield of 53.84 ± 14.48 Nm^3 CH_4·t^{-1} from fresh tobacco under mesophilic conditions using a 16 day degradation period. Nonetheless, using tobacco waste, especially tobacco leaves, as anaerobic digestion feedstock is controversial due to its high nicotine content. A previous research report indicated that tobacco leaves can inhibit the metabolism of anaerobic microbes and cannot be used as a

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mono-feedstock to start up the anaerobic digestion process (Yang et al., 2012a). Nevertheless, the nicotine content in tobacco stalks is much lower than in leaves, so it may be feasible to use tobacco stalks as feedstock for anaerobic digestion. Moreover, some drawbacks to the mono-digestion of crop stalks can be solved by co-digestion with biomass that contains high levels of nitrogen (Mata-Alvarez et al., 2014). Therefore, the anaerobic digestion efficiency of tobacco stalks co-digested with other biomass types is a significant research topic.

However, the existence of tobacco viruses in feedstock plants may lead to virus infected digested slurry, which would prevent the reuse of the digested slurry as organic fertilizer on Solanaceae crop land. Tobacco plants can be attacked by a wide range of diseases, and a great number of viruses can infect them and other Solanaceae crops either naturally or experimentally (Shew and Lucas, 1991). The most harmful tobacco viruses are Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV), Potato virus Y (PVY) and Tobacco etch virus (TEV) (Chatzivassiliou et al., 2004). In particular, TMV is known to be hard to inactivate because it has a very stable structure, even when using ultraviolet disinfection or aerobic composting (Alonso et al., 2013). Anaerobic digestion is an effective pathogen removal method even under mesophilic conditions (Horan et al., 2004; Ward et al., 2008). However, there have been no reports as to whether or not anaerobic digestion could inactivate tobacco viruses.

This study investigated the methane yield and VS removal increase when tobacco (flue-cured tobacco) stalks were co-digested with different biomass feedstocks that are easily found around tobacco growing areas, under mesophilic (35 °C) anaerobic digestion conditions. Subsequently, we chose the best co-digestion group as the feedstock for anaerobic digestion under mesophilic (35 °C) and thermophilic (55 °C) conditions for 20 days, and evaluated TMV and CMV inactivation by anaerobic digestion.

2. Methods

2.1. Feedstock and inoculum collection and preparation

Healthy tobacco stalks without any virus infections (HTS) and tobacco leaves with virus infections (TL) were obtained from a test field and a laboratory at the Tobacco Research Institute of Chinese Academy of Agricultural Sciences (CAAS). Wheat stalks (WS), rape stalks (RS), pig manure (PM) and cow manure (CM) were collected from a local farm in Jintang County, Chengdu, China. All the stalks were cut with a grinder and then sieved with a 40-mesh screen. Pig manure and cow manure were stirred separately by an agitator in order to homogenize the components. The above feedstocks, which were used in the biogas production test, were frozen at −18 °C until needed. Before use, tobacco leaves infected with TMV and CMV for the inhibition test were ground and diluted with distilled water at 20 times the mass of tobacco leaves. The inoculum was the anaerobic sludge collected from anaerobic digestion experiment carried out in a laboratory at the Biogas Institute of Ministry of Agriculture (BIOMA). Characteristics of the feedstock and inoculum before dilution are shown in Table 1.

2.2. Biogas fermentation of tobacco stalks

The digesters were made of plastic with a working weight of 800 g. The fermentation process was under mesophilic conditions (35 °C), which was maintained by a water bath. The mix ratios of the total solid content of feedstock and the inoculum for the different treatments are listed in Table 2. Distilled water was used to fill the digesters and was thoroughly mixed with fresh feedstock. Each treatment was repeated three times. The experiment was stopped 44 days after start up because no significant biogas production was detected for any treatment at that point. Biogas output was analyzed once a day, and total solids (TS) and volatile solids (VS) were tested at the end of the experiment. The methane volume fraction in the biogas was tested every day for the first 12 days. From day 13 to day 32, the methane volume fraction in the biogas was tested once per five days because the biogas output was too low to measure every day. The final test was carried out on the last day of the experiment.

2.3. Viruses inactivation by anaerobic digestion

The digesters were 35 mL working volume serum bottles sealed with rubber plugs. The feedstock was composed of wheat stalk (0.60 g), pig manure (2.15 g), a solution of tobacco leaves infected with TMV (5 mL), solution of tobacco leaves infected with CMV (5 mL) and inoculum (8.78 g) (the best co-digestion group identified by the tobacco stalk fermentation experiment). The feedstock was added to 35 mL distilled water. Control solutions contained the solution of tobacco leaves with TMV (5 mL) and the solution of tobacco leaves with CMV (5 mL).The experiment was operated under mesophilic (35 °C) and thermophilic (55 °C) conditions for 20 days. Each treatment was carried out in triplicate. Biogas output and virus activity were measured at the end of the experiment.

2.4. Analytical methods

TS, VS and TOC (total organic carbon) detection were carried out according to the procedures described in the Standard Methods (APHA, 2012). Cellulose, hemi-cellulose and lignin were detected by a cellulose test instrument (FIWE 3, VELP Scientifica, Innovative Analytical Instruments, Italy). Nicotine and TN (total nitrogen) were detected by an auto-analyzer (SEAL AutoAnalyzer 3, Seal Analytical, Germany). In the tobacco stalk fermentation experiment, biogas output was tested by the water volume replaced by the biogas generated in the digester. Biogas outputs by the substrates were the difference between the biogas output for each treatment and that of the control (44.67 mL in total). For the virus inhibition experiment, biogas output was tested with a milligascounter (MGC-1 V3.2 PMMA, Dr.-ing Ritter ApparatebauGmbh & Co. kg, Germany), and methane content was measured with a gas analyzer (Biogas 401, ADOS GmbH Instrumentation and Control, Germany). MS Excel 2007 and Origin 8.0 were used to analyze the data.

2.5. First-order rate equations

Methane production curves could be modeled by two first-order rate equations (Eqs. (1) and (2)). Eq. (2) gave a better fit result than Eq. (1) because of the heterogeneous feedstock and the presence of both rapidly and slowly degrading fractions (Wellinger et al., 2013).

$$Y = Y_{max}(1 - e^{-kt}) \tag{1}$$

$$Y = Y_{max}\left[1 - Pe^{-k_1^t} - (1 - P)e^{-k_2 t}\right] \tag{2}$$

where Y is the cumulative methane yield at a given time t ($m^3\ CH_4 \cdot kg\ VS^{-1}$), Y_{max} is the ultimate cumulative methane yield ($m^3\ CH_4 \cdot kg\ VS^{-1}$), k is the first-order rate constant, k_1 is the first-order rate constant for the readily degradable substrates, k_2 is the first-order rate constant for less readily degradable substrates, and P is the proportion of readily degradable substrates.

Table 1
Feedstock and inoculum characteristics before dilution.

Parameters	HTS	WS	RS	PM	CM	TL	Inoculum
TS (%)	94.11 ± 0.06	92.63 ± 0.08	94.09 ± 0.17	26.02 ± 0.55	24.32 ± 0.97	12.16 ± 0.34	12.76 ± 0.08
VS (%)	87.93 ± 0.13	75.19 ± 1.26	87.87 ± 0.19	20.83 ± 0.45	20.08 ± 0.84	10.77 ± 0.14	8.03 ± 0.04
Cellulose (%)	56.10 ± 0.24	47.01 ± 0.38	53.78 ± 2.14	9.70 ± 1.05	27.43 ± 1.76	–	–
HC (%)	22.44 ± 0.20	20.65 ± 0.35	18.69 ± 0.34	3.05 ± 0.36	11.84 ± 1.92	–	–
TOC (%)	44.61 ± 3.44	40.57 ± 2.75	43.90 ± 2.10	8.34 ± 1.33	12.07 ± 0.98	–	6.89 ± 0.41
TN (%)	0.83 ± 0.02	0.46 ± 0.02	0.51 ± 0.09	0.84 ± 0.05	0.81 ± 0.04	–	1.25 ± 0.04
C:N	53.75	88.20	86.08	9.93	14.90	–	5.51
Lignin (%)	15.11 ± 2.33	16.33 ± 4.15	13.92 ± 1.97	7.57 ± 1.49	11.72 ± 3.36	–	5.32 ± 0.37
Nicotine (%)	0.26 ± 0.08	–	–	–	–	–	–

HTS is healthy tobacco stalk without virus, WS is wheat stalk, RS is rape stalk, PM is pig manure, CM is cow manure, TL is tobacco leaves with virus.
HC is hemi-cellulose, TOC is total organic carbon, TN is total nitrogen, C:N is carbon nitrogen ratio.

Table 2
Mix ratios for the total solid contents of the different feedstocks and inocula.

	Control	T1	T2	T3	T4	T5	T6	T7	T8	T9
Inoculum (g)	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6
HTS (g)	–	12.8	–	–	–	–	12.8	12.8	12.8	12.8
WS (g)	–	–	12.8	12.8	–	–	12.8	12.8	–	–
RS (g)	–	–	–	–	12.8	12.8	–	–	12.8	12.8
PM (g)	–	–	12.8	–	12.8	–	12.8	–	12.8	–
CM (g)	–	–	–	12.8	–	12.8	–	12.8	–	12.8
C:N	5.51	17.53	16.50	17.62	17.15	18.26	23.17	24.14	23.64	24.59
LC (g)	1.36	3.30	4.42	4.95	4.11	4.64	6.36	6.89	6.05	6.58
I:S	–	1.35	0.78	0.77	0.73	0.72	0.49	0.49	0.47	0.47

HTS is healthy tobacco stalk without virus, WS is wheat stalk, RS is rape stalk, PM is pig manure, CM is cow manure, TL is tobacco leaves with virus, C:N is carbon nitrogen ratio, LC is lignin content, I:S is VS ratio of inoculum to substrate.

2.6. Calculation method of VS degradation and VS removal rate

Suppose all the biodegradable matter in the nine treatments was digested up.

$$VS_{l,\ T6} = VS_{e,\ T6} \times (800 - Q_{T6} \times 1.2912 \times 10^{-3}) \quad (3)$$

$$VS_{l,\ T2} = VS_{e,\ T2} \times (800 - Q_{T2} \times 1.2912 \times 10^{-3}) \quad (4)$$

$$VS_{de,HTS,\ T6} = VS_{l,\ T6} - VS_{l,\ T2} \quad (5)$$

$$VS_{re,\ HTS,\ T6} = VS_{de,\ HTS,\ T6}/VS_{st,\ HTS,\ T6} \times 100\% \quad (6)$$

where $VS_{l,\ T2}$ and $VS_{l,\ T6}$ are the total VS amounts left in T2 and T6 (g), $VS_{e,\ T2}$ and $VS_{e\ T6}$ are the VS mass ratio of the digested liquid of T2 and T6 (%) which were tested at the end of digestion, Q_{T2} and Q_{T6} are the cumulative biogas outputs of T2 and T6 (mL), 1.2912×10^{-3} is the density of biogas ($g{\cdot}mL^{-1}$) (Al Seadi, 2008), $VS_{de,\ HTS,\ T6}$ is the VS degradation for HTS in T6 (g), and $VS_{re,\ HTS,\ T6}$ is the VS removal rate by HTS in T6 (%), $VS_{st,\ HTS,\ T6}$ are the total VS amounts of HTS in T6 when startup (g). The formulas above were used to calculate the VS degradation and the VS removal rates by HTS in T7, T8, and T9.

2.7. TMV and CMV test methods

TMV was tested with an indicator paper stripe (ImmunoStrip ISK 57400/0025, Agdia Inc., USA), by the half-leaf lesion method, and by RT-PCR, while CMV was tested only by RT-PCR. In the half-leaf lesion method, *Nicotianatabacumvar. samsun NN* plants with similar sized leaves were used as the indicator plant species. Inoculation time was 48 h. Sample preparation and test methods were based on proven methodologies (Hull, 2013).

2.8. RT-PCR

Total RNA was extracted from the supernatant of the digested slurry and the control after centrifugation according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com). The degenerative reaction proceeded with a mixture of total RNA, dNTP, Oligo (dT) Primer, and Rnase Free dH_2O in a PCR instrument (Mastercycler nexus flat PCR, Eppendof, Genmany) for 5 min at 65 °C. After shock cooling and centrifugation, 5× PrimeScript TM Buffer, Rnase Inhibitor, PrimeScript TM Rtase, and Rnase Free dH_2O were added to the above mixture, to form the RT liquid. First-strand cDNA was synthesized using the RT liquid at 42–50 °C for 15–30 min, followed by 95 °C for 5 min. The products were then stored at 4 °C until needed. The primer sequences used in this study wereTMV: F:5′-AAAATGAGGGATATGGTC-3′; R:5′-AAACTAACCTATCGTGGA-3′.CMV: F:5′-AGGTCCTAACAGCAATCA-3′;R:5′-ACAATGGTGTTACCGAAG-3′.

First strand cDNA solution was added to a PCR tube containing Premix LA Taq and the corresponding primers. The PCR protocol was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The products were stored at 4 °C. The PCR products were analyzed by electrophoresis on 1% agarose gel, stained by ethidium bromide solution, and photographed under UV illumination (Imagemaster VDS, PharmaciaBiotech, USA).

3. Results and discussion

3.1. Biogas fermentation of tobacco stalks

All the treatments reached peak biogas production on day 3. The largest daily biogas output was 1637.50 mL which came from treatment T8 (HTS + RS + PM). From day 10, another small peak in biogas production, which only belonged to the treatments which contained pig manure (T2, T4, T6, and T8), was detected. The largest daily biogas output for the second peak was also from

treatment T8 (910.00 mL). From day 20, the daily biogas output for all treatments decreased to lower than 100 mL, and this lasted to the end of the experiment. In summary, the anaerobic digestion period could be divided into three stages, first peak stage, second peak stage and a steady stage (Fig. 1). The volumetric percentages for methane contents in the biogas from the nine treatments varied between 50.53% and 68.50%, which confirmed that the anaerobic process was working correctly.

Fig. 1 shows the cumulative methane yield for the nine treatments. It shows that T8 (HTS + RS + PM) had the highest cumulative methane output at 8609.69 mL, whereas T4 (RS + PM) produced the maximum methane yield at 0.315 m^3 CH_4·kg VS^{-1}. The methane yield of tobacco stalk (T1) was 0.133 m^3 CH_4·kg VS^{-1}. Fig. 1 also highlights three more results: (1) the methane yield of RS was higher than that of WS; (2) the methane yield of PM was higher than that of CM; and (3) reduced total solid contents in the fermentation liquid added to the digester at start up led to higher methane yields (except T1 (HTS)).The kinetic coefficients of the nine treatments fitted by Eqs. (1) and (2) are listed in Table 3. As can be seen from Table 3, Eqs. (1) and (2) produced similar R^2 values except for T1. The highest ultimate cumulative methane yield (Y_{max}) was for T4, followed by T2, T8, T6, T5, T9, T3, T7, and T1. With the exception of T3 and T7, the sequence above for ultimate cumulative methane yields was the same as the sequence for experimental methane yield (Fig. 1). Moreover, the feed stocks in T2–T8 contained relatively higher proportions of readily degradable material than T1.

Fig. 2 is the cumulative methane output by tobacco stalk and tobacco stalk co-digested with different biomass feedstocks. Tobacco stalk methane output in T6 was calculated from the total methane output of T6 (HTS + WS + PM) minus that of T2 (WS + PM). Based on the deduction above, the biogas outputs from HTS for T7, T8, and T9 could also be obtained. The cumulative methane output for HTS co-digested with different biomass feedstocks showed an obvious variation tendency from day 21. The cumulative methane outputs of HTS co-digested with WS and PM, WS and CM, and RS and PM were 1954.12 mL, 1680.92 mL, and 1620.57 mL, respectively, which were higher than that of HTS alone (1586.62 mL). However, the cumulative methane output of HTS co-digested with RS and CM only reached 1278.50 mL, which were lower than HTS alone. Therefore, the co-digestion groups in T6, T7 and T8 could increase the biogas output of HTS under mesophilic anaerobic conditions. The methane yield for tobacco stalk in T1 was 0.133 m^3 CH_4·kg VS^{-1}. The largest methane yield for tobacco stalk came from T6, and was 0.163 m^3 CH_4·kg VS^{-1}. The increased HTS methane yield rates in T6, T7, T8, and T9 compared to T1 were 22.6%, 5.9%, 2.1%, and −19.4%, respectively.

The methane yield of tobacco stalk in this study is higher than that in previous works (González-González and Cuadros, 2014; González-González et al., 2013; Meher et al., 1995), and the higher biogas yield could be explained by 3 reasons. Firstly, a higher methane output could be got by batch fermentation rather than by semi-continuous fermentation. Secondly, the feedstock particle size in this study is much smaller than that in previous pilot and industrial scale experiment. Lastly, a suitable carbon–nitrogen ratio from co-digestion of animal manure and plant stalk may improve the biogas potential of feedstock (Table 2). In addition, the methane yield of HTS in this study is lower than the methane yields of other energy crops (Wellinger et al., 2013) and the stalk of traditional crops such as corn stalk, wheat stalk and rice stalk(Sapci, 2013; Ye et al., 2013a; Zhong et al., 2011). Table 1 shows that the lignin content in HTS, WS and RS did not have much difference, and HTS had a better carbon nitrogen ratio (C:N) for anaerobic digestion than WS and RS. Therefore, it is the existence of nicotine, rather than the C:N or lignin content, that adversely affects the biogas yield of HTS as nicotine is harmful to microbial growth (Ye et al., 2013b).

Fig. 3 gives the VS degradation and VS removal rate for HTS and HTS co-digested with different feedstocks. As Fig. 3 shows, the VS degradation and VS removal rate for HTS co-digested with WS and PM in T6 were 6.71 g and 59.10%, respectively. The more cumulative methane yields of HTS, the more VS degradation and VS removal rates. From Table 2 we can see that the C:N, lignin content and VS ratio of inoculum to substrate (I:S) of T7, T8 and T9 did not have much difference. And T7, T8 and T9 showed better C:N for anaerobic digestion compared to T1. But T7, T8 and T9 presented different variations of VS degradation of HTS than T1. Therefore, none of the three factors, namely, C:N, lignin content and I:S constitute the reason for different VS degradation of HTS.

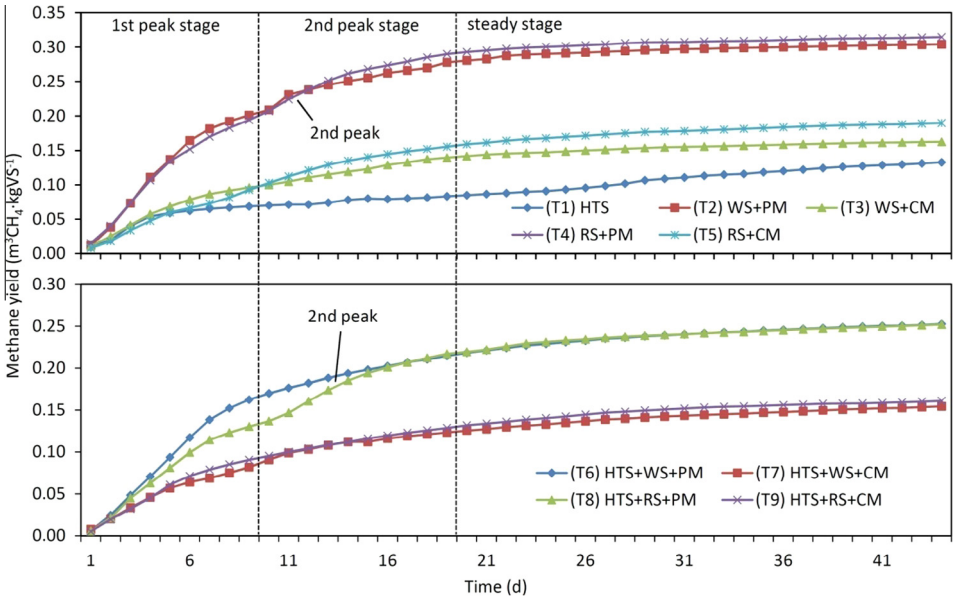

Fig. 1. Cumulative methane yields for tobacco stalk and tobacco stalk co-digested with different biomass feedstocks. HTS is health tobacco stalk, WS is wheat stalk, RS is rape stalk, PM is pig manure, CM is cow manure.

Table 3
Kinetic parameters derived from methane yield modeling.

Parameters		T1	T2	T3	T4	T5	T6	T7	T8	T9
Eq_1	Y_{max} (m^3 CH_4·kg VS^{-1})	0.127	0.306	0.164	0.321	0.198	0.252	0.155	0.262	0.164
	k	0.070	0.119	0.099	0.110	0.076	0.102	0.086	0.083	0.085
	R^2	0.884	0.992	0.997	0.992	0.994	0.989	0.997	0.990	0.996
Eq_2	Y_{max} (m^3 CH_4·kg VS^{-1})	0.162	0.319	0.171	0.329	0.208	0.270	0.169	0.262	0.173
	P	0.231	0.884	0.810	0.915	0.822	0.932	0.760	0.083	0.648
	k_1	0.501	0.128	0.113	0.116	0.083	0.102	0.100	0.990	0.109
	k_2	0.028	0.031	0.033	0.033	0.028	0.010	0.023	0.242	0.039
	R^2	0.965	0.990	0.997	0.990	0.993	0.989	0.997	0.882	0.996

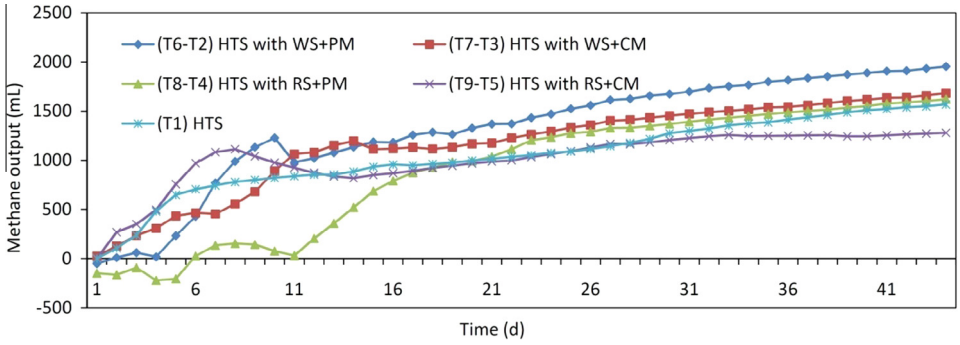

Fig. 2. Cumulative methane outputs for tobacco stalk and tobacco stalk co-digestion with other biomass feedstocks (methane output from tobacco stalk only). HTS is health tobacco stalk, WS is wheat stalk, RS is rape stalk, PM is pig manure, CM is cow manure.

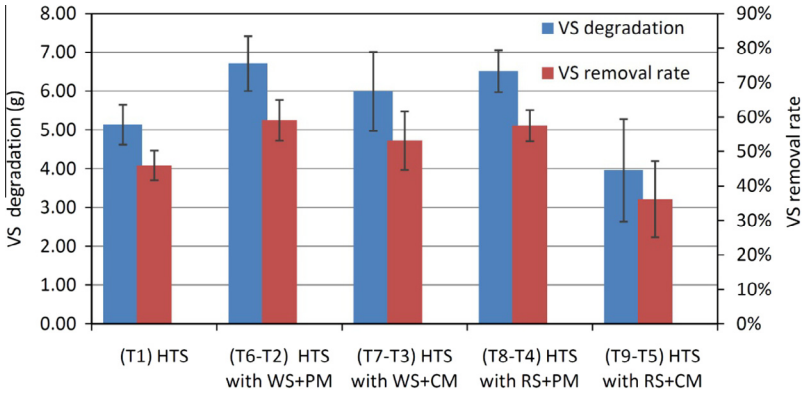

Fig. 3. VS degradation and VS removal rate for tobacco stalk and tobacco stalk co-digested with different biomass feedstocks (tobacco stalk only). HTS is health tobacco stalk, WS is wheat stalk, RS is rape stalk, PM is pig manure, CM is cow manure.

3.2. Inhibition of TMV and CMV by anaerobic digestion

Table 4 Part 1 shows the biogas output and biogas yield of all the treatments in the inhibition experiment. The biogas yields for 35 °C and 55 °C anaerobic digestion were lower than the biogas output for HTS T8. This may due to the higher nicotine content in tobacco leaves than in stalks. Nevertheless, the results showed that the anaerobic processes in this experiment were running as expected because the methane content in the biogas varied in a normal range.

Table 4 Part 2 is the TMV test results for the digested slurry under mesophilic (35 °C) and thermophilic (55 °C) anaerobic digestion conditions obtained using the indicator paper stripe method. TMV was detected in all the treatments, which indicated that TMV cannot be removed completely by mesophilic and thermophilic anaerobic digestions lasting for 20 days. Furthermore, the indicator paper stripe method cannot measure the amount of TMV released, but only tests whether TMV is active or not.

Table 4 Part 3 lists the lesions numbers on the indicator plant leaves inoculated with digested slurry that contained TMV. The control tests showed that heat treatment at 35 °C and 55 °C for 20 days produced similar lesions numbers. The largest lesion number was seen in the 35 °C anaerobic digested slurry treatment 2, whereas the smallest number appeared in the 55 °C anaerobic digested slurry treatments 2 and 3. After 35 °C anaerobic digestion, only treatment 2 showed a slight TMV inhibition, but TMV levels in other 35 °C anaerobic digestion treatments increased. Therefore, only treatments at 55 °C and lasting for 20 days had inhibitory effects on TMV.

RT-PCR is more specific and accurate than the virus test methods mentioned above. As conventional RT-PCR amplificative electrophoretograms describes, the TMV stripes for treatments of 35 °C anaerobic digestion have a similar brightness to treatment of TMV source leaves (control test), namely, there was no inhibitory effect on TMV under 35 °C anaerobic digestion for 20 days. However, treatments of 55 °C anaerobic digestion have relatively

Table 4
Some of experimental results for TMV inhibition by anaerobic digestion.

	Treatment		CK 35 °C	CK 55 °C	AD 35 °C 1	AD 35 °C 2	AD 35 °C 3	AD 55 °C 1	AD 55 °C 2	AD 55 °C 3
Part 1 Anaerobic process	Biogas output (mL)		–	–	392.40	385.86	346.62	395.67	408.75	428.37
	Biogas yield ($m^3 \cdot kg\,TS^{-1}$)		–	–	0.341	0.335	0.301	0.344	0.355	0.372
	Aver. Biogas yield ($m^3 \cdot kg\,TS^{-1}$)		–	–	0.326			0.357		
	Methane content		–	–	57.1%	55.3%	52.0%	51.1%	50.1%	53.9%
Part 2 Indicator paper stripe method	P.1		+	+	+	+	+	+	+	+
	P.2		+	+	+	+	+	+	+	+
	P.3		+	+	+	+	+	+	+	+
Part 3 Half-leaf lesion method	Number of lesion	P.1	25	14	16	49	53	13	6	5
		P.2	30	26	22	67	34	5	4	7
		P.3	33	31	25	55	41	3	6	4
		Aver.	29	24	21	57	43	7	5	5
	Inhibitory rate		–	–	28.41%	−94.32%	−45.45%	70.42%	77.46%	77.46%
	Aver. inhibitory rate		–	–	−37.12%			75.12%		

CK 35 °C and CK 55 °C are control test with heating treatment (35 °C and 55 °C) only, AD 35 °C and AD 55 °C are anaerobic digestion under 35 °C and 55 °C with two parallels.
Part 1 is the anaerobic digestion process parameters.
Part 2 is the virus test result after using the indicator paper stripe method, where "+" shows a positive reaction.
Part 3 is the virus test result after using the half-leaf lesion method, where data shows the lesions numbers. P.1, P.2, P.3 are parallel 1, parallel 2, and parallel 3, respectively.

dark TMV stripes, which show that TMV levels were low. This result makes a strong case for the inhibitory effect of 55 °C anaerobic digestion on TMV. Moreover, all the CMV stripes for 35 °C and 55 °C anaerobic digestion are extremely dark, which proves that 35 °C heat treatment lasting for 20 days can inactivate CMV. Based on the results above, anaerobic digestion can inactivate TMV and CMV under thermophilic conditions.

Many viruses have been inactivated in a short period of time using mesophilic anaerobic digestion (Termorshuizen et al., 2003), which is similar to the CMV results in this study. However, TMV could not be inactivated completely, even under thermophilic anaerobic conditions, which also proves that TMV is more stable than some viruses. Previous research has suggested some factors that may underlie the TMV inactivation mechanism. (1) Some studies indicated that absorption by suspended solids is the key factor controlling TMV removal in aerobic wastewater treatment systems (Zheng et al., 2004a,b). However, this study showed different TMV removals in mesophilic and thermophilic anaerobic digestions that had the same initial TS content in the digester. Therefore, absorption by suspended solids is not the reason for TMV inhibition in this study. (2) TMV is stable in a vacuum with traces of carbon-containing compounds (Alonso et al., 2013), so anaerobic conditions also do not inhibit TMV. (3) Neither mesophilic nor thermophilic conditions could completely inactivate TMV because large amounts of TMV were detected in the control test (solution of tobacco leaves with TMV and CMV at 35 °C and 55 °C) in this study. (4) Microflora, including photo synthetic bacteria and *Chlorella* in wastewater stabilization ponds, have an obvious inhibitory effect on TMV. Therefore microbial activity during anaerobic digestion maybe one of the factors leading to TMV inactivation (Zheng et al., 2004a).

4. Conclusion

The biogas output of tobacco stalks co-digested with different biomass feedstocks, and the inhibition on TMV and CMV through anaerobic digestion were investigated. The maximum methane yield of tobacco stalks at 35 °C was 0.163 $m^3\,CH_4 \cdot kg\,VS^{-1}$, which were from the co-digestion group consisting of tobacco stalks, wheat stalks and pig manure. The three virus test methods showed that CMV could be inactivated by mesophilic and thermophilic anaerobic digestions, whereas TMV could only be inactivated by thermophilic anaerobic digestion. The results suggested that using tobacco stalks as a feedstock for anaerobic digestion, and reusing the digested residue and slurry are feasible.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.04.003.

References

Al Seadi, T., 2008. Biogas handbook. Syddansk Universitet, Esbjerg. <http://www.lemvigbiogas.com/BiogasHandbook.pdf>. (accessed Jan 20, 2015).
Alonso, J., Górzny, M., Bittner, A., 2013. The physics of tobacco mosaic virus and virus-based devices in biotechnology. Trends Biotechnol. 31, 530–538.
APHA, 2012. Standard methods for the examination of water and wastewater. American Public Health Association Washington, DC.
Chatzivassiliou, E.K., Efthimiou, K., Drossos, E., Papadopoulou, A., Poimenidis, G., Katis, N.I., 2004. A survey of tobacco viruses in tobacco crops and native flora in Greece. Eur. J. Plant Pathol. 110, 1011–1023.
Eriksen, M., Mackay, J., Ross, H., 2015. The tobacco atlas. American Cancer Society, the fifth edition, Atlanta. <http://3pk43x313ggr4cy0lh3tctjh.wpengine.netdna-cdn.com/wp-content/uploads/2015/03/TA5_2015_WEB.pdf> (accessed Mar 30, 2015).
González-González, A., Cuadros, F., 2014. Optimal and cost-effective industrial biomethanation of tobacco. Renew. Energy 63, 280–285.
González-González, A., Cuadros, F., Ruiz-Celma, A., López-Rodríguez, F., 2013. Potential application of anaerobic digestion to tobacco plant. Fuel 113, 415–419.
Horan, N., Fletcher, L., Betmal, S., Wilks, S., Keevil, C., 2004. Die-off of enteric bacterial pathogens during mesophilic anaerobic digestion. Water Res. 38, 1113–1120.
Hull, R., 2013. Plant Virology. Academic Press, London.
Kopčić, N., Vuković Domanovac, M., Kučić, D., Briški, F., 2014. Evaluation of laboratory-scale in-vessel co-composting of tobacco and apple waste. Waste Manage. 34, 323–328.
Li, X., Lv, Y., Ma, B., Jian, S., Tan, H., 2011. Thermogravimetric investigation on co-combustion characteristics of tobacco residue and high-ash anthracite coal. Bioresour. Technol. 102, 9783–9787.
Mata-Alvarez, J., Dosta, J., Romero-Güiza, M., Fonoll, X., Peces, M., Astals, S., 2014. A critical review on anaerobic co-digestion achievements between 2010 and 2013. Renewable Sustainable Energy Rev. 36, 412–427.

Meher, K., Panchwagh, A., Rangrass, S., Gollakota, K., 1995. Biomethanation of tobacco waste. Environ. Pollut. 90, 199–202.

Meng, A., Zhang, Y., Zhuo, J., Li, Q., Qin, L., 2014. Investigation on pyrolysis and carbonization of eupatorium adenophorum spreng and tobacco stem. J. Energy Inst. <http://www.sciencedirect.com/science/article/pii/S1743967114202480> (accessed Jan 20, 2015).

Piotrowska-Cyplik, A., Olejnik, A., Cyplik, P., Dach, J., Czarnecki, Z., 2009. The kinetics of nicotine degradation, enzyme activities and genotoxic potential in the characterization of tobacco waste composting. Bioresour. Technol. 100, 5037–5044.

Sapci, Z., 2013. The effect of microwave pretreatment on biogas production from agricultural straws. Bioresour. Technol. 128, 487–494.

Shew, H.D., Lucas, G.B., 1991. Compendium of tobacco diseases. American Phytopathological Society, St. Paul.

Termorshuizen, A., Volker, D., Blok, W., Brummeler, E.t., Hartog, B., Janse, J., Knol, W., Wenneker, M., 2003. Survival of human and plant pathogens during anaerobic mesophilic digestion of vegetable, fruit, and garden waste. Eur. J. Soil Biol. 39, 165–171.

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. 99, 7928–7940.

Wellinger, A., Murphy, J.P., Baxter, D., 2013. The Biogas Handbook: Science, Production and Applications. Elsevier, Cambridge.

Yang, B., Li, Y., Zhang, W., Tian, G., Yang, H., Li, Q., 2012a. Experiment exploration on anaerobic digestion of waste tobacco leaves. J. Yunnan Norm. Univ. 32, 28–32 (in Chinese).

Yang, Z., Zhang, S., Liu, L., Li, X., Chen, H., Yang, H., Wang, X., 2012b. Combustion behaviours of tobacco stem in a thermogravimetric analyser and a pilot-scale fluidized bed reactor. Bioresour. Technol. 110, 595–602.

Ye, J., Li, D., Sun, Y., Wang, G., Yuan, Z., Zhen, F., Wang, Y., 2013a. Improved biogas production from rice straw by co-digestion with kitchen waste and pig manure. Waste Manage. 33, 2653–2658.

Ye, X., Liu, H., Meng, Q., Chen, S., Hu, Z., Sun, S., Ma, J., Yu, X., 2013b. Comparison of chemical composition in stalks of different tobaccos. Tob. Sci. Technol., 76–79 (in Chinese)

Zhang, K., Zhang, K., Cao, Y., Pan, W., 2013. Co-combustion characteristics and blending optimization of tobacco stem and high-sulfur bituminous coal based on thermogravimetric and mass spectrometry analyses. Bioresour. Technol. 131, 325–332.

Zheng, Y., Lin, Q., Xie, L., 2004a. Removal and inactivation of TMV by bacterial-algal ecosystem in a wastewater stabilization pond. Acta Sci. Circumst. 24, 1128–1134 (in Chinese).

Zheng, Y., Lin, Q., Xie, L., 2004b. Removing tobacco mosaic virus from wastewater by active sludge treatment process. Acta Sci. Circumst. 24, 625–632 (in Chinese).

Zhong, W., Zhang, Z., Luo, Y., Sun, S., Qiao, W., Xiao, M., 2011. Effect of biological pretreatments in enhancing corn straw biogas production. Bioresour. Technol. 102, 11177–11182.

Zhong, W., Zhu, C., Shu, M., Sun, K., Zhao, L., Wang, C., Ye, Z., Chen, J., 2010. Degradation of nicotine in tobacco waste extract by newly isolated *Pseudomonas sp*. ZUTSKD. Bioresour. Technol. 101, 6935–6941.

Zi, W., Peng, J., Zhang, X., Zhang, L., Liu, J., 2013. Optimization of waste tobacco stem expansion by microwave radiation for biomass material using response surface methodology. J. Taiwan Inst. Chem. Eng. 44, 678–685.