**Mesophilic and thermophilic anaerobic digestion of aqueous phase generated from hydrothermal liquefaction of cornstalk: Molecular and metabolic** **insights**

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**ABSTRACT**

The critical challenge of hydrothermal liquefaction (HTL) for bio-oil production from biomass is the production of large amounts of aqueous products (HTL-AP) with high organic contents. The present study investigated the anaerobic digestion (AD) performances of HTL-AP under both thermophilic and mesophilic conditions, and molecular and metabolic analysis were conducted to provide insights into the different performances. The results showed that thermophilic AD had lower COD removal efficiency compared to mesophilic AD (45.0 % vs. 61.6%). Liquid chromatography coupled with organic carbon detection and organic nitrogen (LC-OCD-OND) analysis showed that both high molecular weight (HMW) and low molecular weight (LMW) compounds were degraded to some extent and more LMW acids (LMWA) and recalcitrant aromatic compounds were degraded in the mesophilic reactor, which was the main reason of higher COD removal efficiency. Phenyl compounds (e.g. phenol and 2 methoxyphenol), furans and pyrazines were the recalcitrant chemicals detected through GC-MS analysis. Fourier transform ion cyclone resonance mass spectrometry (FT-ICR-MS) analysis demonstrated the complexity of HTL-AP and the proportions of phenolic or condensed aromatic compounds increased especially in the thermophilic effluents. Metabolites analysis showed that the reasons contributing to the differences of mesophilic and thermophilic AD were not only related to the degradation of organic compounds (e.g. benzoate degradation via CoA ligation) in HTL-AP but also related to the microbial autogenesis (e.g. fatty acid biosynthesis) as well as the environmental information processing. In addition, the enrichment of *Mesotoga*, responsible for the high degradation efficiency of LMWA, and *Pelolinea*, involved in the degradation of phenyl compounds, were found in mesophilic reactor, which was consistent with higher removal of corresponding organics.

**Keywords:** Hydrothermal liquefaction aqueous phase; Mesophilic/thermophilic anaerobic digestion; Recalcitrant compounds; Metabolic pathways

1. **Introduction:**

Increasing energy demand has motivated researchers to consider adopting eco-friendly alternative renewable energies. Biomass is the most plentiful source for sustainable renewable energy system, and it can be converted into liquid, solid and gaseous fuels (bio-oil, bio-char, syngas, etc.) through thermochemical technologies (Akhtar and Amin 2011, Chen et al. 2015, Jayaraman and Gökalp 2015, Xu and Lad 2007). Cornstalk is one of the most abundant wet agricultural residues and lignocellulosic biomass worldwide (Zhu et al. 2017). Hydrothermal liquefication (HTL) is a water-based treatment by converting wet biomass into bio-oil and hydro-char (Peterson et al. 2008). Around 20-50% of the organics were transferred into the aqueous phase (HTL-AP) during the HTL process (Chen et al. 2019, Chen et al. 2017). The compositions of HTL-AP were fairly complicated including direct products, intermediates, as well as secondary polymeric compounds such as saccharides, volatile fatty acids, alcohols, cyclopentenones, phenols and protein broken products depending on the feedstocks and reaction conditions (Cao et al. 2017b, Leng et al. 2018). Direct discharge of HTL-AP would waste resources and deteriorate the environment. Appropriate treatment of HTL-AP can reduce pollution as well as mediate energy input burden of the thermochemical process.

Anaerobic digestion (AD) is a promising method applied for HTL-AP treatment with energy recovery in the form of biogas (Chen et al. 2016, Chen et al. 2015, Si et al. 2016). The HTL-AP of straw was used for biogas production under mesophilic conditions giving an average methane yield of 184 mL/g COD and COD removal efficiency of around 53% (Chen et al. 2017). The biogas production from HTL-AP was strongly dependent on the HTL temperatures since less biodegradable organics could be produced at higher HTL temperatures (>290 oC) (Chen et al. 2019, Chen et al. 2017, Si et al. 2018, Usman et al. 2019). Generally, higher HTL temperature was necessary to obtain a higher bio-oil yield (Cao et al. 2017b, Usman et al. 2019). The methane yield of HTL-AP decreased from 286 mL/g COD to 136 mL/g COD when the HTL temperature was increased from 170 °C to 320 °C (Chen et al. 2019). It should be noted that most of recent researches on methane production from HTL-AP were focused on mesophilic digestion (35–37 oC) (Chen et al. 2019, Tommaso et al. 2015, Usman et al. 2019). Considering the high temperature of HTL-AP separated from the HTL mixture products, thermophilic digestion (50–55 oC) might also be applied to convert the HTL-AP. The degradation efficiencies of organics in HTL-AP under both mesophilic and thermophilic conditions is still not known. Previous studies have investigated the mesophilic and thermophilic AD of different organic wastes. It was reported that the methane yields of corn straw and wheat straw at thermophilic condition were higher than the yields at mesophilic condition (Li et al. 2016, Pohl et al. 2012). However, some studies drew opposite conclusions. In one case, it was found that the thermophilic digestion of food waste gave a lower methane yield (448 mL CH4/g VS) compared to the mesophilic digestion (480 mL CH4/g VS) (Zamanzadeh et al. 2016). Furthermore, previous studies comparing mesophilic and thermophilic AD focused mainly on the differences in the removal of COD, the change of biogas yields and the microorganisms. An in-depth understanding of the mesophilic digestion and thermophilic digestion is still lacking (Wirth and Reza 2016, Zamanzadeh et al. 2016).

The components of HTL-AP are fairly complex with a wide range of molecular weights and most of the organic compounds have not been well characterized (Cao et al. 2017a, Wirth et al. 2015). Very few publications have provided insight into the molecular structures in HTL-AP or the relationships between the structures of compounds, microbial communities and their metabolic processes, even at mesophilic condition. Recent analytical techniques including liquid chromatography coupled with organic carbon detection and organic nitrogen (LC-OCD-OND) and Fourier transform ion cyclone resonance mass spectrometry (FT-ICR MS) can help us reveal the detailed molecular structures of the compounds produced in biogas reactors, which would facilitate our understanding of the nature of the reactions in mesophilic and thermophilic AD (Kamjunke et al. 2017, Lu et al. 2018). Metabolic analysis might also provide more information for the different AD performances at mesophilic and thermophilic conditions. As the final products of various biological processes, metabolites hold promise as accurate biomarkers that reflect upstream biological events such as environmental changes (Beale et al. 2016). Therefore, the metabolism of microorganisms might help us to understand the inherent differences of the thermophilic and mesophilic digestion processes. Besides, the metabolomic analysis is mainly used in the culture of pure bacteria or targeted drug metabolism research and the study of a mixed AD system with complex feedstock is still rare (Liu et al. 2017, Wang et al. 2017).

Based on the above considerations, the AD of HTL-AP obtained from cornstalk was investigated under both thermophilic and mesophilic conditions in the present study. The organic compositions and properties that contributed to the digestion difference at both conditions were identified by the combination of various technologies including GC-MS, LC-OCD-OND and FT-ICR-MS. In addition, a pool of metabolites that underwent significant changes in biological systems in response to mesophilic and thermophilic AD were established and the specific metabolic reactions contributed to the difference of mesophilic and thermophilic AD were retrieved from KEGG database. Finally, the microbial communities at both mesophilic and thermophilic conditions were also compared. The present study aimed to provide in-depth understanding of AD of HTL-AP at mesophilic and thermophilic conditions.

1. **Materials and methods**
	1. **HTL-AP feedstock preparation**

Cornstalk was used as the substrate. HTL was carried out in a 3.0 L batch reactor. The HTL reaction temperature was controlled at 300 °C for 60 min, and a high yield of bio-oil could be obtained at similar condition based on a previous study (Zhu et al. 2015). The vessel was removed out and quenched rapidly to room temperature via internal cooling water circulator after 60 min. Subsequently, solid and liquid products were separated by centrifugated at 10000 rpm for 10 min. The supernatant was recovery as HTL-AP and kept at 4 °C until used.

* 1. **Experimental set-up**

Two 1.2L up flow anaerobic sludge blanket (UASB) reactors were made of stainless steel with a working volume of 1.0 L. The reactors were maintained at 35 °C and 55 °C, respectively, using a water jacket. The influent COD was controlled to be 10.0 g/L throughout the experiment. The anaerobic granular sludge obtained from UASB reactor treating cassava stillage in an ethanol plant (Taicang, Suzhou, China) was used as inoculum. The anaerobic granular sludge was pre-cultured by glucose in the UASB reactors at 35 oC and 55 oC. Then, the two reactors were operated with 25%, 50% and 75% of HTL-AP diluted with glucose solution based on a COD basis. The 100% of HTL-AP was applied after long time reactor stability of 75% of HTL-AP operation. The reactors were fed at a flow rate of 250 mL HTL-AP /d corresponding to the hydraulic retention time of 4 days. The pH value of HTL-AP was adjusted to 7.5 by 2M NaOH.

* 1. **LC-OCD-OND analysis**

The HTL-AP and effluents from AD reactors were quantified for the specific molecular weight fractions by LC-OCD-OND system (DOC-LABOR, Karlsruhe, Germany). The organic separation was based on size-exclusion chromatography (SEC) followed by multi detection with organic carbon (OCD), UV-absorbance at 254 nm (UVD) and organic bound nitrogen (OND). The Chrom CALC software (DOC-LABOR, Karlsruhe, Germany) was used for data acquisition and analysis. All the values referred to mass of organic bound carbon (OC). The identified organic fractions included dissolved organic carbon (DOC), hydrophobic organic carbon (HOC), chromatographic dissolved organic carbon (CDOC), inorganic colloids, and additional parameter of SUVA. DOC was determined in the column bypass after in-line 0.45 μm filtration. The organic carbon retained on the column was defined as HOC. Inorganic colloids with negatively charged inorganic polyelectrolytes, polyhydroxides and oxidhydrates of Fe, Al, S or Si were detected by UV light-scattering. CDOC was the OC value obtained by area integration of the total chromatogram.

* 1. **FT-ICR-MS analysis**

HTL-AP and mesophilic and thermophilic effluent samples, which were diluted into the same COD values, were further analyzed using a 9.4 T Bruker apex-ultra FT-ICR-MS equipped with an electrospray ionization source (Bruker 155 Apollo II) in negative mode. Typically, 180 μL/hsamples were injected into the electrospray source and the voltages of capillary was stable to be 4.5 kV. Spectra were improved to 128 scans and mass range of 150−800 Da were considered. Molecular formulas of compounds in AD influent and effluents were calculated using a custom software (Zhang et al. 2012). Solvent blanks and C18 SPE extraction blanks were measured. Methanol blank analyses were performed to check whether the instrument was clean prior to analyzing the samples. Methodologies for FT-ICR MS mass calibration, data acquisition, elemental combinations and double bond equivalence calculation rules through the application of criteria formulas have been described in previous work(Li et al. 2018a). The compound groups discussed in the following part were delineated by elemental ratios, aromaticity index (AI), double bond equivalence (DBE) derived from plants, highly unsaturated and phenolic compounds, lipids, protein/amino sugars, carbohydrates, and lignin according to the previous rules (Li et al. 2018a).

* 1. **Metabolomic analysis**

Five thermophilic (Group T) and five mesophilic (Group M) sludge samples were obtained from the two UASB reactors during the steady-states, respectively, and they were then used for metabolomic analysis. A 40 mg of sample was homogenized in 400 μL water containing 10 μg/mL of L-norvaline as ab internal standard. Following centrifugation at 14000 g and 4 °C for 15 min, a total of 300 μL supernatant was transferred. The extraction was repeated and the supernatants from the two extractions were combined. A 100 μL of combined supernatants and 10 μL of L-norleucine (50 μg/mL) were mixed and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 30 μL of 20 mg/mL methoxyamine hydrochloride in pyridine, and the resulting mixture was incubated at 37 °C for 90 min. A 30 μL of BSTFA (with 1% TMCS) was added into the mixture and derivatized at 70 °C for 60 min prior to GC-MS metabolomics analysis. Quality control (QC) sample pooled from all the samples were prepared and analyzed with the same procedure as those of the experiment samples.

Metabolomics instrumental analysis was performed on an Agilent 7890A gas chromatography system coupled to an Agilent 5975C inert MSD system (Agilent Technologies Inc., CA, USA). A OPTIMA® 5 MS Accent fused-silica capillary column (30 m × 0.25 mm × 0.25μm; MACHEREY-NAGEL, Düren, GERMAN) was utilized to separate the derivatives. Helium (>99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min through the column. Injection volume was 1 μL, and the solvent delay time was 6 min. The initial oven temperature was held at 70 °C for 2 min, ramped to 160 °C at a rate of 6 °C/min, to 240 °C at a rate of 10 °C/min, to 300 °C at a rate of 20 °C/min, and finally held at 300 °C for 6 min. The temperatures of injector, transfer line, and electron impact ion source were set to 250 °C, 260 °C, and 230 °C, respectively. The electron ionization (EI) energy was 70 eV, and data was collected in a full scan mode (m/z 50-600).

For multivariate statistical analysis, the normalized data were imported to SIMCA software (version 14.1, Umetrics, Umea, Sweden), where the data were preprocessed by unit variance (UV) scaling and mean centering before performing principal components analysis (PCA) and orthogonal partial least-squares to latent structures discriminate analysis (OPLS-DA). In order to avoid model over-fitting, a default 7-round cross-validation in SIMCA software was performed throughout to determine the optimal number of principal components.

For univariate statistical analysis, the normalized data (p-value) were analyzed in the “muma” software package in R platform, where the parametric test was performed on the data of normal distribution by Welch’s *t*-test, while the nonparametric test was performed on the data of abnormal distribution by Wilcoxon Mann-Whitney test. The structural identification of differential metabolites was performed as follows. Fold change (FC), was calculated as a binary logarithm of the average mass response (normalized peak area) ratio between Group T vs Group M, where a positive value means that the average mass response of the metabolite in Group T is larger than that in Group Z. The AMDIS software was applied to deconvolute mass spectra from raw GC-MS data, and the purified mass spectra were automatically matched with an in-house standard library including retention time and mass spectra, Golm Metabolome Database, and Agilent Fiehn GC/MS Metabolomics RTL Library.

* 1. **Microbial community analysis**

Thermophilic and mesophilic sludge samples collected from UASB reactors during the steady-states were used for microbial community analysis. Genomic DNA was extracted and PCR was conducted with the primers of ArBa515F and Arch806R (ABI GeneAmp® 9700). The PCR products were purified, quantified, and used for barcoded libraries preparation and then sequenced on an Illumina Miseq platform according to the standard protocols. The high-quality sequences were phylogenetically assigned to taxonomic classification by RDP Classifier (Luo et al. 2013). OTUs clustering and taxonomic assignment were done using Usearch (version 7.0 <http://drive5.com/usearch/>) (clustered at 97%) with multiple sequence comparison. The raw data were submitted to NCBI with accession number SUB5724044.

* 1. **Other analytical methods**

Chemical oxygen demand (COD) was analyzed according to APHA (Association and Washington 1995). The methane content in the biogas and volatile fatty acids in the effluents were measured by gas chromatography based on our previous study (Chen et al. 2017). Methane yield was corrected to standard temperature and pressure (STP) (Chen et al. 2017). All values of methane and biogas were reported at STP throughout the study. GC-MS was also used to characterize the chemical compositions of aqueous samples and the organics were extracted by ethyl acetate according to our previous extraction procedures (Chen et al. 2016). Gas chromatography was performed on a 30 m HP-INNOWax quartz capillary column with 0.25 mm inner diameter (I.D.) and 0.25 mm film thickness with an injection temperature of 250 °C. The column was initially held at 60 °C for 2 min and heated to 250 °C and held there for 10 min. Helium was used as the carrier gas (1.0 mL/min). A NIST Mass Spectral Database was used for compound identification.

1. **Results and Discussion**
	1. **The** **removal efficiencies of** **organics in HTL-AP** **under both mesophilic and thermophilic conditions**

The performances of the reactors are summarized in Table 1. The identified low molecular weight organic acids (C1-C4) were well degraded under mesophilic condition (Table 1). The COD removal efficiencies of HTL-AP at mesophilic (35 oC) and thermophilic (55 oC) conditions were 61.6 % and 45.0 %, respectively. Therefore, it could be inferred that increased digestion temperature decreased COD removal efficiency (Fig. 1) and the average methane yields were 194 mL CH4/g COD under mesophilic conditions and 137 mL CH4/g COD under thermophilic conditions (P < 0.01, ANOVA). However, a previous study showed that the methane yield of cornstalk under thermophilic conditions (325.18 mL/gVS) was higher than the yield under mesophilic conditions (308.63 mL/g VS). Another study also showed thermophilic AD of wheat straw gave a 36% higher methane yield (0.165 L/gVS) compared to AD under mesophilic conditions because higher AD temperature was more favorable for the hydrolysis of straw (Li et al. 2016, Pohl et al. 2012). However, hydrolysis was not the rate-limiting step in the present study since all the organics were solubilized in HTL-AP. The low organic degradation efficiency of thermophilic AD can be related to inefficient degradation of certain organics in HTL-AP by the anaerobic microorganisms. There were still organics left in the effluent in both conditions, which showed the HTL-AP contained some recalcitrant organics. Deficient digestion efficiencies resulting from some inhibitors in HTL-AP were also inferred in previous reports (Chen et al. 2016, Si et al. 2018). Therefore, it is necessary to further clarify the relationships among the recalcitrant compounds, organic removal efficiencies and microbial communities at different AD temperatures.

* 1. **Specific fractions with different molecular weights as revealed by LC-OCD-OND analysis**

DOC, HOC, CDOC, biopolymers (BP, >20 kDa), humic substance (HS, 300-450 Da), building blocks (BB, 300-450 Da), low molecular weight neutrals (LMWN, < 350 Da) and low molecular weight acids (LMWA, < 350 Da) were analyzed before and after mesophilic and thermophilic digestion (Table 2 and Fig. 2). In Fig. 2, the results show that the chemicals of all fractions in HTL-AP were degraded to some extent under both mesophilic and thermophilic conditions. For instance, the concentrations of HOC decreased from 1155 mg/L to 461 mg/L and 607 mg/L, which indicated that part of them might be transformed into hydrophilic compounds or degraded during the AD process. The concentrations of HS decreased from 529 mg/L to 142 mg/L and 147 mg/L under mesophilic and thermophilic conditions, respectively. At the same time, the average molecular weight (310 Da in HTL-AP) was increased to around 430 Da in both effluents (Table 2). This showed that parts of the LMW HS were degraded at mesophilic and thermophilic conditions and there were still HMW recalcitrant HS in HTL-AP that was not degraded due to their polycyclic or heterocyclic aromatic structures. This might be one of the reasons for inadequate COD removal efficiencies reported by AD (Lu et al. 2018). In addition, the aromaticity of HS increased dramatically from 0.87 to 4.88 and to 6.49 after mesophilic and thermophilic digestion, respectively, suggesting that many of the aromatic compounds belonging to HS were recalcitrant compounds and accumulated in AD systems.

For the fraction of LMWA (e.g. lactic acid and benzoic acid), the concentration decreased from 726 mg/L to 59 mg/L after mesophilic digestion, while it only decreased to 349 mg/L after thermophilic digestion (Fig. 2 and Table 2). This implies that LMWA was more efficiently utilized in the mesophilic reactor, which was related to the higher COD removal efficiency. Previous studies also showed that acids with low molecular weights (e.g. VFAs) in other HTL-AP were well digested under mesophilic conditions and the methane yield was strongly correlated to their concentrations (Chen et al. 2019, Lu et al. 2018, Si et al. 2018). LMWN included aldehydes, ketones and alcohols and were slightly accumulated (the remove efficiency was lower than their COD remove efficiency) as their percentages increased from 25% to 35% under mesophilic conditions and to 31% under thermophilic conditions (Xiao et al. 2016). It could be speculated that LMWN contained many recalcitrant compounds and therefore, their percentages increased especially under mesophilic conditions as more easily degraded compounds were digested. The specific molecular weight fractions before and after AD have been identified through LC-OCD-OND analysis and the fractions that affected digestion properties have also been discussed but more information is needed to further understand the differences between mesophilic and thermophilic AD.

* 1. **GC–MS analysis of HTL-AP samples before and after AD**

The aqueous influent and effluent samples of the HTL-AP were also extracted by ethyl acetate to qualitative investigate the variation of chemicals before and after AD under mesophilic and thermophilic conditions using GC-MS. Phenols, ketones and some N-heterocyclic compounds were the main three groups of identified chemicals (Table S2). Phenyl compounds, considered to be potential inhibitor originated from lignin components of cornstalk and they were the main contributor of aromaticity of the HTL-AP (Si et al. 2018). The percentages of phenyl compounds (Table S2) in HTL-AP, thermophilic and mesophilic effluents were 43%, 42% and 36%, while the aromaticity of HS in the above three samples was 0.58, 6.49 and 4.88 respectively (Table 2), suggesting that only part of the organics could be identified through traditional GC-MS analysis. It had been reported that only part of the volatile or semi-volatile organic compounds (15-20 % of the total compounds) could be identified through GC-MS analysis as the oven temperature of the GC was limited (Cao et al. 2017b). Nevertheless, GC-MS analysis revealed that phenyl or other compounds (N-contained compounds) were the recalcitrant compounds especially under the thermophilic condition.

* 1. **FT-ICR-MS analysis of HTL-AP samples before and after AD**

Quantitative and qualitative analysis of the specific components in influent and effluents have been conducted through the LC-OCD-OND and GC-MS analysis. To facilitate our understanding of the nature of organic compounds in HTL-AP and biogas reactors and related biochemical reactions in AD, further molecular properties of the organics were analyzed by FT-ICR-MS analysis. A total of 1488 and 1525 ions were identified from the spectra of mesophilic and thermophilic samples (1599 ions in HTL-AP, Fig. S2), which demonstrated the complexity of HTL-AP. The molecular mass distribution (150−800 Da) of effluents from mesophilic and thermophilic reactors showed distinct differences from HTL-AP (Fig. 3) and moved left for AD effluents especially for the mesophilic effluent, which implied that there was efficient organic degradation by AD under mesophilic condition. The three samples contained heteroatomic CHO and CHON compounds (delineated by elemental formula combinations, Table S1). CHO were the dominant class in HTL-AP influent, while the numbers of CHON compounds were increased after AD (Table S1), which could be due to more N containing organics being accumulated or produced through microbial metabolism in the AD process (Li et al. 2019).

Van Krevelen diagrams that cross-plotted hydrogen to carbon (H/C) ratio and oxygen to carbon (O/C) ratio versus mass plots for mesophilic and thermophilic effluents and HTL-AP influent are shown in Fig. 4 (a, b and c). The intensity data is represented by the size of the dots for the individual molecular formulas in the van Krevelen diagrams. The variation of dots size suggested that the O/C ratio decreased (much lower in mesophilic effluent in Fig. 4 (b)) and it was a deoxidization process during AD as carbon dioxide was produced alongside methane production. The Van Krevelen diagrams also facilitated the assignment of molecular formulas into different broad molecular groups including lipids, proteins/amino sugars, lignin, condensed aromatics, tannin and unsaturated hydrocarbon. The majority of the HTL-AP and AD effluents molecules had H/C ratio between 0.7-1.5 and O/C ratio between 0.1-0.67, which constrained highly unsaturated and phenolic or polyphenols compounds derived from the vascular lignin structure (Li et al. 2018b, Sepehr Shakeri et al. 2012, Yuan et al. 2017). The proportions of this compounds in mesophilic and thermophilic effluents increased compared to the HTL-AP (Fig. 4(d)), suggesting that aromatic compounds might accumulate in agreement with LC-OCD-OND results of aromaticity values in Table 2. It should be pointed out that the percentage of the condensed aromatic compounds in thermophilic effluent was higher than that in mesophilic effluent, suggesting that the aromatic compounds were better degraded in the mesophilic reactor.

Newly produced compounds after mesophilic and thermophilic digestion are indicated in Fig. 5 and might be intermediate by-products of the refractory compounds. The refractory nature of aromatic compounds was also found in the lignin-like region of the van Krevelen diagram after AD degradation in the full scale continuous stirred tank biogas reactor (Sepehr Shakeri et al. 2012). It was also reported that carboxylic and alicyclic compounds within H/C and O/C ratios of 0.7-1.5 and 0.2-0.5 (lignin-like region of van Krevelen diagram.) were the metabolites of organisms or the decomposition products of biomass-related compounds (Hertkorn et al. 2006). Besides, comparing to HTL-AP influent in Fig. 4(a), more chemicals in the regions of lipids and proteins/aliphatic were found in the mesophilic sample (Fig. 4), which should result from the metabolic process of microorganisms (Guo et al. 2018). Comparing with the thermophilic digestion, this lipids and proteins/aliphatic like chemicals might not be well degraded because the metabolic rates were lower at a lower temperature at mesophilic condition. Therefore, metabolomic analysis was necessary to examine the above discussions and gave an idea of how the compounds were digested under mesophilic and thermophilic conditions from a complex network of chemical and biochemical pathways.

* 1. **Metabolomic analysis**

3.3.1 Statistical analysis

Metabolomic analysis was conducted to further illustrate how AD temperature led to different metabolic processes. The metabolome was characterized by processing the data from thermophilic (Group T) and mesophilic (Group M) digestion samples via principle components analysis (PCA). The R2X value representing the goodness of fit for model PCA was 0.826 (>0.5) and it indicated that the model had a high proportion of explanation for the data (Fig. S4(a)). To minimize the effects of background and improve the metabolite discrimination, model OPLS-DA (R2X = 0.824, R2Y = 0.999, and Q2 = 0.994) was established with high goodness of fit and predictability. The significant separation of Group T and Group M in scores plots of PC1 (t [1]) was identified as the existence of potential differential metabolites (Fig. S4(a) and S4(b)).

3.3.2 Discriminating metabolites analysis

Mass spectra were then matched with an in-house standard library including retention time and mass spectra based on the VIP and p values of model OPLS-DA. 54 discriminating metabolites (VIP > 1 and p < 0.05) were screened out and 27 metabolites with negative fold change (FC) values, were prominent in the mesophilic samples and another 27 significant metabolites (positive FC value) were prominent in the thermophilic samples (Table 3). FC was calculated as a binary logarithm of the average mass response (normalized peak area) ratio between Group T vs Group M, where a positive value meant that the average mass response of the metabolite in Group T was larger than that in Group M. Heatmap was used to illustrate the relativity analysis of discriminating metabolites in thermophilic and mesophilic samples (Fig. S5). The distinct color revealed the existence of different metabolites between thermophilic and mesophilic digestion.

It was observed that the metabolites of C2-C5 monocarboxylic, dicarboxylic hydroxyl acids (e.g. 3-hydroxybutyric acid, 2-hydroxyisobutyric acid, and 2-hydroxyadipic acid) and hydroxy benzoic acids (e.g. 2-hydroxyphenylpropionic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and 3-hydroxyphenylpropionic acid) were some common class of metabolites in both mesophilic and thermophilic samples. Conceptually, both thermophilic and mesophilic AD can be described by sequential steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which were accomplished by different guilds of microorganisms (Venkiteshwaran et al. 2015). Therefore, it is readily comprehensible that C2-C5 acids were discriminated under both conditions. Although metabolites of C2-C5 acids were discriminated under both conditions, it needs to be pointed out that most of the short chain organic acids had positive FC (Group T/M) values (number ratio of positive and negative values is 14:1) among the 54 metabolites, indicating the accumulation of C2-C5 metabolites in thermophilic sludge samples. It was also consistent with the high DOC percentage of LMWA in thermophilic effluent (19% vs. 6% in mesophilic effluent in Table 2). Other significant metabolites including furan acid (e.g. 2-furanacetic acid) and medium/long-chain fatty acids (C6-C16 acids e.g. hexanoic acid, stearic acid, phytanic acid and palmitic acid) had negative FC (Group T/M) values, showing that they had high contents in the mesophilic reactor (Group M) (Table 3).

3.3.3 Metabolic pathway analysis

The discriminating metabolites were compared with the metabolic pathway in KEGG to illustrate the metabolic differences of HTL-AP under mesophilic and thermophilic conditions. 15 pathways relating to 366 compounds were identified upon the metabolome views of metabolites in the thermophilic and mesophilic samples (Fig. S6 and Table S2). The abundances of certain metabolites were significantly changed as shown in the metabolic network by comparison of mesophilic and thermophilic samples, and they were involved in aromatic compounds degradation (benzoate and polycyclic aromatic hydrocarbon degradation), fatty acid metabolism (biosynthesis, elongation, and degradation), and other biological process of membrane transport (e.g. ABC transporters) (Fig. 6) by comparing with metabolic pathways in KEGG. Taking phenyl compounds as an example, phenol, 3-ethylphenol and 2,6-dimethoxyphenol, which were the main phenolic constituents in influent, still existed after thermophilic digestion while only degraded phenolic compounds (intermediate products) such as 3-methylbenzoic acid, benzoic acid, and 2-hydroxyphenylpropionic acid were found after mesophilic digestion (Table 3). The original phenyl compounds in HTL-AP and the corresponding metabolites after processes using both sets of conditions are listed specially in Table S4. It can be concluded that the degree of phenyl compounds degradation at mesophilic condition was higher than that at thermophilic condition through benzoate degradation via CoA ligation and polycyclic aromatic hydrocarbon degradation. It has been previously demonstrated that the degradation of phenolic compounds was restricted at thermophilic conditions, while these compounds were mineralized by the mesophilic microbial cultures (Levén and Schnürer 2005). The lower AHS value in mesophilic effluent (4.88 vs. 6.48 in Table 2) also showed that some aromatic compounds were well degraded at mesophilic condition comparing with thermophilic condition.

Furthermore, it could be inferred that there were metabolic differences between mesophilic and thermophilic conditions with the variations on metabolites of fatty acids derived from lipids (Fig. 6). More metabolites relating with medium/long-chain fatty acids were identified in mesophilic reactor and the lipids were also identified significant but they were negligible in the HTL-AP and thermophilic effluent through FT-ICR-MS analysis (Fig. 4), This suggesting that the discriminate metabolites should be resulted from the activities of mesophilic anaerobic microorganisms. According to the comparison with metabolic pathway in KEGG, microbial autogenesis of fatty acid biosynthesis concerned with metabolomics of tricarballylic acid, hexanoic acid and stearic acid were responsible for the their prominent at mesophilic condition. Lu et. al also observed that the long chain fatty acids and alcohols (e.g. 3,7,11,15-tetramethylhexadecanol in Table 3) were mainly fragmentation of cytoplasm, extracellular substances and cell membrane and during AD process, most of which could be digested or transformed to other intermediate products (Lu et al. 2018). An increased process temperature in general had a positive effect on the metabolic rate of the microorganisms (Hou et al. 2018). The high digesting temperature at thermophilic condition might be conducive to the degradation of these substances.

Based on the metabolomic analysis, the metabolic pathways of other compounds (e.g. furan degradation) and environmental information processing (e.g. ABC transporters) also contributed to the difference of metabolic pathways and degradation efficiencies although it was still difficult to describe (Lu et al. 2018). The main reason was that the interactions between microorganisms and HTL-AP compositions were too complex and the actually matched number (Hits value) of significant metabolites was very limited and the pathway impact value calculated from pathway topology analysis was not higher. For instance, furans which were acknowledged to be inhibitory compounds of AD process were degraded (at least partly) in mesophilic reactor as its metabolite of 2-furanacetic acid had the lowest FC value (-5.05 in Table 2) but only one metabolite was matched by comparing with KEGG (Fig. 5). Metabolomics combined with community structure analysis might be used to further explore the metabolic shifts for HTL-AP digestion at mesophilic and thermophilic conditions.

* 1. **Microbial community analysis**

The above results indicated that different metabolic pathways lead to different degradation efficiencies which might involve diverse microorganisms at mesophilic and thermophilic conditions (Chen et al. 2016, Luo et al. 2016). Therefore, sludge samples obtained from both the mesophilic and thermophilic reactors were further used for microbial community analysis. The diversity of both bacterial and archaeal communities in mesophilic reactor was higher than that in thermophilic reactor as reflected by the Shannon diversity and CHAO1 richness values (Table S3). According to ecology principles, microorganisms with high diversity had strong biological abilities which is a key factor in preserving stability of an ecosystem (Finlay et al. 1997). Therefore, the higher microbial diversity in mesophilic reactor might contribute to a high degradation ability of the complex organics in HTL-AP. The taxonomic classification of bacterial sequences by RDP classifier was shown in Fig. 6. A total of 30 phyla were identified. *Firmicutes* (11.8-30.3%), *Synergistetes* (23.9-28.0%), *Thermotogae*(22.8-24.0%), *Proteobacteria* (11.8-13.2%), and *Chloroflexi* (2.7-10.1%) were the dominant phyla in both mesophilic and thermophilic digestion effluents samples, which were consistent with previous reports in anaerobic reactors (Chen et al. 2016, Luo et al. 2016, Sundberg et al. 2013).

The abundance of phylum *Firmicutes* (11.8% vs. 30.8%) significantly decreased while phyla *Chloroflexi* (10.1% vs. 2.7%) and *Bacteroidetes* (6.1% vs. 0.5%) were accumulated in the mesophilic reactor compared to the thermophilic reactor (Fig. 7). At the genus lever, *Mesotoga* in phylum *Thermotogae* and *Pelolinea* in phylum *Chloroflexi* were accumulated in the mesophilic reactor. *Mesotoga,* with dominant abundance of 24.0% in the mesophilic reactor, was reported to efficiently ferment short-chain fatty acids and carbohydrate to acetic acid (Nesbø et al. 2012). This was consistent with the lower LMWA value of the mesophilic effluent in Table 2 and higher COD removal rate in Fig. S1. The genus *Pelolinea* accumulated in the mesophilic reactorwas reported to ferment glucose into VFAs and hydrogen and it was also involved in the inhibitory phenolic compounds degradation (Rosenkranz et al. 2013, Takeshi et al. 2006). This could be one of the reasons that the aromaticity of mesophilic effluent was lower than the thermophilic effluent (4.88 vs. 6.49 in Table 1) and higher COD remove efficiency and methane yield was obtained in the former.

The noteworthy accumulations of genera *Thermacetogenium*, *Gelria*, and *Syntrophaceticus* in phylum *Firmicutes* in thermophilic sample was reported to be very versatile and participate in the degradation of several complex organic residues, such as lipids, carbohydrates and proteins (Martin-Gonzalez et al. 2011). Besides, the contribution of relatively low abundant bacterial populations was also worthy of being discussed. Compared with mesophilic sample, the enrichment ofgenera *Syntrophobacter* (6.7 % vs. 5.4 %) and *Syntrophaceticus* (1.9 % vs. 0 %) in the thermophilic reactor were reported to be involved in the degradation of fatty acids via syntrophic oxidizing activity and coupled with methanogenesis (Amha et al. 2017, Wu et al. 2016). This could facilitate the decomposition of microbial byproducts under the thermophilic condition as the FT-ICR-MS and metabolomic analysis shown in Fig. 4 and Table 3. Besides, very high percentages (around 69%) of sequences especially for mesophilic sample, were unclassified at the genus level classification. This could be attributed to the complex components in HTLWW as well as the high bacteria diversity, after all, most of biogas reactor's communities are still uncharacterized (Bassani et al. 2015).

For the taxonomic classification of archaea, differences between the relative abundances among the mesophilic and thermophilic reactor were also observed. *Methanosaeta* and *Methanolinea* were the dominant genera in mesophilic reactor while the dominant genera in thermophilic reactor were*Methanothermobacter* and *Methanosarcina* (Fig. 8). For the mesophilic digestion, the overwhelming sequences withrelative abundances of 56 % were assigned to genus *Methanosaeta*. The microorganisms belonging to *Methanosaeta* were strictly aceticlastic methanogens (Chen et al. 2016), and it had higher affinity for acetate and grew better with lower acetate concentrations (Gang et al. 2016). The vigorous acetoclastic methanogens could consume acetate quickly and kept the VFAs content low in the mesophilic effluent (Table 1). The dominant methanogens in the thermophilic reactor were hydrogenotrophic methanogens (*Methanothermobacter, Methanobacterium, Methanolinea*, and *Methanosarcina*). The decline of *Methanosaeta* and increase of *Methanosarcina* (hydrogenotrophic and acetoclastic methanogens) in thermophilic reactor might indicate that there existed a competitive growth between these two acetoclastic methanogens and different methanogenic pathways were conducted under the mesophilic and thermophilic conditions. The relatively higher concentration of acetate in thermophilic reactor might facilitate the growth of *Methanosarcina* as they had lower affinity for acetate (Venkiteshwaran et al. 2015). It could be concluded that different AD temperatures could drive different environmental information processing and developed different microorganisms and metabolic functions, which resulted in different organic degradation efficiencies.

1. **Implications**

The present study provided an in-depth understanding of mesophilic and thermophilic AD of HTL-AP, which was derived from a representative biomass (cornstalk). The molecular, microbial and metabolic analysis revealed the intrinsic differences in organic degradation, microbial community and metabolic pathways of mesophilic and thermophilic AD. Future studies should focus on mesophilic AD of HTL-AP, and it is determined by the nature of the complex constitutes in HTL-AP, which requires diverse microbial community for the degradation of the organics. In order to further improve the AD biodegradability of HTL-AP, granular activated carbon can be added in AD process, which can enhance the growth of slow-growing and synthrophic microbes to better degrade various organics (Zhao et al. 2017). Furthermore, the present study showed metabolic analysis was a powerful tool for elucidating the changes of microbial pathways. LC-MS-based metabolomic analysis is suggested to be applied instead of GC-MS analysis as more metabolites could be determined through the former method (Usman et al. 2019). Besides, some algorithms need to be applied or developed to calculate the data and evaluate the metabolic pathway activities based on the metabolomics results.

1. **Conclusion**

The present study showed that increased AD temperature in fact decreased COD removal efficiency (from 61.6% to 45.0 %). HMW and LMW compounds were degraded to some extent during both mesophilic and thermophilic processes as measured by LC-OCD-OND and FT-ICR-MS. More LMWA and recalcitrant aromatic compounds were degraded in the mesophilic reactor, which was the main reason for higher COD removal efficiency under mesophilic conditions. Phenyl compounds (e.g. phenol and 2 methoxyphenol), furans and pyrazine were the recalcitrant chemicals. Metabolites analysis showed that the reasons contributed to the difference of mesophilic and thermophilic digestions were not only related to the degradation of organic compounds in HTL-AP but also related to the microbial autogenesis as well as the environmental information processing. The enrichment of bacteria e.g. *Mesotoga* and *Pelolinea* might be responsible for the higher COD remove efficiency in the mesophilic reactor.

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**Supporting Information**

Supporting information includes Tables and Figures as noted in the text.

**Abbreviations**AD: Anaerobic digestion

BB: Building blocks

BOM: Biodegradable organic matter

COD: Chemical oxygen demand

CDOC: Chromatographic dissolved organic carbon

DOC: Dissolved organic carbon

Effluent: the cornstalk HTL-AP after AD

FT-ICR MS: Fourier transform ion cyclone resonance mass spectrometry

HTL: Hydrothermal liquefication

HTL-AP: Hydrothermal liquefication aqueous phase

HMW: High molecule weight

HOC: Hydrophobic organic carbon

HS: Humic substances

Influent: the cornstalk HTL-AP before AD

LMW: Low molecule weight

LC-OCD-OND: Liquid chromatography coupled with organic carbon detection and organic nitrogen

LMWA: Low molecular weight acids

LMWN: Low molecular weight neutrals

OC: Organic carbon

ROM: Refractory organic matter

STP: Standard Temperature and Pressure

UASB: Upflow anaerobic sludge blanket

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