

A Combined Process for Efficient Biomethane Production from Corn Straw and Cattle Manure: Optimizing C/N Ratio of Mixed Hydrolysates

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A novel combined process, consisting of thermal-alkali pretreatment, enzymatic hydrolysis, and anaerobic digestion (AD) was utilized to methanize corn straw (CS) and cattle manure (CM) efficiently. This study aimed to identify the carbon/nitrogen (C/N) ratio of the mixed hydrolysates of CS and CM that maximized methane production in an AD reactor. Additionally, pretreatment conditions for CS and CM were evaluated. The optimum condition of pH 10 was 80 °C and 3 h of thermal-alkali pretreatment to produce 42% of hydrolysis efficiency, while a further enzymatic process increased the efficiency to 72%. The C/N ratio was optimized during the co-digestion of the mixed hydrolysates, and better performances were obtained with a C/N ratio of 11 to 30 having specific methane production from 180 to 280 mL/g COD_{added}. The maximum methane production reached to 280 mL/g COD_{added} at the C/N ratio of 20. Approximately 75% of the total organic matter from the liquid fractions of mixed hydrolysates was converted to methane. Trace elements in CM hydrolysates may also promote the methane yield. This community structure change was proposed to be an internal response for different C/N ratio adaptation. An inappropriate C/N ratio may cause accumulation of free ammonia or volatile fatty acids, which would inhibit methanogens, but not affect the acidogens.

Keywords: Thermal-alkali pretreatment; Enzymatic hydrolysis; Mixed hydrolysates; C/N ratio optimization; Anaerobic digestion

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INTRODUCTION

In recent years, renewable energy recovery from organic waste has received considerable attention because of the growing demand of energy and increasing pollution (Ding *et al.* 2012; Zhang *et al.* 2016a; Tsapekos *et al.* 2017). Methane is a promising alternative energy carrier due to its high-energy yield (39829 KJ/m³) (Song and Zhang 2015). Methane is generated using the anaerobic digestion (AD) of crop residues and livestock manure, which contain a tremendous potential of energy; both lignocellulosic agricultural residues and nutrient-rich cattle manure are abundantly available (Zhai *et al.* 2015; Awais *et al.* 2018). China is a large agricultural country and has the world's largest straw residues. The straw yield is over 800 million tons per year. Corn straw (CS) is one

of the major straw residues in China and contributes about 35% of the total amount (Li *et al.* 2014; Wei *et al.* 2015). Although traditionally CS has been treated by various methods such as energy production, animal feed, and return to agriculture soils, the utilization rate of CS is still less than 50% to 60% (Yuan *et al.* 2015; Tsapekos *et al.* 2017). The yield of cattle manure (CM) from livestock farms is over 382 million tons per year, which accounts for about a third of the total yield of livestock manure (Li *et al.* 2014). However, the improper disposal (such as burning) of CM wastes the resource and pollutes the environment (Fu *et al.* 2015). These wastes have great potential for producing renewable energy and could play an increasingly important role in replacing limited fossil fuels.

The AD process for biogas production offers a potential means of converting agricultural biomass into a renewable energy source, which meets the growing energy needs and reduces environmental concerns (Zhang *et al.* 2015; Mehryar *et al.* 2017; Awais *et al.* 2018). However, there are quite a few problems inherent in the AD of agricultural biomass, such as poor utilization of substrate and low digestion efficiency. The complex and recalcitrant structure of lignocellulosic materials are difficult to be directly utilized by fermentative bacteria during the hydrolysis process (Khatri *et al.* 2015; Wang *et al.* 2015). The hydrolysis reaction is being seen as a rate-limiting process in the AD of lignocellulosic materials (Fu *et al.* 2015). Pretreatments such as thermal-alkali, anaerobic/aerobic composting, steam-explosion, and mechanical methods have been investigated by researchers to promote hydrolysis (Odnell *et al.* 2016; Yang *et al.* 2017). Among these pretreatments, thermal-alkali pretreatment increases methane production of the straw residues efficiently. Thermal-alkali pretreatment opens the chemical bonds between lignin and the other carbohydrate fractions (such as cellulose, hemicellulose, and protein) in raw materials (Krishania *et al.* 2013). Furthermore, mechanical pretreatment is an efficient way to increase the surface area and decreases the crystallinity of lignocellulose, which can accelerate the hydrolysis (Kalamaras and Kotsopoulos 2014).

After pretreatment, the remaining organic solids can be accessed by specific hydrolytic enzymes, producing large amounts of saccharides and other compounds from cellulose and protein into liquid hydrolysates (Wang *et al.* 2015; Zhang *et al.* 2016a). For example, Nkemka and Murto (2013) conducted the AD of liquid hydrolysates from crop stalk after pre-hydrolysis in an anaerobic reactor, which is usually used for organic wastewater treatment. Most studies pay more attention to applying enzymatic hydrolysis to crops for bioethanol production (Abada *et al.* 2018; Awais *et al.* 2018; Shokrkar *et al.* 2018). However, the application of enzymatic hydrolysis to agricultural lignocellulosic wastes and animal manure for biogas production is rarely reported. A combined process of enzymatic hydrolysis with AD will have a better future for obtaining bioenergy from these bio-wastes.

The nutrient and composition of fermentation substrates is vital to achieve an effective and steady biogas production (Risberg *et al.* 2013). Generally, the proper carbon/nitrogen (C/N) ratio for the AD of bio-wastes should be 20 to 30 (Krishania *et al.* 2013; Hassan *et al.* 2017). The AD of high-N substrates, such as livestock and poultry manure, usually results in ammonia accumulation and nutrition imbalance (Zhang *et al.* 2015). In contrast, the AD of high-C substrates, such as crop straw, eventually causes volatile acid accumulation and inhibits the activity of methanogens, which decreases the digestion efficiency (Zhang *et al.* 2015). Compared to adding N-containing chemicals, such as ammonium salt, co-digestion with livestock manure is regarded as a useful way for regulating nutrition (Li *et al.* 2015). In fact, high solid co-digestion has been extensively applied in recent years. Biogas production from the co-digestion of crop straw with

livestock manure is increased more than that from the mono-digestion (Zhou *et al.* 2012; Song and Zhang 2015). However, the research on effect of the C/N ratio on the co-digestion of pre-hydrolyzed crop straw and animal manure has not been reported to the best of our knowledge.

The aim of this study was to identify the C/N ratio of mixed hydrolysates that maximizes methane production in a combined process. Additionally, the pre-treatment conditions for CS and CM were also examined. Firstly, the two-step pretreatment (consisting of thermal-alkali pretreatment and enzymatic hydrolysis) was applied on CS and CM, respectively. Secondly, the mixed substrates were prepared with certain proportions of liquid CS and CM hydrolysates and then fed into an expanded granular sludge blanket (EGSB) reactor for producing methane. Process evaluation parameters such as chemical oxygen demand (COD) yield after pre-hydrolysis, COD removal efficiency, volatile fatty acids (VFAs), ammonia, nutrients, and methane production were investigated in the anaerobic digestion period. The impact of different C/N ratios of mixed hydrolysates on microbial communities and dominant species were analyzed during the EGSB reactor operation.

EXPERIMENTAL

Raw Materials

Raw CS was obtained from a corn field using a maize harvester. Raw CS was dried by oven-drying at 105 °C until at a constant weight. After that, the CS was chopped into about 2-mm-long pieces. These chopped pieces were further ground to less than 1 mm in size and stored in a refrigerator at 4 °C for later use. The mechanical pretreatment can increase the surface area in contact with NaOH alkali during the thermal-alkali pretreatment. Raw CM was collected from a livestock farm in Yancheng, Jiangsu province, China. The CM was filtered using 5 mm stainless steel mesh (Dongmai: mesh-number-4 Nanjiang, China) to remove large particles (such as wood, undigested debris and other garbage) and stored in plastic bucket at -18 °C before being used.

Thermal-alkali Pretreatment

A thermal-alkali pretreatment with NaOH was performed to the CS and CM respectively in order to break down the structure of the raw materials and to increase the solubilization of lignocelluloses, hemicellulose, and protein. Four different pH values (8, 9, 10, and 11) were used in this process. First, 10 kg of raw CS and 10 kg of raw CM were taken into a stainless-steel tank with a working volume of 50 L, respectively. Then, 10 L of water was added to each stainless-steel tank followed by stirring the mixer at a constant speed of 40 rpm. Then, NaOH was added to the stainless-steel tanks and in the amount of 4.3×10^{-5} , 4.2×10^{-4} , 2.2×10^{-3} , 8.7×10^{-2} g and 1.1×10^{-5} , 3.9×10^{-4} , 2.1×10^{-3} , 8.5×10^{-2} g based on the biomass solids (per kg of raw CS and raw CM) to achieve four different pH, respectively. The reactor was equipped with heating systems and the temperatures were kept at the 60 °C for 4 h, 70 °C for 3 h, 80 °C for 3 h, and 90 °C for 2 h (Fig. 1).

Enzymatic Hydrolysis

Raw CS contains higher cellulose and hemicellulose and lower protein, whereas raw CM contains higher protein and cellulose and lower hemicellulose (Li *et al.* 2015; Wang *et al.* 2015).

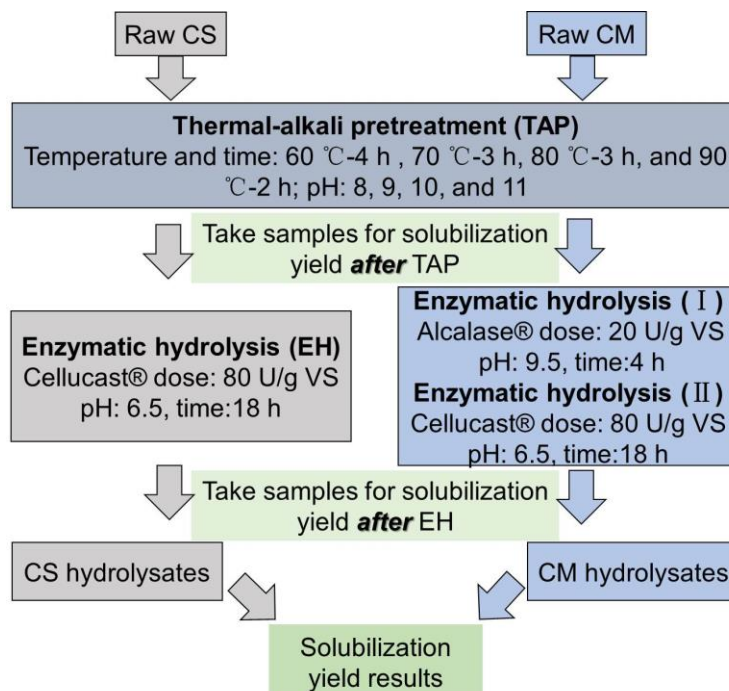


Fig. 1. Scheme of solubilization yields of raw CS and CM hydrolysis process

Alkali applications can break down the structure of cellulose and crude protein and hydrolyze hemicellulose (Ramos-Suárez *et al.* 2017). Two kinds of enzymes (Cellucast® and Alcalase® provided by Novozymes (China) Biotechnology Co., Ltd, Tianjin) were used for the enzymatic hydrolysis after thermal-alkali pretreatment (Fig. 1). The optimum experiment conditions of Cellucast® and Alcalase® were pH (5.5 to 6.5), temperature (50 to 60 °C), time (4 h), and pH (8.5 to 9.5), temperature (55 to 80 °C), time (18 h), respectively.

Table 1. Characterization of Raw CS/CM and CS/CM Hydrolysates

Parameters (g/kg)	Raw CS	Raw CM	CS Hydrolysates	CM Hydrolysates
Total Solids (TS)	251.2 ± 8.5	322.3 ± 10.8	85.4 ± 5.4	102.2 ± 6.8
volatile Solids (VS)	227.4 ± 4.5	258.4 ± 8.4	76.3 ± 4.3	63.2 ± 4.4
Cellulose	54.6 ± 5.2	62.6 ± 4.2	11.6 ± 5.2	15.2 ± 7.6
Hemicellulose	61.7 ± 4.4	73.3 ± 6.3	15.6 ± 6.9	17.4 ± 5.8
Lignin	35.3 ± 3.2	45.4 ± 4.7	N.D. ^a	N.D.
Glucose	N.D.	N.D.	4.6 ± 1.1	0.9 ± 0.1
Xylose	N.D.	N.D.	6.0 ± 1.3	0.8 ± 0.2
Arabinose	N.D.	N.D.	3.0 ± 0.8	0.5 ± 0.1
Total Carbohydrates	2.3 ± 6.3	15.3 ± 2.2	84.4 ± 6.3	67.4 ± 5.7
Total Organic Carbon (TOC)	N.A. ^b	N.A.	53.4 ± 4.1	39.8 ± 3.8
Total Nitrogen (TN)	9.2 ± 2.7	N.A.	1.3 ± 0.2	5.3 ± 0.7
Organic Nitrogen (ON)	N.A.	N.A.	N.A.	1.2 ± 0.6
Ammonia Nitrogen (AN)	0.1 ± 0.1	1.5 ± 0.7	0.2 ± 0.1	1.6 ± 0.8
Free AN (FAN)	N.D.	0.2 ± 0.1	N.D.	0.1 ± 0.1
Protein	37.4 ± 4.6	78.5 ± 7.2	N.A.	7.5 ± 2.2
Total Phosphorus (TP)	1.2 ± 0.7	N.A.	0.5 ± 0.3	0.8 ± 0.4

a: Not detectable; b: Data not available.

The pH was adjusted by adding HCl or NaOH. After the enzymatic hydrolysis, the liquid and solid fraction were separated by a multifilament filter cloth (Yongning model no: PP2400 Zhejiang, China), and the obtained hydrolysates were fed into the EGSB reactor. Results of the characterization of the raw CS/CM and CS/CM hydrolysates are shown in Table 1.

Anaerobic Digestion Tests

A lab scale EGSB reactor was used for the AD in this study. The plexiglass-made EGSB reactor was 60 mm in diameter and 120 cm high with a total volume of 5.0 L and 3.0 L of working volume. The operational temperature was stable at 35 ± 2 °C by an automatic thermostat (Shinko model no: PCD-33A, Osaka, Japan). A peristaltic pump (Longer model no: BT100-2J, Baoding, China) introduced hydrolysates from CS and CM continuously into the EGSB reactor at the column bottom. A gas-washing device collected the gas that was generated at the column top of the EGSB reactor. The scheme diagram of the experimental setup of the combined process is shown in Fig. 2.

The EGSB reactor was initially inoculated with 3 L of anaerobic granular sludge with biomass VSS of 3.57 g/L (VSS/TSS (Volatile suspended solids/Total suspended solids) = 0.92). Twenty-one different compositions of the CS and CM hydrolysates based on volume were designed. Resulting C/N ratios due to different compositions of the CS and CM hydrolysates are evaluated and described in Table 2.

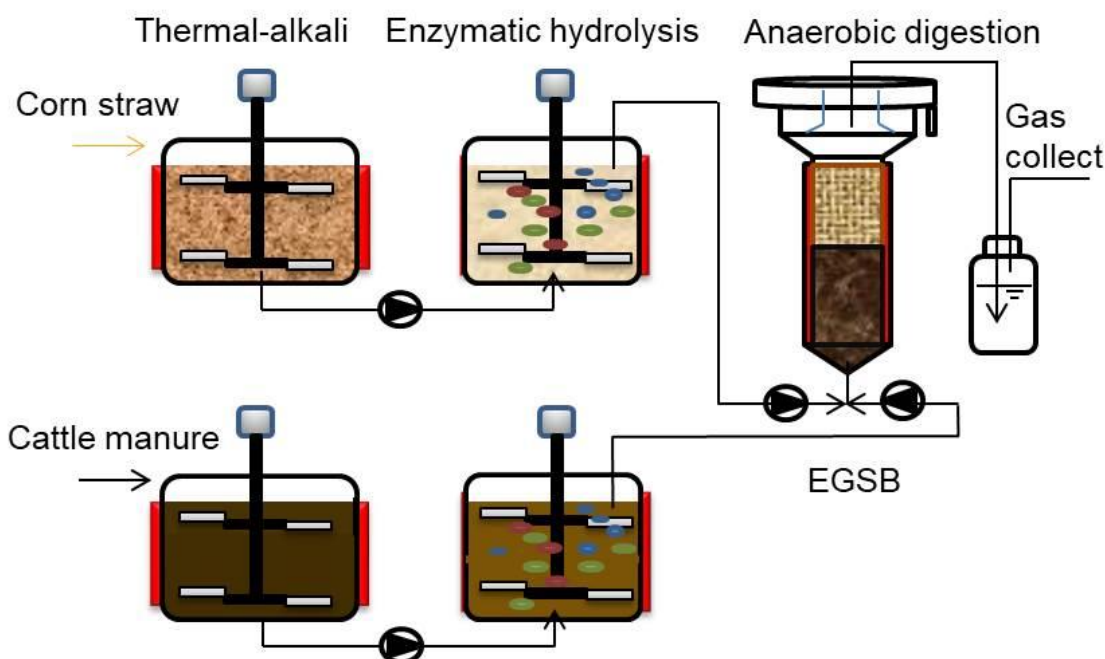


Fig. 2. Scheme diagram of the experimental setup of the combined process

Microbial Community Analysis

The details for the DNA extraction, 16S rRNA gene PCR amplification and Illumina MiSeq sequencing, and data analysis are available in the method described by Yuan *et al.* (2015). The sampling information is shown in Table 3.

Table 2. Anaerobic Digestion Operating Conditions and Performance Details

Run Stage	Composition ratio CS:CM	C/N Ratio	COD (g/L)	COD Removal (%)	pH	VFAs (g/L)	AN (g/L)	FAN (g/L)	CH ₄ Yield (mL/g COD _{added})
Stage I (1-15)	1.00:0.00	46.40 ± 4.21	113.00 ± 8.12	30.67 ± 2.13	6.50 ± 0.50	0.16 ± 0.10	0.63 ± 0.13	0.03 ± 0.01	91 ± 8
Stage I (16-30)	0.95:0.05	44.44 ± 5.63	111.75 ± 7.23	31.34 ± 6.54	5.70 ± 0.70	0.16 ± 0.08	0.78 ± 0.10	0.03 ± 0.01	105 ± 11
Stage I (31-45)	0.90:0.10	42.48 ± 3.14	110.50 ± 8.54	34.22 ± 3.35	6.30 ± 0.50	0.17 ± 0.09	0.92 ± 0.23	0.04 ± 0.01	117 ± 9
Stage I (46-60)	0.85:0.15	40.52 ± 6.86	109.25 ± 6.66	40.56 ± 3.75	5.50 ± 0.40	0.17 ± 0.07	0.94 ± 0.22	0.04 ± 0.01	123 ± 10
Stage II (61-75)	0.80:0.20	38.56 ± 5.23	108.00 ± 7.45	42.44 ± 4.43	5.80 ± 0.60	0.16 ± 0.08	1.01 ± 0.13	0.05 ± 0.01	137 ± 11
Stage II (76-90)	0.75:0.25	36.60 ± 4.24	106.75 ± 6.4	45.55 ± 4.64	5.80 ± 0.50	0.16 ± 0.11	1.04 ± 0.11	0.05 ± 0.01	143 ± 9
Stage II (91-105)	0.70:0.30	34.64 ± 5.74	105.5 ± 5.64	51.60 ± 5.66	6.20 ± 0.80	0.15 ± 0.09	1.08 ± 0.14	0.06 ± 0.01	167 ± 10
Stage II (106-120)	0.65:0.35	32.68 ± 5.43	104.25 ± 6.03	55.98 ± 5.17	6.50 ± 0.40	0.14 ± 0.12	1.12 ± 0.13	0.06 ± 0.01	178 ± 12
Stage II (121-135)	0.60:0.40	30.72 ± 3.76	103.60 ± 6.85	58.58 ± 6.15	6.60 ± 0.50	0.13 ± 0.08	1.18 ± 0.09	0.07 ± 0.01	183 ± 10
Stage III (136-150)	0.55:0.45	28.76 ± 5.25	101.75 ± 5.1	60.34 ± 5.53	6.80 ± 0.90	0.11 ± 0.07	1.26 ± 0.14	0.07 ± 0.01	195 ± 9
Stage III (151-165)	0.50:0.50	26.80 ± 2.56	100.50 ± 4.65	62.89 ± 6.25	7.10 ± 0.80	0.10 ± 0.08	1.32 ± 0.15	0.08 ± 0.02	207 ± 8
Stage III (166-180)	0.45:0.55	24.84 ± 2.94	99.25 ± 5.83	69.70 ± 7.12	7.00 ± 0.30	0.10 ± 0.06	1.36 ± 0.13	0.08 ± 0.01	222 ± 11
Stage III (181-195)	0.40:0.60	22.88 ± 3.53	98.00 ± 5.35	73.22 ± 5.56	7.20 ± 0.50	0.09 ± 0.05	1.38 ± 0.12	0.08 ± 0.02	266 ± 9
Stage III (196-210)	0.35:0.65	20.92 ± 3.24	96.75 ± 4.98	75.65 ± 7.37	7.50 ± 0.40	0.08 ± 0.03	1.47 ± 0.38	0.09 ± 0.01	280 ± 12
Stage IV (211-225)	0.30:0.70	18.96 ± 1.96	95.50 ± 5.66	72.44 ± 6.25	7.40 ± 0.60	0.08 ± 0.04	1.54 ± 0.38	0.09 ± 0.01	272 ± 9
Stage IV (226-240)	0.25:0.75	17.00 ± 1.57	94.25 ± 7.48	72.76 ± 5.53	7.40 ± 0.50	0.09 ± 0.03	1.60 ± 0.11	0.10 ± 0.02	265 ± 10
Stage IV	0.20:0.80	15.04 ± 2.18	93.00 ± 5.47	73.11 ± 4.63	7.40 ± 0.40	0.09 ± 0.02	1.67 ± 0.28	0.11 ± 0.01	268 ± 12

(241-255)									
Stage IV (256-270)	0.15:0.85	13.08 ± 2.04	91.75 ± 6.46	73.51 ± 7.35	7.70 ± 0.80	0.09 ± 0.03	1.71 ± 0.31	0.12 ± 0.02	270 ± 10
Stage IV (271-285)	0.10:0.90	11.12 ± 1.27	90.50 ± 4.65	52.22 ± 5.63	8.40 ± 0.50	0.13 ± 0.05	1.84 ± 0.54	0.13 ± 0.02	180 ± 11
Stage IV (286-300)	0.05:0.95	9.16 ± 1.37	89.25 ± 5.24	45.77 ± 7.85	8.80 ± 0.50	0.16 ± 0.03	1.83 ± 0.54	0.14 ± 0.04	100 ± 8
Stage IV (301-315)	0.00:1.00	7.20 ± 1.18	88.00 ± 5.33	45.30 ± 5.38	8.70 ± 0.80	0.16 ± 0.04	2.15 ± 0.54	0.14 ± 0.03	97 ± 9

Table 3. The Sampling Conditions in Different Stages

Sample Name	Sampling Time	Respective C/N Ratio	CH ₄ Yield (mL/g COD)	COD Removal (%)
MH1	Stage I (Day 60)	40.52 ± 6.85	123 ± 10	40.56 ± 3.75
MH2	Stage II (Day 135)	30.72 ± 3.76	183 ± 10	58.58 ± 6.15
MH3	Stage III (Day 210)	20.92 ± 3.24	280 ± 12	75.65 ± 7.37
MH4	Stage IV (Day 315)	7.20 ± 1.18	97 ± 9	45.30 ± 5.38

Analytical Methods

The pH value was directly measured using a pH meter (Mettler-Toledo, Model No: FE20, Shanghai, China). The TSS, VSS, TS, VS, COD, Ammonia Nitrogen (AN), and total alkalinity were determined according to the APHA standard methods (2005). The TOC and TN were analyzed with a total organic carbon analyzer (Elementar, Model No: Liqui TOC II, Hanau, Germany). The contents and composition of the VFAs were analyzed by a gas chromatograph (Shimadzu, Model No: GC-2010 Plus, Kyoto, Japan) with a flame ionization detector and a Stabilwax DA capillary column (Restek Corporation, PA, USA). The samples of liquid hydrolysates were centrifuged at 10000 rpm for 10 min at room temperature and filtered through a 0.45 μm fiberglass filter for COD, AN, TOC, TN, and VFAs analysis. The chemical composition of the CS and CM before and after pretreatment was determined by the method described by Van Soest *et al.* (1991) using a raw fiber determination extraction system (Lai-Heng, Model No: L-807, Beijing, China). The biogas was pretreated in a desiccant-filled water trap and analyzed using a gas chromatography (Agilent, Model No: 6890, Santa Clara, CA, USA) equipped with a thermal conductivity detector (TCD) to measure the methane (CH₄) content.

The solubilization yield was determined using the VS (Volatile Solids) of the supernatant and the total slurry after pretreatment, using the following equation,

$$\text{Solubilization Yield \%} = (\text{VS}_S/\text{VS}_T) \times 100\% \quad (1)$$

in which VS_S is the VS of the supernatant (%), VS_T is the VS of the total slurry (%).

RESULTS AND DISCUSSION

Thermal-alkali Pretreatment and Enzymatic Hydrolysis

The time, pH value, and temperature of thermal-alkali pretreatment influence the solubilization yield of CS and CM before enzymatic hydrolysis. An experimental design was employed in this study as shown in Fig. 1 and Table 4 to examine the influence of each time under different temperatures and pH values. After thermal-alkali pretreatment, the range of the solubilization yield of CS and CM was respectively 33 to 52% and 37 to 46% at pH of 8 and 9, whereas 55 to 61% and 45 to 64% were at a pH of 10 and 11. The enzymatic hydrolysis for CS contributed 7% to 10% of solubilization at 4 h at 60 °C and 3 h at 70 °C conditions, while there was 11% to 16% more solubilization generated from enzymatic hydrolysis at 3 h at 80 °C and 2 h at 90 °C conditions. The results indicated that the thermal-alkali pretreatment for CS (3 h at 80 °C and 2 h at 90 °C, pH 10 and 11) resulted in hydrolysis efficiency ranging from 27% to 42%, while a subsequent enzymatic reaction increased the efficiency to 72%. The effect of different thermal-alkali conditions on solubilization of CM was close to CS. Thermal-alkali conditions of pH 11 and 90 °C contributed little more solubilization than the condition of pH 10 and 80 °C. Therefore, the condition of pH 10, 3 h, and 80 °C was selected for the thermal-alkali pretreatment.

Table 4. Solubilization Yields of CS and CM Responding to Different Conditions

CS Solubilization Yields (%)	60 °C 4 h		70 °C 3 h		80 °C 3 h		90 °C 2 h	
	a. TAP	a. EH	a. TAP	a. EH	a. TAP	a. EH	a. TAP	a. EH
pH=8	33.43	40.29	35.56	43.17	37.28	48.43	40.43	52.33
pH=9	41.28	48.33	48.76	55.23	51.53	61.14	52.11	62.27
pH=10	55.28	62.20	56.24	66.87	56.40	72.23	55.43	67.56
pH=11	56.67	61.15	58.47	66.56	60.35	72.27	61.15	71.72
CM Solubilization Yields (%)	60 °C 4 h		70 °C 3 h		80 °C 3 h		90 °C 2 h	
	a. TAP	a. EH	a. TAP	a. EH	a. TAP	a. EH	a. TAP	a. EH
pH=8	37.14	50.33	40.43	52.41	38.26	54.30	42.22	55.55
pH=9	41.23	53.43	43.60	55.33	45.24	56.13	46.27	57.65
pH=10	45.38	72.56	48.65	73.65	58.45	80.20	61.63	79.34
pH=11	61.44	72.54	63.32	69.81	63.35	76.47	64.28	75.21

a. TAP: After Thermal-alkali pretreatment; a. EH: After Enzymatic hydrolysis

Thermal-alkali pretreatment followed by enzymatic hydrolysis was an efficient combined pretreatment for CS and CM. The concentrations of the main compounds contained in the CS and CM hydrolysates are shown in Table 1.

The presence of lignin in the hydrolysates can limit its use for producing methane by anaerobic digestion (Wang *et al.* 2015; Ramos-Suárez *et al.* 2017). After enzymatic treatment, the lignin-containing solid fraction was separated by a multifilament filter cloth. Lignin was obviously not found in the CS and CM hydrolysates. The TS and VS of CS hydrolysates were about 34% and 33% of raw CS. More than 80% of cellulose and 75% of hemicellulose were converted to carbohydrates after the hydrolysis process. Meanwhile, the TS and VS of the CM hydrolysates were about 31% and 24% of raw CM. Nearly 90% of the proteins in raw CM were decomposed in the hydrolysis process. In addition, the conversion rate of cellulose and hemicellulose were similar to CS.

Effects of Different C/N Ratios of Mixed Hydrolysates on AD Performance

The C/N ratio, as an indispensable parameter of AD, regulates the nutrient balance. The C/N optimization for the AD of mixed hydrolysates is thus an important issue to be addressed. The operational periods at different C/N ratios ranging from 46.4 to 7.20 were applied to the EGSB reactor for 340 days. The operation of the EGSB was outlined in four stages. The HRT involved in all stages was 1.5 days. The effects of different C/N ratios of mixed hydrolysates on AD performance are shown in Table 2 and Fig. 3. At stage I (1 to 60 days), the EGSB was initially operated at C/N ratios from 46.40 to 40.5 and achieved 40.6% of COD removal. After day 60, VFAs accumulated up to 0.17 g/L and the methane yield dropped to 123 mL/g COD_{added}. The results showed that VFAs accumulation caused a decrease in pH from 6.5 to 5.5 at this high C/N ratios stage (Table 2). An acidification environment could lead to the inoculation sludge washout and deterioration of the AD process. AN is a nitrogen source for maintaining the activity and growth of methanogens and is a pH-controlling agent for neutralizing the accumulated VFAs (Zhang *et al.* 2015). A lower methane yield at a higher C/N ratio suggested that a nitrogen deficiency could have limited the specific methanogenic activity (Hassan *et al.* 2017).

At stage II (61 to 135 days), when the C/N ratio of the EGSB reactor was decreased from 38.6 to 30.7, the COD removal increased from 42.4 to 58.6%. The VFAs decreased to 0.13 g/L and the methane yield increased to 183 mL/g COD_{added}. The VFAs in the digester effluent were mainly constituted of propionate and acetate, which contributed about 85% of TOC (data not show). The inoculation sludge quantity was steady in the EGSB reactor at this stage. At stage III (136 to 210 days), as the C/N ratio decreased from 28.8 to 20.9, the COD removal increased from 60.3 to 75.7%. The methane yield increased significantly from 195 to 280 mL/g COD_{added}. This stage doubled the methane yield compared with stage I. Meanwhile, the VFAs decreased to 0.08 g/L and acetate was the main form. For an efficient AD process, the VFAs usually maintained at a lower level, since the VFAs generated from acidification phase can be utilized by methanogens. The neutral pH was beneficial to the growth of methanogens. In this stage, the pH was about 7 (Table 2), suggesting that improvement of C/N ratio helped to maintain stable pH and buffering ability. Former studies have shown that the C/N ratio ranging from 20 to 30 is the optimum parameter for the AD process (Habiba *et al.* 2009; Wang *et al.* 2012).

At stage IV (211 to 315 days), when the C/N ratio was reduced from 19.0 to 13.1 (211 to 270 days), the EGSB reactor still maintained 72.4 to 73.5% removal of COD. The production of methane was stabilized at about 270 mL/g COD_{added}. When the C/N ratio was further reduced from 11.1 to 7.20 (271 to 315 days), the COD removal decreased from 52.2 to 45.3%. On the 285th day, the VFAs returned to 0.16 mg/L but the methane yield decreased to 97 mL/g COD_{added}. FAN and AN are the two main forms of inorganic ammonia nitrogen in the hydrolysates. FAN has been regarded as the major inhibitor in an AD reactor (Li *et al.* 2015). FAN could diffuse into microorganisms, causing potassium metabolism disturbance (Zhang *et al.* 2016b; Gao *et al.* 2015). FAN does not have a significant effect on acidogens but it exerts a stronger inhibition effect on methanogens (Tsapekos *et al.* 2017; Zhou *et al.* 2012). At stage IV, the maximum FAN concentration was up to 0.14 g/L, which was close to the inhibition level of 0.15 g/L (Gao *et al.* 2015). Accumulated ammonia generated in anaerobic degradation of organic nitrogen in the CM hydrolysates would cause a rise in pH. Thus, the pH increased from average 7.40 to 8.70 at this stage. A higher pH would have a negative impact on methane yield. Therefore, it can be concluded that the AD system with a low C/N ratio was inhibited by the accumulation of FAN instead of VFAs. In this study, the methane yield of 180 to 280 mL/g

COD_{added} is observed within the C/N range of 11 to 30, which is wider than that of 20 to 30 in previous studies (Habiba *et al.* 2009; Wang *et al.* 2012; Krishania *et al.* 2013; Hassan *et al.* 2017). The maximum methane production reached to 280 mL/g COD_{added} at the C/N ratio of 20.

In order to find other factors that affect the AD of the mixed hydrolysates, the trace elements in CS and CM hydrolysates were tested. The result showed that the trace elements content was higher in CM hydrolysates than that in CS hydrolysates (Table 5). It is an efficient way to enhance AD of lignocellulosic wastes by adjusting micronutrients. In this study, the results show that as the C/N ratio decreased from 30 to 10, the COD removal efficiency and methane yield increased significantly. Therefore, it can be inferred that by co-digestion with CM hydrolysates, the added trace elements increased efficiency of the AD of CS hydrolysates. Zhang *et al.* (2016b) also found similar results that trace elements in CM such as Mg, Ca, Co, Zn, etc also contribute to the improvement in methane yield.

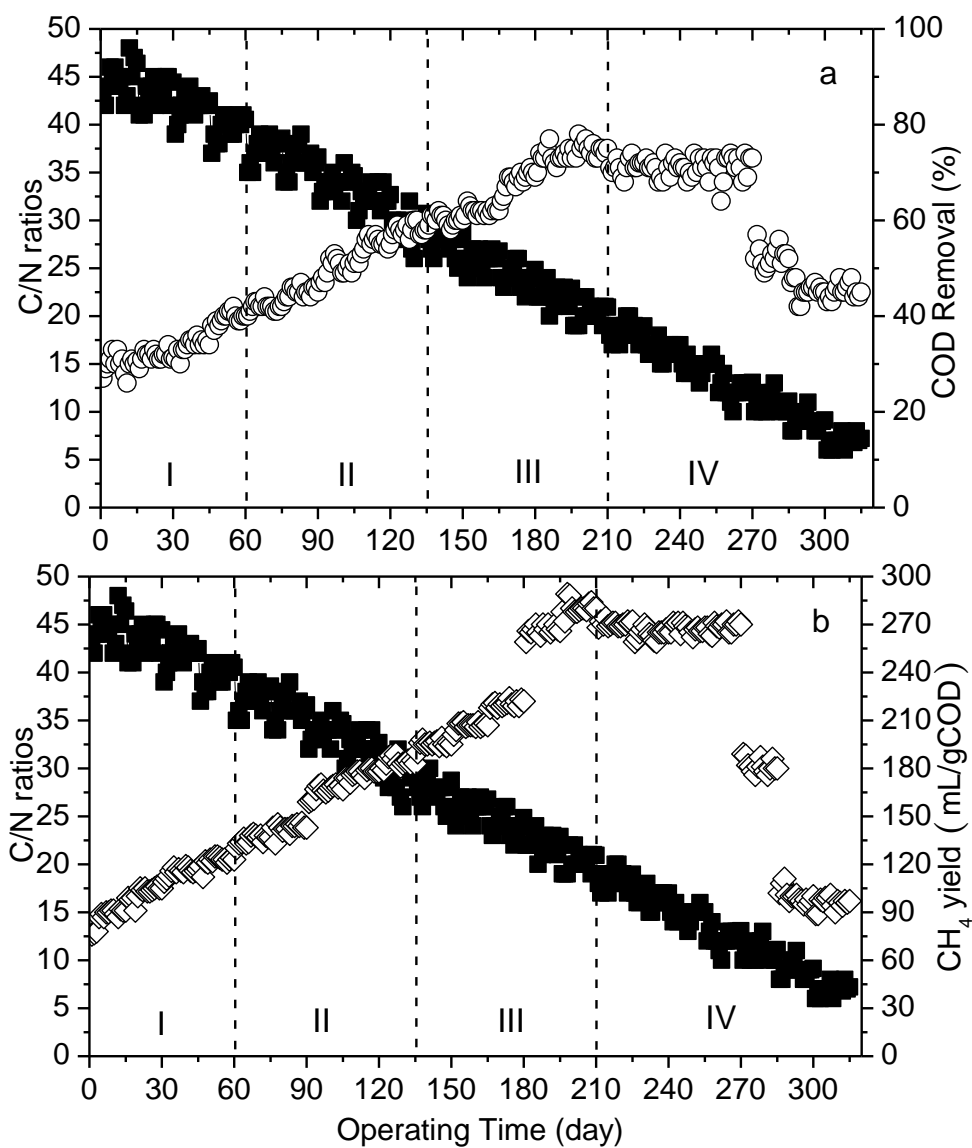


Fig. 3. The AD performance of mixed hydrolysates under various C/N ratios. (a) C/N ratios (■), COD Removal (○); (b) C/N ratios (■), CH₄ yield (◇)

Table 5. Trace Elements in CS/CM Hydrolysates and Mixed Hydrolysates in different stages

Elements	CSH	CMH	CCH			
			Stage I (90th day)	in Stage II (135th day)	in Stage III (210th day)	In Stage IV (300th day)
Na	0.11 ± 0.03	0.31 ± 0.08	0.14 ± 0.02	0.19 ± 0.07	0.24 ± 0.09	0.30 ± 0.11
K	7.67 ± 1.18	14.20 ± 2.11	8.65 ± 1.13	10.28 ± 1.86	11.91 ± 2.16	13.87 ± 2.67
Mg	2.13 ± 0.55	8.21 ± 1.78	3.04 ± 0.59	4.56 ± 0.88	6.08 ± 1.12	7.91 ± 1.33
Ca	4.17 ± 0.35	27.7 ± 5.32	7.70 ± 1.23	13.58 ± 2.45	19.46 ± 3.78	26.52 ± 4.15
Mn	0.77 ± 0.12	3.58 ± 0.89	1.19 ± 0.12	1.89 ± 0.14	2.60 ± 0.56	3.44 ± 1.01
Fe	0.78 ± 0.14	2.98 ± 0.87	1.11 ± 0.12	1.66 ± 0.09	2.21 ± 0.75	2.87 ± 0.98
Co	0.83 ± 0.14	0.43 ± 0.08	0.77 ± 0.06	0.67 ± 0.14	0.57 ± 0.06	0.45 ± 0.07
Zn	28.4 ± 0.94	69.2 ± 5.44	34.52 ± 5.18	44.72 ± 4.42	54.92 ± 5.14	67.16 ± 5.18

CSH: CS Hydrolysates; CMH: CM Hydrolysates; CCH: CS and CM Hydrolysates; The unit for Ca, Mg, K, Na and Fe was g/kg TS. The unit for Zn, Co and Mn was mg/kg TS.

Microbial Community Structure and Dominant Species Analysis

Anaerobic digestion and C/N optimization is a complex process. It is related to a certain C/N ratio and to the substrates utilizing the character of the microbial communities. This change in community structure can reflect an internal response for different C/N ratio adaptation. Each of the samples, MH1 (sample name), MH2, MH3, and MH4, were collected in the EGSB reactor in stage I (Day 60), stage II (Day 135), stage III (Day 210), and stage IV (Day 315), respectively (Table 3). The sequence numbers in the four samples of MH1, MH2, MH3, and MH4 were 19456, 20187, 22470, and 20527, respectively. The relative operational taxonomic units (OTUs) number comparison of the four samples were MH1 > MH2 > MH3 > MH4. To examine the dynamic response of the microbial communities at the four different stages, the classified OTUs were analyzed at the family level (Fig. 4). Meanwhile, to identify the function of the dominant species, the classified OTUs were analyzed at the genus level (Table 6).

In stage I, sample MH1 was collected at a higher C/N ratio of 40.5. The results showed the relatively high abundances for Anaerolineaceae (19.31%), Clostridiaceae (18.2%), and Spirochaetaceae (17.1%) families in the EGSB reactor (Fig. 4). The *Anaerolinea* (10.1%), *Anaerobacter* (17.3%), and *Spirochaeta* (5.13%) species as acidogens were enriched in the EGSB reactor and capable of converting complex organic carbon sources (*i.e.* hemicellulose and xylose) into VFAs (*i.e.* acetate and propionate) (Yamada and Sekiguchi 2009; Yuan *et al.* 2014) (Table 6). Accumulated VFAs generated by the acidogens would inhibit the methanogens. The *Methanobacterium* (4.23%) and *Methanomethylovorans* (4.53%) species only showed relatively lower abundance (Table 6). Sun *et al.* (2010) found that when the C/N ratio was higher than 40, acidogens was dominant, and the methane production had a certain influence.

In stage II, the relative abundances for Anaerolineaceae (15.9%), Clostridiaceae (14.5%), and Spirochaetaceae (14.6%) families were decreased with the C/N ratio

decreasing from 40.5 to 30.7 (Fig. 4). However, the relative abundances for the Methanobacteriaceae (19.0%) and Methanosarcinaceae (15.5%) families were obviously increased at this stage (Table 6). The *Anaerolinea* (5.21%), *Anaerobacter* (5.02%), and *Spirochaeta* (3.22%) species still dominated in the EGSB reactor at this stage. The *Methanobacterium* (13.2%) and *Methanomethylovorans* (9.53%) began to be enriched, and methane was fully produced in this stage (Table 6). In stage III, the relative abundances for Anaerolineaceae (17.6%), Clostridiaceae (15.1%), and Spirochaetaceae (12.2%) families remained stable with the C/N ratio decreasing from 30.7 to 20.9 (Fig. 4). Meanwhile, the relative abundances for the Methanobacteriaceae (22.4%) and Methanosarcinaceae (11.8%) families were still increased (Fig. 4). The *Anaerolinea* (10.1%), *Anaerobacter* (17.3%), *Spirochaeta* (5.13%), and *Cloacibacillus* (3.41%) species were also dominant in this stage. The *Methanobacterium* (16.2%) and *Methanomethylovorans* (12.1%) reached a high point and methane was produced at a high level in this stage (Table 6). The compositions of the communities and predominant genus in stage III were similar to those in stage II. These results are in good agreement with those reported by Wang *et al.* (2012), who also found a higher abundance of methanogens at the C/N ratio of 20 to 30.

Table 6. Phylogenetic Classification of the 16S rRNA Gene Sequences at Genus Level (relative abundance > 1%) in the MH1, MH2, MH3, and MH4

Phylum	Family	Genus (%)	MH1	MH2	MH3	MH4
<i>Chloroflexi</i>	Anaerolineaceae	<i>Anaerolinea</i>	10.06	5.21	4.38	11.55
<i>Chloroflexi</i>	Anaerolineaceae	<i>Thermomicrobia</i>	1.05	2.11	3.96	2.45
<i>Chloroflexi</i>	Anaerolineaceae	<i>Levilinea</i>	1.09	2.91	1.47	2.01
<i>Firmicutes</i>	Clostridiaceae	<i>Anaerobacter</i>	17.33	5.02	8.03	15.06
<i>Firmicutes</i>	Clostridiaceae	<i>Clostridium</i>	3.98	1.42	2.29	2.01
<i>Firmicutes</i>	Acidaminococcaceae	<i>Megamonas</i>	3.22	2.23	3.11	1.12
<i>Firmicutes</i>	Peptostreptococcaceae	<i>Acetoanaerobium</i>	1.21	1.34	1.01	2.21
<i>Firmicutes</i>	Eubacteriaceae	<i>Acetobacterium</i>	1.35	1.55	1.15	1.39
<i>Spirochaetae</i>	Spirochaetaceae	<i>Spirochaeta</i>	5.13	3.22	3.76	6.11
<i>Spirochaetae</i>	Spirochaetaceae	<i>Lewinella</i>	1.55	1.98	2.13	3.11
<i>Spirochaetae</i>	Spirochaetaceae	<i>Aureispira</i>	2.98	1.11	2.78	3.33
<i>Synergistetes</i>	Synergistaceae	<i>Cloacibacillus</i>	3.41	4.49	6.43	4.39
<i>Synergistetes</i>	Synergistaceae	<i>Synergistes</i>	2.48	2.92	4.06	4.43
<i>Synergistetes</i>	Synergistaceae	<i>Thermovirga</i>	1.49	1.18	2.39	1.72
<i>Euryarchaeota</i>	Methanobacteriaceae	<i>Methanobacterium</i>	4.23	13.21	16.21	7.78
<i>Euryarchaeota</i>	Methanosarcinaceae	<i>Methanomethylovorans</i>	4.53	9.53	12.14	5.21
<i>Bacteroidetes</i>	Porphyromonadaceae	<i>Macellibacteroides</i>	1.05	3.25	2.33	2.55
<i>Proteobacteria</i>	Desulfomicrobiaceae	<i>Desulfomicrobium</i>	2.51	4.21	3.84	4.66
<i>Proteobacteria</i>	Desulfobulbaceae	<i>Desulfobulbus</i>	2.25	2.65	3.94	5.11

In stage IV, the relative abundances for Anaerolineaceae (22.1%), Clostridiaceae (18.7%), and Spirochaetaceae (14.3%) families were increased with the C/N ratio decreasing from 20.92 to 7.2 (Fig. 4). However, the relative abundances for the Methanobacteriaceae (9.69%) and Methanosarcinaceae (6.74%) families were decreased. The *Anaerolinea* (11.55%), *Anaerobacter* (15.1%), and *Spirochaeta* (6.11%) species were obviously dominant in the EGSB reactor. The *Methanobacterium* (7.78%) and *Methanomethylovorans* (5.21%) species began to drop gradually in this stage (Table 6). Vivekanand *et al.* (2017) found that a lower abundance of methanogens was observed when using manure as single substrate at a lower C/N ratio. A lower C/N ratio would bring a higher level of ammonia. The *Methanobacterium* and *Methanomethylovorans*, respectively,

belong to hydrogenotrophic and acetotrophic methanogens (Rodríguez *et al.* 2012). Previous findings demonstrated that acetotrophic methanogens were less tolerant to ammonia stress than hydrogenotrophic methanogens (Tsapekos *et al.* 2017).

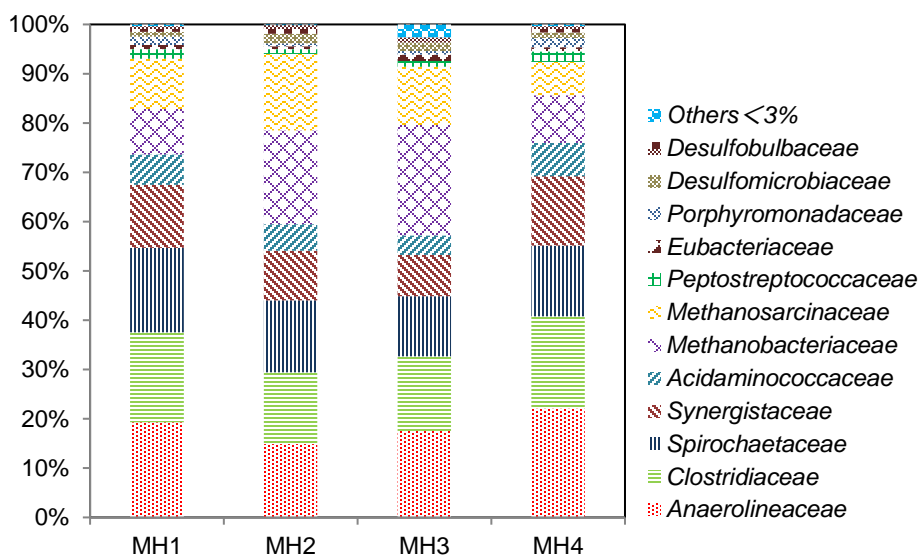


Fig. 4. Microbial community structure of the EGSR reactor at the family level in different stages

CONCLUSIONS

1. This study proposed a novel combined process to methanize CS and CM efficiently. Thermal-alkali pretreatment and enzymatic hydrolysis helped to break down the structure of the cellulose, hemicellulose, and protein, which were further converted into small organic molecules in the liquid hydrolysates.
2. The CS and CM hydrolysates could be transformed into methane via AD in an EGSR reactor. Co-digestion of the CS and CM hydrolysates was optimized based on the C/N ratio, and better performances were obtained at the C/N ratio of 11 to 30, which is wider than that of 20 to 30 in previous studies.
3. In the different stages of C/N ratio, the dominant microbial communities of acidogens slightly changed. The dominant status of methanogens was observed during stage II and stage III, coinciding with the optimum C/N ratio. Accumulated FAN and VFAs would inhibit methanogens, but they would not affect the acidogens.
4. This study represents a new try, and the approach has significant meaning and is worth studying deeply, although the economic advantage is yet unclear. The digestion kinetics and energy balance will be investigated at the optimized C/N ratio in the future work.

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